10-10-2003

Transcriptional inhibition of type I collagen gene expression in scleroderma fibroblasts by the antineoplastic drug ecteinascidin 743.

Natalia Louneva  
*Thomas Jefferson University*

Biagio Saitta  
*Thomas Jefferson University*

David J Herrick  
*Thomas Jefferson University*

Sergio A. Jimenez  
*Thomas Jefferson University*

Follow this and additional works at: [https://jdc.jefferson.edu/medfp](https://jdc.jefferson.edu/medfp)

Part of the [Rheumatology Commons](https://jdc.jefferson.edu/medfp)

**Let us know how access to this document benefits you**

**Recommended Citation**


https://jdc.jefferson.edu/medfp/183

This Article is brought to you for free and open access by the Jefferson Digital Commons. The Jefferson Digital Commons is a service of Thomas Jefferson University's [Center for Teaching and Learning (CTL)](https://www.jefferson.edu/ctl). The Commons is a showcase for Jefferson books and journals, peer-reviewed scholarly publications, unique historical collections from the University archives, and teaching tools. The Jefferson Digital Commons allows researchers and interested readers anywhere in the world to learn about and keep up to date with Jefferson scholarship. This article has been accepted for inclusion in Department of Medicine Faculty Papers by an authorized administrator of the Jefferson Digital Commons. For more information, please contact: JeffersonDigitalCommons@jefferson.edu.
Transcriptional Inhibition of Type I Collagen Gene Expression in Scleroderma Fibroblasts by the Antineoplastic Drug Ecteinascidin 743*

Natalia Louneva†, Biagio Saitta‡§, David J. Herrick¶ and Sergio A. Jimenez||

From the †Department of Medicine, Division of Rheumatology, and the ¶Department of Dermatology and Cutaneous Biology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107.

We previously showed that COL1A1 expression is up-regulated at the transcriptional level in systemic sclerosis (SSc) fibroblasts and that the CCAAT-binding factor (CBF) is involved in this increased expression. Ecteinascidin 743 (ET-743) is a chemotherapeutic agent that binds with sequence specificity to the minor groove of DNA and inhibits CBF-mediated transcriptional activation of numerous genes. Therefore, we examined the effects of ET-743 on the increased COL1A1 expression in SSc fibroblasts. The drug caused a potent and dose-dependent inhibition of type I collagen biosynthesis, which reached 70–90% at 700 pM without affecting cell viability. The same drug concentration caused 60–80% reduction in COL1A1 mRNA levels. The stability of the corresponding transcripts was not affected. In vitro nuclear transcription assays demonstrated a 54% down-regulation of COL1A1 transcription. Transient transfections with COL1A1 promoter constructs containing the specific CBF binding sequence into SSc cells previously treated with 700 pM ET-743 failed to show an effect on COL1A1 promoter activity. Furthermore, ET-743 did not affect the binding of CBF or Sp1 transcription factors to their cognate COL1A1 elements. However, treatment with 700 pM ET-743 of stably transfected NIH 3T3 cells expressing a human type II procollagen gene under the control of the human COL1A1 promoter caused a greater than 50% reduction in the production of type II procollagen and a similar decrease in the corresponding type II procollagen transcripts. These results indicate that ET-743 is a potent inhibitor of COL1A1 transcription. However, this effect cannot be explained by a direct effect on CBF binding to the COL1A1 promoter. Although the exact mechanisms responsible for the transcriptional inhibition of COL1A1 by ET-743 are not apparent, our observations suggest that the drug may be an effective agent to decrease collagen overproduction in SSc and other fibrotic diseases.

* This work was supported by National Institutes of Health (NIH) Grant AR19816 (to S. A. J.) and by a Translational Research Grant from Thomas Jefferson University (to B. S.). We thank Dr. Darwin J. Prockop for the COL1A1/COL2A1 NIH 3T3 stably transfected cells. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Present address: Coriell Institute for Medical Research/UMDNJ, 403 Haddon Ave., Camden, NJ 08103.

‡ Present address: Department of Medicine, Division of Rheumatology, Thomas Jefferson University, Philadelphia, PA 19107-5541. Tel.: 215-503-5042; Fax: 215-923-4649; E-mail: Sergio.Jimenez@jefferson.edu.

The abbreviations used are: SSc, systemic sclerosis; CBF, CCAAT-binding factor; ET-743, ecteinascidin 743; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CAT, chloramphenicol acetyltransferase; WT, wild type; MT, mutant; NF, nuclear extracts; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Inhibition of Fibroblast COL1A1 Expression by Ecteinascidin 743

40401

The activity of the COL1A1 promoter from different species including chickens, rats, mice, and humans (14) further emphasizes its importance in the regulation of collagen gene expression. Thus, selective targeting and inhibition of CBP-mediated activation of the type I collagen genes could provide a successful approach to control the process of fibrosis.

Recent studies of ecteinascidin 473 (ET-473) have shown its ability to be a selective inhibitor of transcription of several genes and that CBF is one of its targets (15, 16). ET-473 is a DNA-alkylating agent extracted from the marine tunicate Ecteinascidia turbinata that has demonstrated potent antineoplastic activity against various human tumors including ovarian and colorectal cancers and advanced sarcoma (17). Numerous clinical trials are currently under way, and the mechanism of antineoplastic action of this unique agent is under intense investigation. Although the precise mechanisms of ET-473 action have not been elucidated completely, it appears that it interferes with the function of CBP in the transcription of numerous genes. There is evidence that ET-473 does not directly block CBP binding to DNA, but instead, it appears to interfere with the ability of CBP to recruit and associate with histone acetylases that are typically required for full gene promoter function. This sequence-specific effect has been demonstrated in genes such as the multidrug resistance gene, MDR-1, and the gene encoding heat shock protein 70, HSP70 (15, 16).

Our previous studies were aimed at defining the role of CBF and other transcription factors in type I collagen regulation under normal conditions and in pathologic fibrosis (9). Here, we have extended these studies by examining the inhibitory effects of ET-473 on type I collagen gene expression in SSc, the prototype of fibrotic diseases. Our data show that the drug causes potent inhibition of type I collagen biosynthesis and that these effects are largely due to a reduction of COL1A1 transcription. Our results provide strong support to the notion that such a gene transcription targeting approach may prove promising for the treatment of SSc, a currently fatal condition, and of numerous other diseases characterized by pathologic fibrosis.

EXPERIMENTAL PROCEDURES

Culture of Human Dermal Fibroblasts—Dermal fibroblast cell lines were established from patients with diffuse SSc of recent onset and rapid progression, as previously reported (4, 5, 9). All patients studied were followed at the Scleroderma Center of Thomas Jefferson University and fulfilled published criteria for the classification of SSc (18). Only untreated cases were studied to avoid spurious results caused by various therapeutic agents. In all cases, the cell lines were obtained from full thickness skin biopsies surgically excised from the leading edge of clinically apparent SSc lesions. Control fibroblasts were obtained from age- and sex-matched individuals undergoing surgical procedures for unrelated purposes. For all studies, only early passage fibroblasts (≤10) were employed to avoid possible phenotypic changes during extended subculture. Cells were routinely maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Invitrogen), 1% vitamins, 2 mM L-glutamine, and antibiotics and were plated in 6-well plates, grown to confluence, and then treated with 700 nM ET-743 for 24 h. At the end of the incubation, cells were washed with cold saline and 600 μl of serum-free medium containing the same ET-743 concentrations was added. The cultures were incubated with 4 μCi/mL 14C-labeled proline (ICN Biomedical, Costa Mesa, CA). After 3 h, the media were harvested from each well, and the cells were trypsinized and counted. Aliquots of media were taken for immunoprecipitation. The volume of medium for each immunoprecipitation assay was normalized according to the number of cells present in each well. The medium was incubated with the type I collagen rabbit polyclonal antibody (Rockland, Gilbertsville, PA). A standard curve was established that related the inhibition of color development to the concentration of added human type I collagen. The concentration of type I collagen in the culture media was analyzed in duplicate and determined directly from the standard curve, which was linear between 30 ng/ml and 2 μg/ml type I collagen.

Northern Hybrdizations—Fibroblast collagen biosynthesis was examined essentially as described previously (20). Briefly, normal and SSc fibroblasts were plated in 6-well plates, grown to confluence, and then incubated with 700 nM ET-743 for 24 and 48 h, and then the media were changed and 600 μl of serum-free medium containing the same ET-743 concentrations was added. The cultures were labeled with 4 μCi/ml [3H]UTP (3000 Ci/mmol) for 24 h, and the antigen-antibody complexes were precipitated overnight with Protein G-agarose (Roche Applied Science). The precipitates were resuspended in 2× SDS sample buffer containing β-mercaptoethanol and boiled for 5 min. Samples were electrophoresed in 6% polyacrylamide gels, and the radioactive bands were visualized employing a Storm PhosphorImager and analyzed with ImageQuant version 5.1 software (Amersham Biosciences).

Northern Hybrdizations—Fibroblasts were grown to confluence in 100-mm dishes and treated with ET-473 under the same conditions as those used for the protein experiments. Total RNA was isolated by an RNeasy Mini Kit (Qiagen, Valencia, CA), and aliquots (6 μg/well) were electrophoresed on formaldehyde 1% agarose gels. The RNA was then transferred to Hybond N+ filters (Amersham Biosciences) and hybridized to 32P-radiolabeled human cDNA for COL1A1, COL2A1, and GAPDH. The filters were analyzed using densitometry equipment and software (ImageQuant version 5.1 software; Amersham Biosciences). The hybridization signals were normalized to those of GAPDH.

Assessment of Steady-state mRNA Levels and mRNA Stability—Confluent SSc fibroblasts were incubated for 24 h with or without ET-473 (700 pm), and mRNA synthesis was inhibited by the addition of 1 μg/ml a-amanitin as described previously (21). Cells were harvested every 6 h following the addition of α-amanitin, and total RNA was isolated and analyzed by Northern hybridizations using specific cDNA probes for COL1A1 and GAPDH. All data were normalized for the levels of GAPDH mRNA.

In Vitro Nuclear Transcription Assay—The transcription rates of COL1A1 were measured by an in vitro nuclear run-off assay. Confluent SSc fibroblasts were cultured in T-175 flasks in the presence of isopropl alcohol and then treated with 700 nM ET-473 for 24 h. The cells were harvested 6 h following the addition of α-amanitin, and total RNA was isolated and analyzed by Northern hybridizations using specific cDNA probes for COL1A1 and GAPDH. All data were normalized for the levels of GAPDH mRNA.

The transcription rates of COL1A1 were measured by an in vitro nuclear run-off assay. Confluent SSc fibroblasts were cultured in T-175 flasks in the presence of isopropl alcohol and then treated with 700 nM ET-473 for 24 h. The cells were harvested 6 h following the addition of α-amanitin, and total RNA was isolated and analyzed by Northern hybridizations using specific cDNA probes for COL1A1 and GAPDH. All data were normalized for the levels of GAPDH mRNA.
untreated cells. The results are shown as means ± S.E. of three independent experiments. Open bars, control; dotted bars, 70 μM ET-743; horizontally striped bars, 140 μM ET-743; criss-crossed bars, 700 μM ET-743; hatched bars, 3 mM ET-743. B, photomicrograph of cultured fibroblasts; −, untreated cells; +, cells treated with 700 μM ET-743 for 72 h.

A

B

FIG. 1. Effects of ET-743 on normal and SSc fibroblast viability and morphology. Cytotoxicity of ET-743 was tested by a MTT-based cytotoxicity assay as described under “Experimental Procedures.” A, values shown represent the percentage of viable cells following 24, 48, or 72 h of treatment with a given drug concentration compared with untreated cells. The results are shown as means ± S.E. of three independent experiments. Open bars, control; dotted bars, 70 μM ET-743; horizontally striped bars, 140 μM ET-743; criss-crossed bars, 700 μM ET-743; hatched bars, 3 mM ET-743. B, photomicrograph of cultured fibroblasts; −, untreated cells; +, cells treated with 700 μM ET-743 for 72 h.

Transient Transfections of Human Dermal Fibroblasts—Cells were transfected employing the FuGene 6 kit (Roche Applied Science) as described previously (9). Control and SSc fibroblasts were plated and cultured to 85% confluence in 60-mm dishes and then transfected with different cell lines. The concentration of CAT was determined directly from the standard curve of CAT protein. The protein concentration of the extracts was determined using the BCA protein assay kit (Pierce). Radioactive probes were generated by phosphorylating the 5′-ends with polynucleotide kinase (Roche Applied Science) and [γ-32P]ATP (ICN, Costa Mesa, CA). Binding reactions containing 10 μg of nuclear extracts, 4 μg of poly(dI-dC), and 5–8 × 104 cpm (−0.2 ng) of radiolabeled probes were incubated for 15 min at room temperature in a buffer containing 40 mM KCl, 15 mM HEPES, pH 7.9, 1 mM EDTA, 0.5 mM dithiothreitol, 1 mM MgCl2, and 5% glycerol in a total volume of 25 μl. To determine the in vitro effects of ET-743 on CBP and Sp1 binding to their corresponding binding sites in the COLIA1 promoter, the probes were incubated with either 700 μM or 3 μM of the drug for 10 min, and then nuclear extracts were added to the mixture for an additional 15 min. To test the specificity of transcription factor binding to the DNA probes, competition experiments using a 150-fold molar excess of wild type (WT) or mutated (MT) oligonucleotides were performed. Oligonucleotides containing consensus WT sequences for Sp1 and CBP and
Inhibition of Fibroblast COL1A1 Expression by Ecteinascidin 743

**Experimental Procedures.** Confluent fibroblasts were incubated in T-175 flasks in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and L-ascorbic acid (40 µg/ml) with or without 700 pM ET-743 for 24 h, and then α-amanitin (1 µg/ml) was added to arrest further transcription. Cells were harvested at the intervals shown following the addition of α-amanitin, total RNA was isolated, and equal amounts were analyzed by Northern hybridizations and densitometry as described under “Experimental Procedures.” Densitometric analyses are shown in arbitrary densitometric units, and the results represent two independent experiments.

**Fig. 4. Effects of ET-743 on COL1A1 mRNA steady-state levels.** Confluent cultures of two normal (N1 and N2) and two SSc (S2 and S3) cell lines were incubated for 48 h with or without 700 pM ET-743, and COL1A1 mRNA levels were determined by Northern hybridization analysis. C, representative autoradiograph of Northern blot. D, densitometric analysis of Northern blots. The results represent two independent experiments, and values are expressed as a percentage relative to untreated fibroblasts.

**Fig. 5. Effects of ET-743 on the stability of the COL1A1 transcripts.** Confluent fibroblasts were incubated in T-175 flasks in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and L-ascorbic acid (40 µg/ml) with or without 700 pM ET-743 for 24 h, and then α-amanitin (1 µg/ml) was added to arrest further transcription. Cells were harvested at the intervals shown following the addition of α-amanitin, total RNA was isolated, and equal amounts were analyzed by Northern hybridizations and densitometry as described under “Experimental Procedures.” Densitometric analyses are shown in arbitrary densitometric units, and the results represent two independent experiments.

**Western Blot Analysis—**For Western blot analysis of type II procollagen production by control or ET-743-treated NIH 3T3 stably transfected cells, the cell culture medium was removed and precipitated in a buffer containing 0.4 M NaCl, 25 mM EDTA, 0.4% NaN₃, 0.1 M Tris-HCl, pH 7.4. Equal aliquots of the samples were boiled for 5 min in SDS buffer with 1% β-mercaptoethanol and then applied to 6% polyacrylamide gels and electrophoresed as described previously (19, 20). The separated proteins were transferred to nitrocellulose membranes, and following transfer, the membranes were stained with 0.5% Ponceau S to assure equal loading and transfer of the proteins. The transferred proteins were reacted for 2 h with a 1:500 dilution of SJ 441 antibody, which is a polyclonal rabbit antibody specific for the COOH-terminal telopeptide of human type II collagen (28). After incubation with horseradish peroxidase-conjugated anti-
Effect of ET-743 on Collagen I Production and Biosynthesis

The effects of ET-743 on collagen I expression were examined in four different cell lines of SSC and two different cell lines of normal fibroblasts treated for up to 72 h with 70, 140, or 700 pM ET-743. The results are shown in Fig. 2. ET-743 reduced type I collagen production in both types of cells in a dose-dependent manner. At concentrations of 70 and 140 pM, ET-743 collagen production was reduced by an average of 50 and 65%, respectively, in four SSC cell lines (S1–S4) and by an average of 30 and 40%, respectively, in two normal fibroblast cell lines (N1 and N2). The higher ET-743 concentration (700 pM) had a more potent inhibitory effect (~70–90%) in both normal and SSC fibroblasts. The experiments were carried out in triplicate, and the variation between samples was 5–10%. Biosynthetic studies in SSC fibroblasts treated with 70, 140, 700, and 3000 pM ET-743 showed a strong inhibition of production of newly synthesized type I procollagen (Fig. 3A). At a concentration of 700 pM, ET-743 caused an 85% inhibition during a 24-h treatment (Fig. 3A, lane 4) and greater than 90% inhibition during a 48-h treatment (Fig. 3A, lane 9). Studies using 700 pM ET-743 were also performed in other SSC (Fig. 3D) and normal fibroblast (Fig. 3C) cell lines with similar results.

ET-743 Down-regulation of COL1A1 Steady-state mRNA Levels—To investigate the effects of ET-743 on COL1A1 mRNA levels, confluent cultures of one SSC fibroblast cell line (S1) were treated for 48 h with 70, 140, and 700 pM ET-743. A 75% reduction of COL1A1 transcripts was observed during 48-h treatment with 700 pM ET-743 (Fig. 4, A and B), whereas the control GAPDH mRNA levels were not affected. Two other SSC fibroblast cell lines (S2 and S3) and two normal fibroblast cell lines (N1 and N2) were incubated with 700 pM ET-743, and COL1A1 mRNA was analyzed following 48 h of treatment (Fig. 4, C and D). A 58% reduction of COL1A1 was observed in the two SSC fibroblast cell lines (Fig. 4C, lanes 7 and 8). Inhibition of up to 40% was observed for COL1A1 mRNAs in the two normal fibroblast cell lines (Fig. 4D, lanes 3 and 4). Further studies showed that ET-743 did not affect the stability of the COL1A1 mRNA in either normal or SSC dermal fibroblasts (Fig. 5).
ET-743 Causes a Reduction in COL1A1 Transcription Rates—To determine whether the changes in COL1A1 mRNA levels resulted from the modulation of the transcription rates of the corresponding gene, \textit{in vitro} transcription assays were performed in SSc fibroblasts treated with 700 pM ET-743 (Fig. 6). Three experiments were performed using nuclei from SSc cells. The results showed that COL1A1 transcription was down-regulated by an average of 50%. The level of COL1A1 transcription inhibition was similar to the level of inhibition of mRNA steady-state levels found by Northern hybridization (see Fig. 4). The transcription of the control GADPH was not affected by ET-743 treatment.

COL1A1 Promoter Activity Is Not Affected by ET-743 in Transient Transfections—Transient transfection experiments were performed to determine whether the COL1A1 promoter region that contains the CBF response element was involved in the transcriptional down-regulation of COL1A1 activity observed in the \textit{in vitro} nuclear run-off assays. Various COL1A1 promoter constructs were transfected into normal and SSc fibroblasts, and then the cells were treated with 700 pM ET-743. When concentrations of the drug shown in the \textit{in vitro} transcription experiments to cause inhibition of COL1A1 transcription (700 pM) were employed, the promoter activity of the COL1A1 constructs spanning −804 to −84 bp was not down-regulated (Fig. 7).

To determine whether the transcriptional down-regulation of COL1A1 expression by ET-743 was due to competition of CBF binding to its cognate elements within COL1A1, the interaction of ET-743 with gene promoter sequences known to be involved in the regulation of COL1A1 transcriptional activity was investigated. Also, the effects of ET-743 on binding of the regulatory transcription factors Sp1 and CBF to COL1A1 proximal promoter sequences were examined. Nuclear extracts from untreated and ET-743 (700 pM)-treated fibroblasts were examined by an electrophoretic gel mobility shift assay with consensus oligonucleotides for Sp1 and CBF and with DNA fragments from the COL1A1 promoter containing Sp1 or CBF binding sites. As shown in Fig. 8, nuclear extracts from fibroblasts treated with ET-743 displayed the same level of Sp1 and CBF binding activity to consensus Sp1 or CBF oligonucleotides as untreated cells (Fig. 8, A and C). The specificity of the binding was confirmed by specific displacement and inhibition of binding employing an excess of unlabeled oligonucleotides. Similar binding assays with oligonucleotides corresponding to Sp1 and CBF binding sites from the COL1A1 promoter also failed to show any differences between untreated and ET-743-treated cells (data not shown).

A direct interaction of ET-743 with Sp1 or CBF binding sites preventing or blocking the subsequent binding of the transcription factors was then examined. For this purpose, oligonucleotides containing Sp1 and CBF consensus sequences were incubated \textit{in vitro} with ET-743, and DNA-protein complex formation was analyzed by an electrophoretic gel mobility shift assay. As shown in Fig. 8, B and D, ET-743 at a 700 pM concentration and at 3 nM did not change the pattern of transcription factor binding obtained without the drug. The results for Sp1 consensus oligonucleotide are shown in Fig. 8B, and those for CBF are shown in Fig. 8D. Similar results were obtained with oligonucleotides corresponding to the Sp1 and CBF sequences from the COL1A1 promoter (data not shown).
COL1A1 Promoter Activity Is Affected by ET-743 in Stably Transfected NIH 3T3 Cells—Experiments with stably transfected cells were performed to confirm the transcriptional down-regulation of COL1A1 activity by ET-743 observed in the in vitro nuclear run-off assay. NIH 3T3 cells stably transfected with a cosmid containing a chimeric COL1A1/COL2A1 gene, encompassing a 2-kb SphI/HindIII fragment from the 5′-end of the human COL1A1 gene, which included the promoter region (−560 bp), the first exon (222 bp), and 1218 bp of the first intron, and a full-length type II procollagen gene were employed. The stable transfectants were prepared by Ala-Kokko et al. (26) and express effectively the cartilage-specific type II procollagen gene under the control of COL1A1 regulatory elements. Confluent cultures of NIH 3T3 cells expressing type II procollagen were treated with 0.7 nM ET-743 for 48 h, and medium proteins were assayed for type II procollagen by Western blot (Fig. 9). Separate experiments indicated that these conditions did not cause cytotoxic effects on the stable transfectants (data not shown). Collagen II protein production by these cells displayed a significant decrease (−50%) in response to ET-743 treatment (Fig. 9A, lanes 3 and 5) compared with control untreated cells (Fig. 9A, lanes 4 and 6). In order to confirm that the data at the transcription level, total RNA was isolated from treated and untreated cells, and Northern hybridizations with a COL2A1 cDNA were performed (Fig. 9, C and D). The level of inhibition of COL2A1 mRNA was similar to the level of inhibition of type II procollagen production found by Western blot, and both levels of inhibition were close to those of COL1A1 transcription inhibition observed in the nuclear run-off assays (Fig. 6).

DISCUSSION

Here, we describe the first demonstration that the antineoplastic drug ET-743 is a potent inhibitor of the expression of COL1A1 in normal human dermal fibroblasts and, more importantly, that the drug also inhibits the exaggerated overexpression of this collagen gene in SSF fibroblasts. These effects were apparent both at the protein (Figs. 2 and 3) and at the mRNA levels (Fig. 4). Notably, these effects occurred without affecting the viability and morphology of normal and SSc fibroblasts (Fig. 1). We also show that the drug is effective at low concentrations (70–700 pm), much lower than those required to exert antineoplastic effects (15, 16). With a run-off assay employing isolated fibroblast nuclei, we determined that at this low concentration (700 pm), ET-743 has a direct transcriptional effect on COL1A1 (Fig. 6).

Previous studies on the mechanisms of the antineoplastic effects of ET-743 demonstrated that the drug at much higher concentrations than those employed here (10–50 nM) inhibits promoter activation of MDR-1 and HSP-70 genes (15, 16). It was also shown that ET-743 at high concentrations (3 nM) suppressed okadaic acid induction of the JUN B CAAT box promoter and that CBF and possibly Sp1 were involved in this process (23). Therefore, we analyzed whether CBF or Sp1 are involved in the transcriptional inhibition of ET-743 on COL1A1. We performed gel shift analysis of the interaction of Sp1 and CBF oligonucleotides to examine the binding activity of Sp1 and CBF present in fibroblast nuclear extracts from untreated or ET-743 (700 pm)-treated cells. Nuclear extracts from treated fibroblasts yielded normal CBF and Sp1 protein binding activities, since there was not any detectable quantitative or qualitative change in the Sp1 and CBF complexes detected by an electrophoretic gel mobility shift assay (Fig. 8). However, it is necessary to mention that these experiments may be limited by the size or sequence of the oligonucleotides employed and, according to Hurley et al. (24), by the stability of ET-743-DNA complexes. The stability of these complexes is governed by DNA target sequences, since under some conditions the drug is not capable of forming optimally stable bonds with its corresponding recognition sites in the DNA leading to the formation of unstable products. The same authors in other work (25) demonstrated that ET-743 can migrate from a nonfavored bonding sequence (5′-AGT) to the favored sequence site (5′-AGC) and that the ET-743-AGT adduct is less stable, displays a higher degree of molecular motion, and produces dif-

FIG. 9. Western and Northern blot analysis of type II collagen and type II procollagen mRNA levels in stably transfected NIH 3T3 cells expressing the human type II procollagen gene under control of the COL1A1 promoter. Cells were treated 48 h with 0.7 nM of ET-743, A, immunoblotting with anti-collagen II antibody of culture medium Lane 1, type II collagen as positive control; lane 2, medium from NIH 3T3 cells that were not transfected as negative control; lanes 3 and 4, medium from ET-743-treated (lane 3) and untreated cells (lane 4) from experiment 1. Lanes 5 and 6, medium from ET-743-treated (lane 5) and untreated (lane 6) cells from experiment 2. Equal loading of the gel was confirmed by Ponceau S staining of the transfer membrane (data not shown). B, densitometric analysis of Western blots shown in arbitrary densitometric units as means ± S.E. of three independent experiments. C, a representative Northern hybridization of total RNA from ET-743-treated and untreated NIH 3T3 cells with a type II procollagen cDNA. D, densitometric analysis of Northern blots shown in arbitrary densitometric units. The results represent the averages of two independent experiments, which differed from each other by less than 10%.
ferent conformational changes in DNA than the more stable alkylation product ET-743-AGC.

Our studies employing transient transfections of COL1A1 constructs containing CBF and Sp1 binding sites showed that in the picomolar concentration range ET-743 is unlikely to target CBF- or Sp1-mediated transcription. Therefore, the mechanisms responsible for the transcriptional down-regulation of COL1A1 caused by the drug do not appear to be related to competition or prevention of CBF or Sp1 binding to their corresponding sites within the collagen gene promoter. Thus, the putative mechanisms of the drug on COL1A1 transcription remain elusive but could include interactions of ET-743 with DNA sites close to transcription factor binding sites within COL1A1 or to sites recognized by other transcriptional factors. Alternatively, the observed effects could be due to reversible ET-743 alkylation products that, when formed at this low concentration of the drug, are not stable, and the drug could then interact with other preferable, more stable sequences. The experiments employing stably transfected NIH 3T3 cells expressing a COL1A1/COL2A1 hybrid gene indicate that ET-743 exerts a negative effect on COL1A1 transcription at very low concentrations (0.7 nM) without causing cytotoxic effects. A comparison of these results with those obtained employing transient transfections of the human dermal fibroblasts suggests that the drug may be more effective within a more permissive gene context, perhaps due to the formation of shorter gene sequences employed in the transient transfection experiments. Alternatively, it is possible that ET-743 might prevent association of histone acetylases with the gene, thus precluding its full promoter function. Therefore, our studies show that ET-743 displays transcriptional inhibition on COL1A1 at very low concentrations, an effect that cannot be explained appropriately by a direct CBF-mediated mechanism. Thus, although the precise mechanism(s) by which ET-743 affects COL1A1 transcription in normal and SSc dermal fibroblasts remains to be determined, the results described here indicate that the drug may represent a novel and potentially effective therapy for SSc and other fibrotic diseases.

Acknowledgment—The expert assistance of Kate Salmon in the preparation of the manuscript is gratefully acknowledged.

REFERENCES
Transcriptional Inhibition of Type I Collagen Gene Expression in Scleroderma Fibroblasts by the Antineoplastic Drug Ecteinascidin 743
Natalia Louneva, Biagio Saitta, David J. Herrick and Sergio A. Jimenez

doi: 10.1074/jbc.M301964200 originally published online July 24, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M301964200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 28 references, 12 of which can be accessed free at http://www.jbc.org/content/278/41/40400.full.html#ref-list-1