

Trabectedin (ET-743) promotes differentiation in myxoid liposarcoma tumors

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Abstract

Differentiation is a complex set of events that can be blocked by rearrangements of regulatory genes producing fusion proteins with altered properties. In the case of myxoid liposarcoma (MLS) tumors, the causative abnormality is a fusion between the *CHOP* transcription factor and the *FUS* or *EWS* genes. *CHOP* belongs to and is a negative regulator of the large CAAT/enhancer binding protein family whose α , β , and δ members are master genes of adipogenesis. Recent clinical data indicate a peculiar sensitivity of these tumors to the natural marine compound trabectedin. One hypothesis is that the activity of trabectedin is related to the inactivation of the *FUS-CHOP* oncogene. We find that trabectedin causes detachment of the *FUS-CHOP* chimera from targeted promoters. Reverse transcription-PCR and chromatin immunoprecipitation analysis in a MLS line and surgical specimens of MLS patients *in vivo* show activation of the CAAT/enhancer binding protein-mediated transcriptional program that leads to morphologic changes of terminal adipogenesis. The activity is observed in cells with type 1 but not type 8 fusions. Hence, the drug induces maturation of MLS lipoblasts *in vivo* by targeting the *FUS-CHOP*-mediated transcriptional block. These data provide a rationale for the specific activity of trabectedin

and open the perspective of combinatorial treatments with drugs acting on lipogenic pathways. [Mol Cancer Ther 2009;8(2):449–57]

Introduction

Several human cancers are caused by a block in differentiation and accumulation of cellular precursors. Myxoid liposarcomas (MLS) are a specific histologic subtype within the family of adult soft tissue sarcomas that are tumors of mesenchymal origin. MLS accounts for a third of liposarcomas. More specifically, the major liposarcoma subtypes identified by morphologic and cytogenetic criteria are (a) well differentiated/de-differentiated, (b) usual myxoid/round cell, and (c) pleomorphic (1). Specifically, >90% of usual myxoid/round cell liposarcomas (MLS/RCLS) carry a t(12;16) (q13;p11) chromosome rearrangement resulting in a fusion between the NH₂-terminal part of *FUS* and the full-length *CHOP* (2). The causative role of the *FUS-CHOP* fusion in the initiation of MLS/RCLS has been shown (3–5). The *FUS* gene is constitutively active and codes for a nRNA-binding protein, whose NH₂-terminal part contains an autonomous transcriptional activation domain required for the full oncogenic potential of the chimera (6). *CHOP*, also termed as DDIT3, GADD153, and C/EBP ζ , is a member of the CCAAT/enhancer binding protein (C/EBP) transcription factor family. Originally identified as a gene induced by treatment of cells with DNA-damaging agents, it became clear that it takes part in many processes that involve a response to noxious stimuli, particularly the endoplasmic reticulum stress response. Its expression is tightly regulated and the protein is also implicated in developmental programs. At the molecular level, it affects G₁-S cell cycle progression, growth arrest, and apoptosis (7, 8). One of the important roles of *CHOP* is to heterodimerize with other members of the family, serving as a dominant negative protein by altering their transcriptional potential (7). Within the constitutively active *FUS-CHOP* chimeras, *CHOP* retains the heterodimerization and DNA-binding domains and it is thus proficient in shortcutting the normal C/EBP activities (9, 10). Among the activities of this important class of transcription factors, genetic and biochemical experiments established the key role of three members C/EBP α , β , and δ in adipocyte differentiation. C/EBP β and C/EBP δ play redundant roles in the early phases of commitment, whereas C/EBP α becomes active and important in the later phases leading to terminal differentiation (7).

A total of nine variants of *FUS-CHOP* transcripts have been described in MLS (11), but three are overwhelmingly recurrent: fusion of exon 7 (type 1, ~20% of cases), exon 5 (type 2, ~60%), and exon 8 (type 3, ~10% of cases) of *FUS*, all to exon 2 of *CHOP* (12).

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Surgery alone or in combination with radiotherapy is the main means of treatment for localized MLS. However, ~40% of patients relapse and chemotherapy is given to patients with advanced/unresectable disease. Interestingly, a recent case series analysis of patients with MLS/RCLS treated with trabectedin showed a high percentage of tumor responses with a certain degree of a tumor control over time (13).

Trabectedin (ET-743, Yondelis) is a marine alkaloid isolated from the tunicate *Ecteinascidia turbinata*, cytotoxic against a variety of tumor cell lines *in vitro* and human tumor xenografts *in vivo* (14). It binds to the minor groove of DNA with some degree of sequence specificity and forms covalent adducts by reacting with the N-2 of guanine to its carbinolamine moiety (15). The mechanisms of action of trabectedin seem to be unique and still poorly understood. It induces a DNA damage response through pathways that are not traditional for alkylating agents (16–19). In parallel, a second mechanism pertains to a role in transcriptional interference. The induction of the *HSP70* and *MDR1* promoters and of other inducible genes is effectively prevented by pharmacologic doses of trabectedin (20–22). Gene expression analysis with microarray technology also identified selected groups of genes that are modulated in different cell lines (23).

The surprising finding on the sensitivity of liposarcoma tumors carrying a specific translocation involving a negative regulator of adipogenesis prompted us to investigate the mechanism of action of trabectedin related to this differentiation pathway. We used reverse transcription-PCR (RT-PCR) and chromatin immunoprecipitation (ChIP) assays with two lines carrying different *FUS-CHOP* translocations, as well as *in vivo* examination of patients treated with the drug. A prodifferentiation effect of trabectedin emerged.

Materials and Methods

Cell Cultures and Cytotoxic Assay

MLS cell lines 402-91 and 1765, both expressing the *FUS-CHOP* fusion protein, were described in detail (24). All cell lines were cultured in RPMI supplemented with 2 mmol/L L-glutamine, penicillin 100 units/mL, streptomycin 100 µg/mL, and 10% FCS. Drug stock solutions were prepared in DMSO at a concentration of 10 µmol/L. The stock solution was then diluted in cultured medium (RPMI). The cytotoxic effect of trabectedin was evaluated by a standard sulforhodamine B assay. Cells were stained with sulforhodamine B 7 d after treatment and absorbance was determined at 540 nm using a plate reader (Labsystem Multiskan MS).

RT-PCR Analysis, Total and Nuclear Extract Preparation, and Western Blot Analysis

Total RNAs were extracted using RNA-Easy kit (Qiagen) and retrotranscribed using SuperScript II (Invitrogen). Semiquantitative PCRs were done after normalizing all the cDNAs for glyceraldehyde-3-phosphate dehydrogenase control. The RT-PCR primers used are reported in

Supplementary Table S1.⁵ Gels were scanned with a Typhoon 4000 instrument and values within the linear range of amplifications were plotted as ratio over the untreated sample.

Nuclear extracts were prepared as described (25). Total extracts were prepared in radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% NP40, 0.5% DOC, 0.1% SDS]. Thirty micrograms of protein extracts were used in Western blots with antibodies against CHOP (Santa Cruz), FUS (Bethyl Laboratories), NF-YB (Diagenode, B), C/EBPβ (Santa Cruz), C/EBPα (Active Motif), caspase-3 (Cell Signaling Technology-CST), cleaved caspase-7 (CST), Lamin A/C (CST), and actin (Sigma). Horseradish peroxidase-conjugated secondary antibodies (GE Healthcare) and the PDS chemiluminescence system (Genespin, I) were used for detection.

Chromatin Immunoprecipitation

ChIP assays were done as previously described (26) with 5 µg of the following antibodies: NF-YB (Diagenode, B), CHOP (Santa Cruz), FUS (Bethyl Laboratories), C/EBPβ (Active Motif), and Flag control antibody (Sigma). The immunoprecipitated DNAs were analyzed by semiquantitative PCR with Taq DNA Polymerase (Genespin, I) and primers described in Supplementary Table S1.⁵

Immunostainings

402-91 cells were fixed in 2% paraformaldehyde for 20 min at room temperature and then permeabilized (0.2% Triton-0.1% bovine serum albumin in PBS) for 30 min at room temperature. Incubation with the primary antibody (anti-C/EBPα-CST) was done overnight at 4°C in a wet chamber. Rhodamine-conjugated secondary antibody (Sigma) was incubated at room temperature for 1 h and 4',6-diamidino-2-phenylindole (Sigma) staining was done before mounting. Confocal images were obtained with a Leica TCS SP2 AOBS microscope (Cimaina) with a ×40 objective.

Differentiation Assays

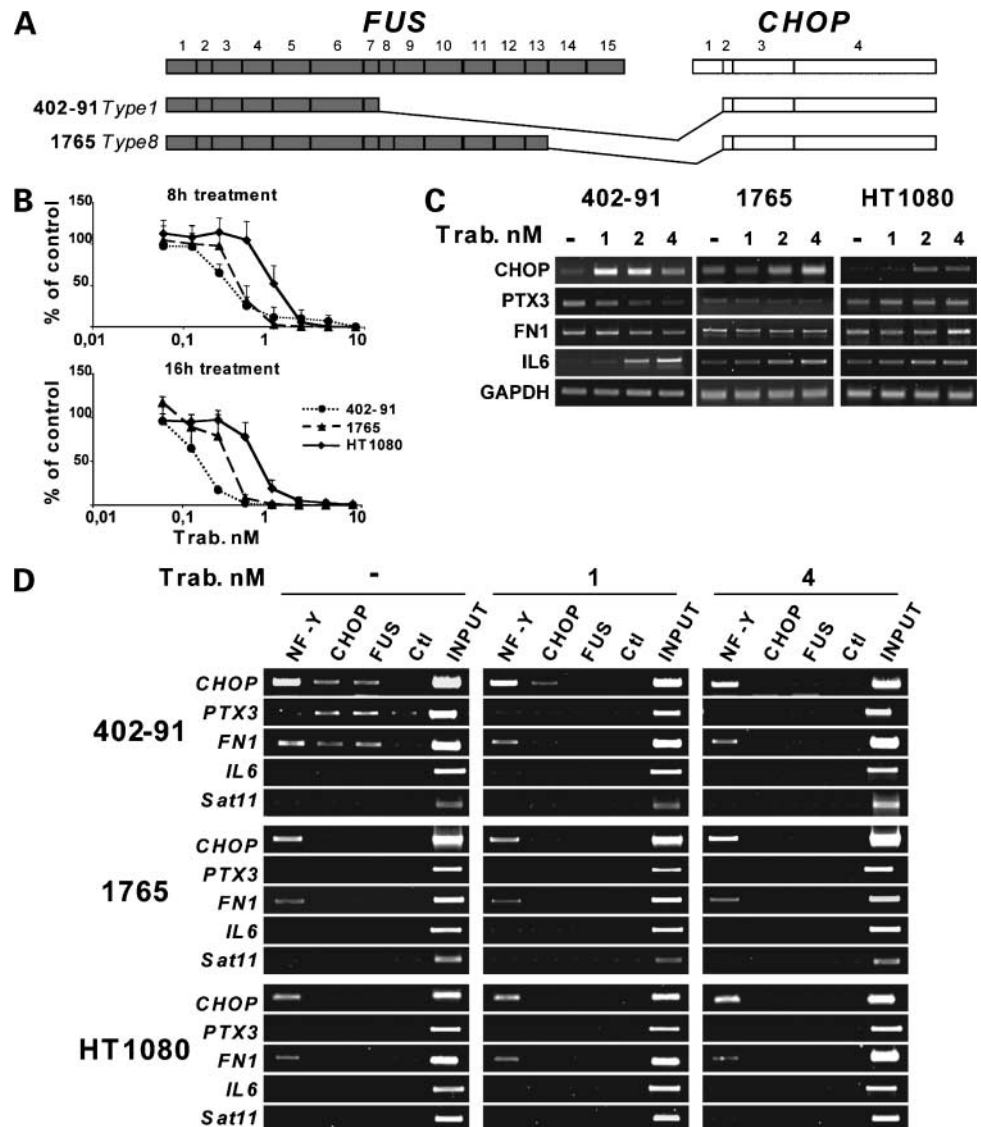
One day postconfluent cells were fed for 2 d with DMEM supplemented with 10% FCS, 10 µg/mL insulin, 1 µmol/L dexamethasone, and 0.5 mmol/L 3-isobutyl-1-methyl-xanthine (Sigma) or treated with trabectedin or Adriamycin. At the end of treatments, the medium was changed and cells were maintained for 1 or 2 d in DMEM containing 10% FCS and 10 µg/mL insulin. After three washes with PBS, cells were fixed for 2 min with 3.7% formaldehyde and then washed once with PBS. Oil Red-O (Sigma) 0.5% in isopropanol was diluted 3:2 with water, filtered, and incubated with fixed cells for 1 h at room temperature. Cells were then washed thrice with water and visualized by light microscopy.

In vivo Assays

Surgical specimens derived from two patients before and after treatment with trabectedin were selected. Patient LA (a 47-year-old man) had a usual MLS, treated before surgery with five cycles of trabectedin. Patient TM

⁵ Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

Figure 1. Effects of trabectedin on MLS cell lines. **A**, schematic representation of *FUS-CHOP* translocations in MLS 402-91 and 1765 cell lines. **B**, cytotoxic assay on 402-91, 1765, and HT1080 treated with increasing concentrations of trabectedin (*Trab.*) for 8 or 16 h. Each point is the mean of six replicates. **C**, MLS 402-91, MLS 1765, and HT1080 cells were treated for 16 h with the indicated concentrations of trabectedin and RT-PCR analysis were done for the genes indicated using glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as internal standard. **D**, ChIPs from untreated cells and cells treated with 1 and 4 nmol/L trabectedin for 16 h with α -NF-YB, α -CHOP, α -FUS, or α -Flag (*CtI*) antibodies. Semiquantitative PCRs were done with primers specific for the promoters indicated. *INPUT*, DNA used as a positive control.



(a 55-year-old woman) had a round cell MLS, excised after 10 cycles of trabectedin. Immunohistochemistry was done on 2- μ m cut sections of a representative paraffin block of formalin-fixed tumoral tissue derived from surgical specimens selected after microscopic evaluation. C/EBP α (Active Motif) and C/EBP β (Santa Cruz) immunodecorations were obtained with antigen retrieval [15 min at 95°C in 10 mmol/L citrate buffer (pH 6)]. The slides were developed with the UltraVision LP Volume Detection System (LabVision Corp.).

Results

Transcription of *FUS-CHOP* Targets Is Directly Affected by Trabectedin

We assayed trabectedin sensitivity of the 402-91 and 1765 MLS cell lines carrying type 1 and type 8 *FUS-CHOP* fusions, respectively (Fig. 1A), by comparing with the

HT1080 fibrosarcoma line. Among the cell lines, 402-91 are the most sensitive and HT1080 are four times more resistant, with 1765 being intermediate (Fig. 1B). We evaluated by RT-PCR (Fig. 1C) the expression of genes specifically regulated by *FUS-CHOP*, according to previous microarray analysis (27). In 402-91 cells, all genes were significantly altered already at low doses, whereas only *CHOP* and *IL-6* were modulated, at high doses, in 1765 and HT1080 (quantification of the data is reported in Supplementary Data 1). To verify whether the transcriptional changes were primary events, we monitored binding of *FUS-CHOP* to these promoters by ChIP with anti-*CHOP*, anti-*FUS*, and two control antibodies: an irrelevant anti-Flag and one against the widespread CCAAT-factor NF-Y (Fig. 1D). Note that no antibody was available to recognize specific chimeras and it was therefore essential to monitor both *CHOP* and *FUS* in the same ChIPs. Importantly, binding of neither *FUS* nor *CHOP* was

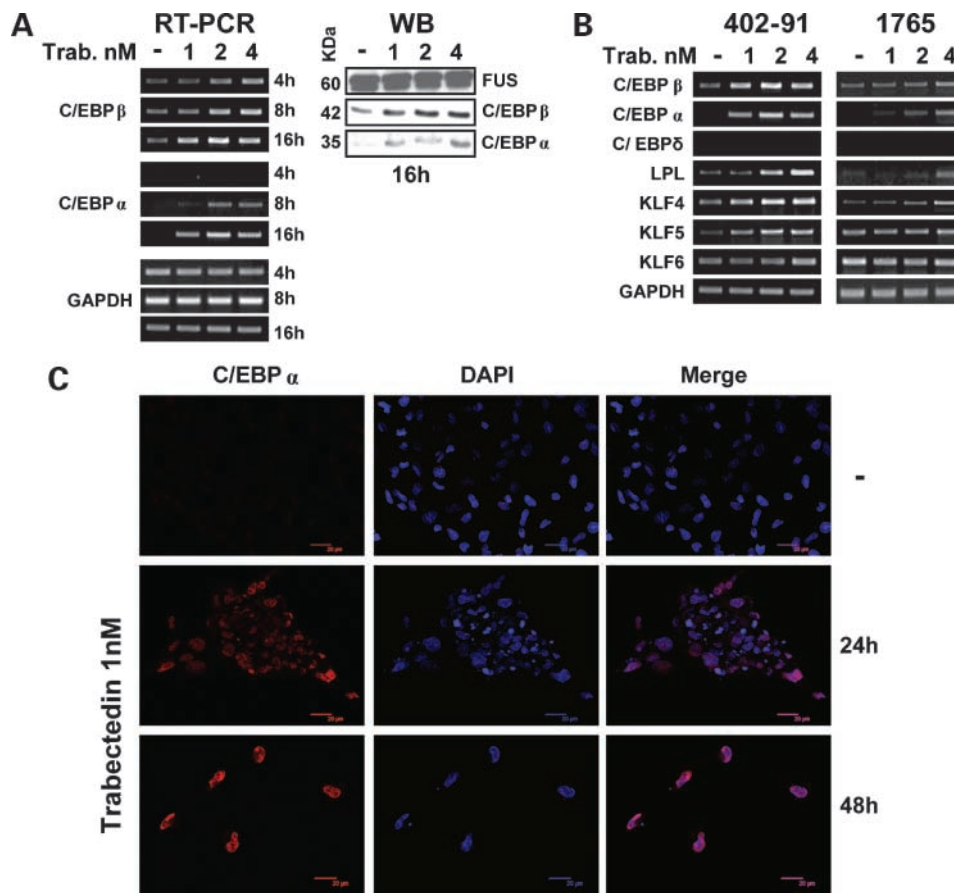


Figure 2. Analysis of adipocytic markers. **A.** MLS 402-91 cells were treated with different concentrations of trabectedin and harvested after 4, 8, and 16 h. Semiquantitative RT-PCR analysis was done for C/EBP β and C/EBP α . Western blot analysis was done on samples after 16 h treatment with antibodies against FUS, C/EBP β , and C/EBP α . **B.** Semiquantitative RT-PCR analysis with primers specific for genes involved in adipogenesis on 402-91 and 1765 cells treated for 16 h with the indicated concentrations of trabectedin. **C.** 402-91 cells were exposed to 1 nmol/L trabectedin for 16 h and then fixed for immunostaining with anti-C/EBP α 24 and 48 h later. Nuclear staining with 4',6-diamidino-2-phenylindole (DAPI) and the merge between C/EBP α (red) and 4',6-diamidino-2-phenylindole (blue).

detected in HT1080 nor in 1765, on any promoter, whereas both antibodies were positive in untreated 402-91 on the *CHOP*, *PTX3*, and *FN1* promoters. This association was decreased by trabectedin treatment, already at 1 nmol/L. An indication that our ChIPs detect the chimera is evidenced by the lack of FUS binding in the absence of CHOP, in any of the promoters or cell lines tested. The positive control NF-Y was bound to intended targets in all conditions and unaffected by trabectedin, ruling out unspecific effects of the drug. We conclude that trabectedin induces the removal of the type 1 FUS-CHOP chimera from promoters, altering their functionality.

The fate of *FUS-CHOP* expression after trabectedin was followed by RT-PCR and Western blot analysis. The chimeric RNAs are stable in 402-91 and 1765 cells (Supplementary Data 2). There was a decrease of the chimeric protein expressed in 402-91, but not in 1765, at high drug concentrations. No effect was observed on the normal FUS protein in the two cell types. We conclude that the removal of the chimera from DNA at low doses of trabectedin detailed in ChIP is not caused by a down-modulation of the type 1 FUS-CHOP.

Trabectedin Treatment Activates an Adipogenic Cascade in MLS Cells

C/EBPs play key roles in adipogenesis (7, 28) and trabectedin treatment might affect adipogenesis. MLS cells

were thus evaluated for differentiation markers and transcription factors involved in the cascade. RT-PCRs and Western blot analysis show that C/EBP β is present in 402-91 and increases after treatment. C/EBP α is undetectable in untreated cells and is rapidly induced at the mRNA level by trabectedin (Fig. 2A and B). Extracts were prepared from cells treated with the indicated amounts of trabectedin for 16 hours and assayed in Western blots: C/EBP β accumulates and C/EBP α , which is not present in untreated cells, is induced (Fig. 2A, right). We also monitored other genes of the lipogenic pathways: C/EBP δ , which is genetically redundant with C/EBP β , remains unexpressed; *KLF4* and *KLF5*, also involved in differentiation (29), but not the *KLF6* homologue, were up-regulated in 402-91 (Fig. 2B). *LPL*, a marker of terminal adipocyte differentiation, was equally activated. 1765 showed a different pattern: no increase of *KLF5* and increase of C/EBP β , C/EBP α , *KLF4*, and *LPL*, but only at highest trabectedin dose (quantification of the data is reported in Supplementary Data 1). Finally, we performed confocal immunofluorescence analysis of C/EBP α in 402-91 before and after treatment with low doses of trabectedin. C/EBP α -positive cells are present only after treatment at 24 and 48 hours (Fig. 2C). We conclude that trabectedin induces a transcription program of adipogenesis, specifically in 402-91 cells, by inducing the master genes of this process.

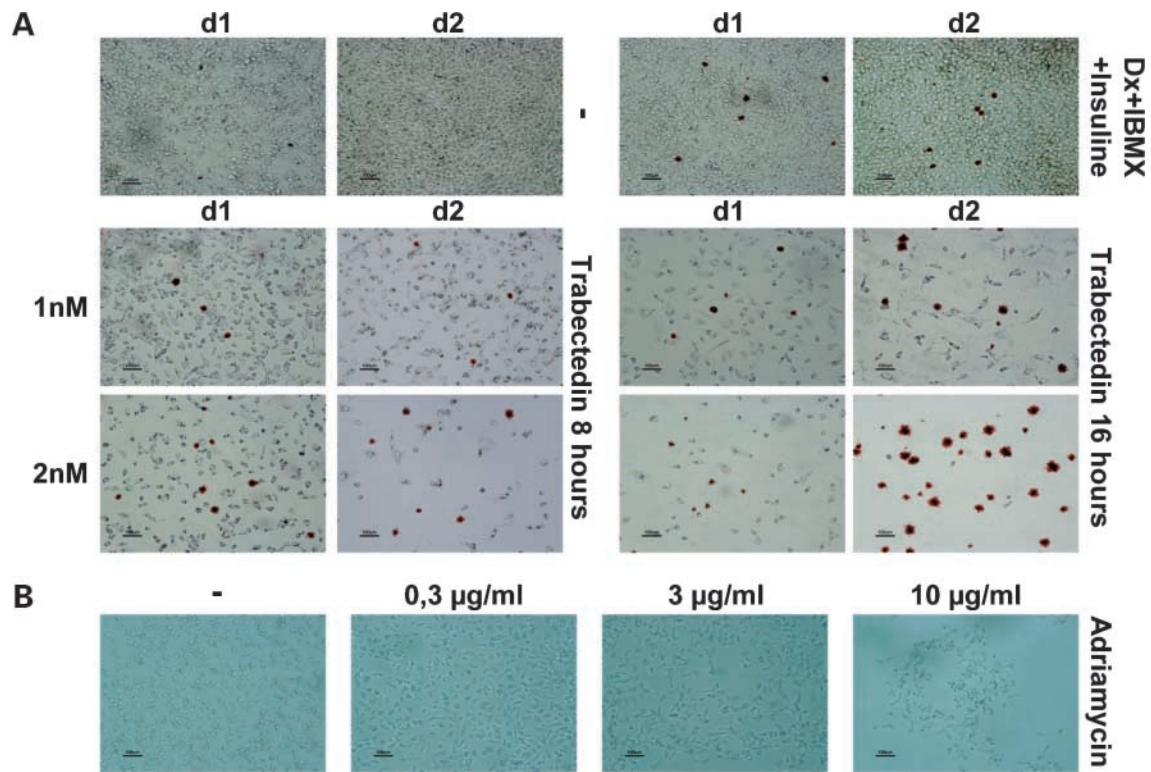


Figure 3. Differentiation of MLS 402-91 is induced by trabectedin treatment. **A**, 1 d postconfluent MLS 402-91 cells were exposed to 1 or 2 nmol/L trabectedin for 8 or 16 h or induced to differentiate using the standard protocol (*Dx+IBMX+Insulin*). One (*d1*) or two (*d2*) days after treatments, cells were stained with Oil Red-O and visualized by light microscopy. **B**, oil Red-O staining of MLS 402-91 cells exposed to different concentration of Adriamycin overnight.

Because of the effect on lipogenic markers, we evaluated whether accumulation of lipids occurs in MLS cells treated with the drug through Oil Red-O staining. Positive staining was visible 1 to 2 days after addition of trabectedin to 402-91 (Fig. 3A), but not to 1765 cells (Supplementary Data 3). Trabectedin was comparatively more potent in inducing lipid accumulation than standard treatments with Insulin/

IBMX/Dex. As a further control, 402-91 cells were treated with cell cycle arresting doses of the DNA-damaging Adriamycin and no positivity was observed even after high doses (Fig. 3B). These results confirm the molecular data and we conclude that trabectedin is specific for type 1 MLS cells in activating markers of postmitotic adipocytes and accumulation of lipids.

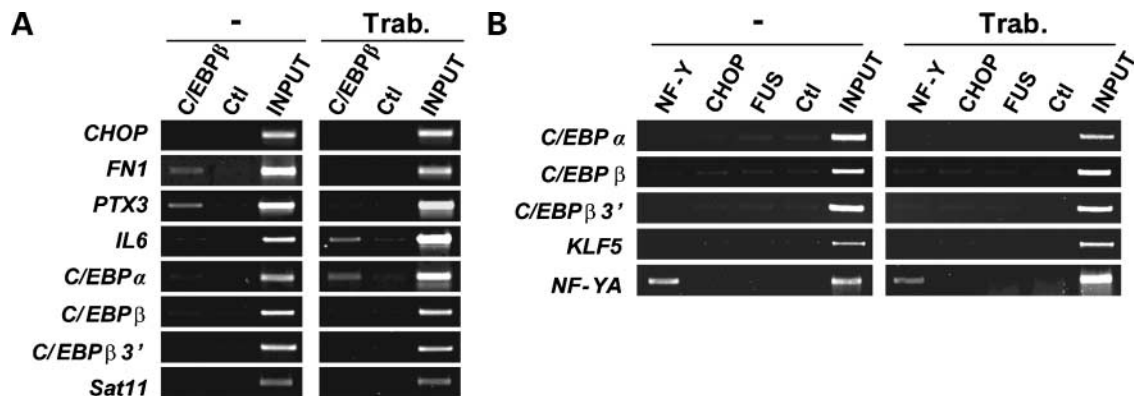


Figure 4. Analysis of C/EBP β binding *in vivo*. **A**, ChIP analysis of 402-91 cells untreated and treated with 4 nmol/L trabectedin for 16 h with α -C/EBP β or α -Flag (*CtI*) antibodies. PCRs were done for the promoters. **B**, ChIPs with antibodies were assayed on promoters of genes involved in adipocyte differentiation. The NF-Y factor was used as a positive control of the ChIP on the NF-YA promoter.

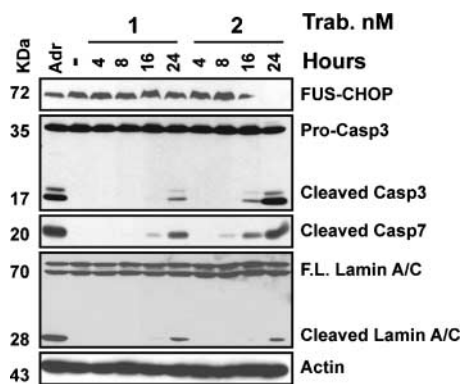


Figure 5. Analysis of apoptosis induction of 402-91. MLS 402-91 cells were treated with different concentrations of trabectedin (1 and 2 nmol/L) and harvested after 4, 8, 16, and 24 h. Positive control cells were treated with 1 μ mol/L Adriamycin and extracts were prepared after 24 h. Total extracts were subjected to Western blot with the indicated antibodies against apoptosis markers and CHOP. Antibody against actin was used as loading control.

C/EBP α is crucial for cell cycle exit and terminal differentiation of adipocytes and its activation is triggered by the binding of C/EBP β to its promoter (7, 28). We verified whether this would be the case, and indeed ChIP analysis on 402-91 showed increased binding of C/EBP β to the C/EBP α promoter after treatment (Fig. 4A). The *IL6* promoter was also bound after treatment and we conclude that C/EBP β induction results in the binding to and activation of C/EBP α and *IL6* (see RT-PCR of Fig. 1). Interestingly, *PTX3* and *FN1* are bound by C/EBP β only before, but not after, treatment: Both are down-regulated transcriptionally (RT-PCR of Fig. 1C). FUS-CHOP was not present on functionally important elements of the C/EBP α , C/EBP β , or *KLF5* promoters, suggesting that they are not under direct regulation of the chimera (Fig. 4B). Together with the ChIPs (Fig. 1D), this suggests that the chimera is sequestering C/EBP β on genes other than those required for adipogenesis, thus preventing progression of this pathway.

The 4',6-diamidino-2-phenylindole staining of IFs (Fig. 2C) and the Oil Red-O experiments (Fig. 3A) suggest that there might be apoptotic events in MLS cells treated with trabectedin. We performed dose-response experiments at different time points and assayed in Western blots with specific antibody markers typical of apoptosis: cleaved forms of caspase-3, caspase-7, and Lamin A/C (Fig. 5). Untreated 402-91 cells showed only the pro-caspase isoforms. Extracts from Adriamycin-treated cells served as positive control and had strong bands corresponding to cleaved forms of all apoptotic proteins, as expected; 402-91 were slightly positive with 1 nmol/L trabectedin at 24 hours, but robust signals were visualized with 2 nmol/L. Note the lack of effect of the drug on the levels of FUS-CHOP chimera at 1 nmol/L and the drop at late time points at 2 nmol/L (see also Supplementary Data 2). Overall, these data indicate that trabectedin treatment induces dose- and time-dependent apoptotic events in 402-91; however, at low trabectedin doses, the appearance of apoptotic signals is

mild and posterior to the induction of differentiation markers (Figs. 2 and 3).

Trabectedin Induces Maturation of MLS Tumors *In vivo*

Next, we evaluated by RT-PCR and immunohistochemistry the adipocytic markers in two patients representative of the usual MLS (Fig. 6A) and of the round cell subtype (Fig. 6B). Note that molecular and cytogenetic fluorescence *in situ* hybridization analyses of LA (MLS) and TM (RCLS) revealed type 2 and type 1 FUS-CHOP transcripts, respectively. After treatment with trabectedin, TM showed a small increase in C/EBP β expression and a dramatic change in all differentiation markers. LA, instead, had high basal levels of LPL, FABP4, and C/EBP β . C/EBP α was induced after treatment (data quantification in Supplementary Data 1). A strong immunoreactivity for C/EBP α and C/EBP β was present in the nuclei of all cells after treatment (Fig. 6A and B), with a particularly impressive increase in TM. In both cases, a drastic change in cellular morphology was evident, consistent with a progressive transition from immature, nonlipogenic/spindle cells to mature univacuolated/multivacuolated lipoblasts (Supplementary Data 4). Finally, because of the induction of some apoptosis in 402-91 cells, we performed Western blot analysis with caspase-3, caspase-7, and Lamin A/C antibodies. No cleavage was manifested on any of the markers in LA or TM (Fig. 6C). These data confirm the results obtained with the 402-91 MLS line and indicate that trabectedin leads to differentiative changes of MLS tumors *in vivo*.

Discussion

We investigated the molecular mechanisms underlying the activity of the anticancer drug trabectedin in MLS/RCLS, an effort prompted by the exceedingly high antitumor activity noticed in MLS/RCLS among sarcomas (13). We find that the drug induces a cascade of events leading to the activation of differentiation in a cell line characterized by the type 1 FUS-CHOP fusion, and in type 1 and type 2 MLS patients.

Several fusion proteins generated by recombinatorial events in somatic cells are known to cause a wide variety of tumors. In many cases, it has been formally proven that the resulting fusions are true oncogenic entities capable of dramatically altering the cellular strategy. In general, they block cells at a particular stage, preventing terminal differentiation and maintaining their proliferative potential. One such paradigmatic example is represented by the RAR fusions in promyelocytic leukemias. In these tumors, resulting from accumulation of myelomonocytic precursors, treatment with pharmacologic doses of retinoic acids and derivatives leads to remission in a large cohort of patients with PML-RAR fusions, by inducing differentiation through removal of the negative transcriptional block (30). The fusion of *CHOP* with different genes generates products that are causative of the MLS. Under normal conditions, *CHOP* is induced by different types of noxious stimuli—DNA damage and endoplasmic

reticulum stress—conditions in which a normal differentiation program is put on hold, or outrightly inhibited, and making it possible for apoptotic mechanisms to ensue (31, 32). It is clear that the normal functions are altered by the addition of FUS (8–10). Considering that CHOP is not expressed under normal conditions and that it is a negative regulator of C/EBP α and C/EBP β , it is understood that normal C/EBP functions are subverted by the constitutive chimeras (9, 10). Our data support this model and suggest that FUS-CHOP targeting of promoters plays a role in this process. In particular, the most likely pathogenetic cause of MLS tumors is the inactivation of the lipogenic terminal differentiation programs induced by C/EBPs through interactions with the chimera.

Two mechanisms of action of trabectedin have been hypothesized—induction of DNA damage, impinging on specific and unique DNA repair pathways (16–19), and transcriptional interference (20–22). One of the most striking features of the drug is that DNA damage is generally believed to be very mild, compared with other compounds that form alkylating adducts with DNA. The potent activity of trabectedin in a large set of tumors

suggests that both pathways are involved and indeed the strong connections between these functions could very well influence each other. Indeed, the dose responses in sarcoma lines (Fig. 1B) show differential behavior; yet, even the fibrosarcoma HT1080 are sensitive in the low nanomolar range. A certain degree of apoptosis detailed in 402-91 cells (Fig. 5) is a further indication of toxicity. These are best seen at drug concentrations and at times above those sufficient to observe differentiation. It is possible that the DNA-damaging effects are essential to induce a general apoptotic response and that this might explain the pleiotropic effects. However, recent clinical data strongly suggest a more exquisite mechanism of action of trabectedin specifically in MLS tumors. We find that the drug induces a cascade of events leading to the activation of differentiation in 402-91 (Fig. 3) and in tumor cells *in vivo* (Fig. 6). There is a downplay of the chimera at the protein level, but kinetic and dose-response analyses indicate that removal of the chimera from promoters largely precedes FUS-CHOP overall decrease. The drug induces activation of the C/EBPs (Fig. 2), master regulators of terminal differentiation, causing morphologic changes *in vitro* (Fig. 3) and *in vivo* (Fig. 6). Thus, trabectedin is a specific

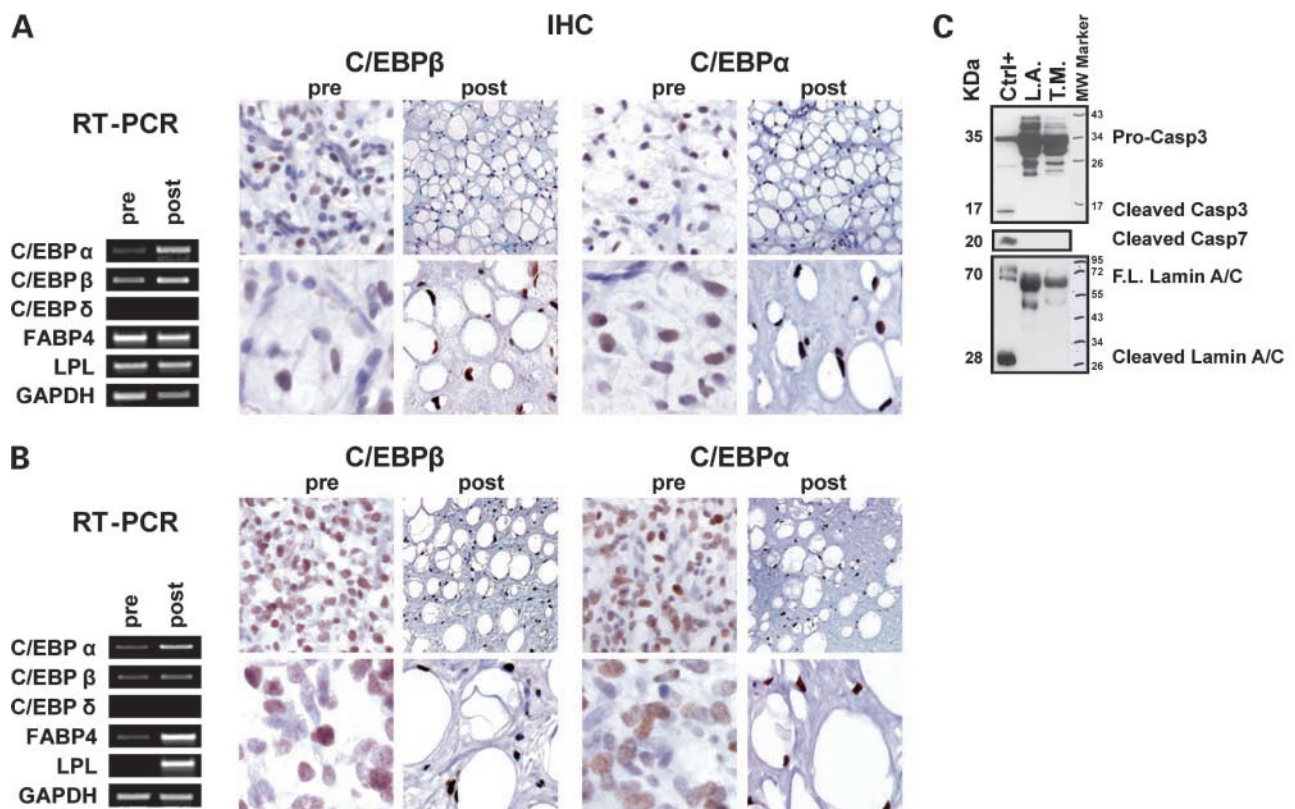


Figure 6. Trabectedin treatment leads to differentiation of MLS tumors. **A**, patient LA (usual-type MLS, type 2 chimera). Semiquantitative RT-PCR analysis and immunophenotyping performed on samples obtained from patients before and after trabectedin treatment. *Left*, RT-PCR analysis for genes involved in adipogenesis. Immunohistochemistry (IHC) was done with antibodies specific against C/EBP β and C/EBP α . Bottom panels are a higher magnification of the top panels. **B**, same as **A**, except that we used materials from patient TM (RCLS, type 1 FUS-CHOP). **C**, total extracts obtained from LA and TM patients were subjected to Western blot with antibodies against the apoptosis markers. As a positive control for the markers, we used extracts of HeLa cells treated with Adriamycin.

prodifferentiative compound for MLS cells. Consistent with this, tumor responses *in vivo* were marked by cellular depletion with no obvious sign of apoptosis (Fig. 6) and evidence of, or increase in, mature lipoblasts.

There is an additional important level of selectivity in the cellular systems considered: Trabectedin acts on type 1, not on the longer type 8, chimeras *in vitro*. Like the two patients analyzed here, most responders in the clinical studies carried type 1 or 2 chimeras, which were previously shown to be biologically equivalent (33). On the contrary, patients with the least common, longer type 3 translocation did not respond to trabectedin (13). This is mirrored in our study by the differential behavior of 1765 cells with the longer type 8 fusion. Our data suggest that 402-91 and 1765 are blocked at a different stage of differentiation, with the former being more differentiated, as evidenced by higher basal levels of C/EBP β and LPL. In turn, this is apparently due to a different genomic strategy of the chimeras, as shown by gene targeting in our ChIP analysis and expression data. We therefore propose a model in which the shorter types 1 and 2 chimeras allow cellular progression to a later stage of differentiation, which immediately precedes terminal differentiation, whereas types 3 and 8 arrest maturation at an earlier stage that is not susceptible to switch on master genes of cell cycle exit and differentiation such as C/EBP α . Along this line of reasoning, it will be interesting to compare gene expression profiles of the different cell lines and, possibly, of patients with different chimeras.

The fine molecular details causing the observed elimination of FUS-CHOP from the targeted promoters is unclear. The DNA-binding capabilities of the drug might enable it to displace the fusion by competition and the chimera, inappropriately released from DNA, could in the long term be prone to degradation. An indication along this line is the fact that a DNA-binding defective analogue carrying an OH to H substitution in the active drug site shows little activity on 402-91 cells.⁶ On the other hand, it is unclear why trabectedin would allow the full binding and activity of normal C/EBP β and C/EBP α dimers to their adipogenic targets. This is best exemplified by the recruitment of C/EBP β on the C/EBP α promoter (Fig. 4), which is expected based on past studies on the C/EBP α promoter functionality (7). It should be noted that the C/EBP β promoter is already active transcriptionally, both in 402-91 and in MLS tumors *in vivo*, and the drug improves transcriptional efficiency; however, it is not clear whether by improving elongation and/or mRNA stability. Subtle differences in binding of the different C/EBP members and with the FUS-CHOP chimera would have to be invoked in a competition-type of scenario. Clearly, establishing the genomic binding sites of FUS-CHOP and of the drug is vital but beyond our possibilities at the moment. Appropriate antibodies in genome-wide ChIP-based assays are required to shed light on this.

⁶ M. D'Incalci, unpublished data.

Finally, our data have additional relevant clinical implications. The first concerns the development of analogues with MLS-specific prodifferentiative behaviors, with minimal toxicity on other cell types. Essentially, the 402-91 cells can be used as a readout system to aim at widening the window between the prodifferentiative and proapoptotic effects. In clinical terms, that would help increase the therapeutic efficacy and decrease toxicity. The second relates to peroxisome proliferator activated receptor γ , a nuclear receptor critical for adipocyte differentiation (34). It is already present in MLS tumor cells and indeed remains so after treatment with trabectedin (not shown). Intriguingly, histologic changes similar to those observed with trabectedin were reported in MLS patients treated with troglitazone, a peroxisome proliferator activated receptor γ agonist drug (35), although its antitumor activity has not been established. Therefore, the assays shown here could help develop combinatorial strategies with peroxisome proliferator activated receptor γ agonists. A third potential implication is based on the knowledge that translocations involving C/EBP genes and the IGH locus are causative of some acute lymphoblastic leukemias (28, 36). Although the mechanism seems to be different, leading to dysregulation of the protein, and not to a fusion oncogene, the specific antitumor activity of trabectedin for the C/EBP pathway could also be assayed in these cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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