INHIBITION OF COLLAGEN GENE EXPRESSION IN SYSTEMIC SCLEROSIS DERMAL FIBROBLASTS BY MITHRAMYCIN

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ABSTRACT

Introduction. The antitumor antibiotic mithramycin is also a potent inhibitor of fibrosis following glaucoma surgery. This drug displays high affinity binding to GC rich sequences in DNA, including those present in the promoter of the gene encoding the \( \alpha_1 \) chain of type I collagen (COL1A1).

Objective. To evaluate the effects of mithramycin on COL1A1 expression in systemic sclerosis fibroblasts.

Methods. Confluent cultures of dermal fibroblasts from patients with recent onset diffuse systemic sclerosis were treated with mithramycin \textit{in vitro}. Cell viability and protein expression were examined using fluorescence and confocal imaging. Type I collagen production was analyzed by confocal imaging and metabolic labeling. COL1A1 messenger RNA levels and stability were assessed by Northern hybridizations and COL1A1 transcription was examined by transient transfections.

Results. Treatment of systemic sclerosis fibroblasts with 10 nM to 100 nM did not cause significant cytotoxicity. Type I collagen biosynthesis decreased 33-40\% and 50-70\% in cells cultured with 10 nM and 100 nM mithramycin, respectively. Mithramycin at 50 nM decreased COL1A1 mRNA levels by 40-60\%. The effects of mithramycin on collagen gene expression were mediated by transcriptional and post-transcriptional mechanisms as shown by a reduction of COL1A1 promoter activity and by a decrease in the stability of these transcripts, respectively.

Conclusions. Mithramycin causes potent inhibition of collagen production and gene expression in systemic sclerosis dermal fibroblasts \textit{in vitro} in the absence of cytotoxic effects. These results suggest that this drug may be an effective treatment for the fibrotic process which is the hallmark of systemic sclerosis.

INTRODUCTION

Systemic sclerosis is a disease of unknown etiology that is characterized by the excessive deposition of collagen and other extracellular matrix proteins in skin and several internal organs, microvascular fibroproliferative lesions, and humoral and cellular immunologic abnormalities (1-3). The precise mechanism(s) involved in the tissue fibrosis are unknown although this process is clearly responsible for most of the clinical manifestations of the disease. Indeed, the severity of systemic sclerosis symptoms and mortality are determined by the extent and the degree of tissue fibrosis. Extensive recent efforts have been devoted to the study of the mechanisms involved in the pathologic increase of collagen gene expression in systemic sclerosis. Although these have not been entirely elucidated, it has been shown that fibroblasts from affected skin in culture produce excessive amounts of various collagens (4) and display increased transcription rates of the corresponding genes (5). The modulation of collagen transcript stability may also be altered in systemic sclerosis as shown by the increased stability of collagen mRNA in systemic sclerosis dermal fibroblasts cultured in tridimensional lattices (6). The most abundant extracellular matrix protein in systemic sclerosis affected tissues is type I collagen. Type I
collagen is assembled from two $\alpha_1$ and one $\alpha_2$ chains that are encoded in COL1A1 and COL1A2, respectively. The transcriptional activity of the two genes is coordinated under most physiological and pathological conditions. The transcriptional regulation of these genes results from tightly regulated interactions between transcription factors and regulatory elements contained within the promoters and first introns of the collagen genes (4). It has been suggested that in the case of COL1A1 expression one of the most important regulatory transcription factors belongs to the Sp1 family of DNA binding proteins. It has been demonstrated that Sp1 plays an important role in the pathogenesis of fibrosis and increased Sp1 binding activity (7) and phosphorylation (8) have been observed in fibroblasts cultured from affected systemic sclerosis skin. Therefore, interference with Sp1 binding to its cognate elements in the COL1A1 gene may allow to modulate its increased transcriptional activity. One of the possible ways is to compete for the Sp1-specific GC-rich binding site in COL1A1. Previous studies have shown that drugs capable of binding to GC-rich regions of DNA such as mithramycin, doxorubicin, and mitoxantrone may inhibit the formation of the Sp1-DNA complex (9). The expression of collagen genes can also be regulated at the post-transcriptional level since changes in the stability of their corresponding mRNA also contribute to its turnover and steady-state levels. Indeed, it has been shown that cortisol and other glucocorticoids regulate $\alpha_1$(I) and $\alpha_2$(I) procollagen steady-state mRNA levels by decreasing the stability of their corresponding transcripts (10). Furthermore, interferon $\gamma$, one of the most potent inhibitors of collagen production, in addition to its transcriptional effects reduces the half life of $\alpha_1$(I) and $\alpha_1$(III) procollagen mRNA (11).

Given the important role of Sp1 in the regulation of type I collagen gene expression and its potential participation in the abnormally increased expression of collagen genes in systemic sclerosis, the aim of this study was to examine the effect of mithramycin, a potent inhibitor of Sp1 binding to DNA, on COL1A1 gene expression and transcriptional activity in fibroblasts from patients with systemic sclerosis. We also examined the effects of the drug on the stability of COL1A1 transcripts. The results show that mithramycin is a potent inhibitor of COL1A1 gene expression in systemic sclerosis dermal fibroblasts causing profound inhibition of the production of the corresponding protein. These effects appear to be mediated by both transcriptional and post-transcriptional mechanisms and render the drug a potentially effective therapeutic agent for the severe fibrotic process in systemic sclerosis.

**METHODS**

*Cell cultures.* Fibroblasts were obtained from biopsies from the leading edge of the affected forearm skin (volar surface) of patients with diffuse systemic sclerosis of recent onset as described previously (5,12). None of the patients had been treated previously with immunosuppressants or with drugs known to affect collagen metabolism. The cells were cultured in Eagle’s MEM supplemented with 10% fetal bovine serum (FBS), 1% vitamins, 2 mM glutamine and antibiotics (Cellgro, Mediatech, Inc., Hendron, VA). When the cells reached confluency, they were incubated at 37°C in a 5% CO$_2$ humidified atmosphere. The cell cultures were supplemented with 50 $\mu$g/ml ascorbic acid for 24 h prior to initiation of the experiments to optimize their level of collagen production. Fibroblasts were used for experiments between passages 4-8. Mithramycin (Sigma, St. Louis, MO) was dissolved in distilled water and the solution stored at 4°C until use. Initially, six systemic sclerosis and four normal cell lines were screened employing two concentrations of mithramycin ($10^{-5}$ M and $10^{-7}$ M). The results
obtained were similar for all cell lines. From these cell lines we examined 4 systemic sclerosis cell lines by confocal microscopy studies. From these four cell lines we selected the two systemic sclerosis cell lines which exhibited the highest levels of type I collagen production for more detailed study since we wished to examine the effects of the drug on pathologic fibroblasts which were clearly collagen overproducers.

**Cell viability assessment.** Equal numbers of systemic sclerosis fibroblasts were plated in 35 mm dishes and cultured to confluency in 10% MEM as described above. The cultures were then treated with various concentrations of mithramycin for 48 h. Cell counting was performed in duplicates using a fluorescence microscope. To visualize the nuclei the cells were labeled with Hoechst 33422 (1 μg/ml). Phase contrast and Hoechst-fluorescence images were acquired as described previously (13). An equal number of areas (10 fields, each 0.16 mm²) were examined in each dish. To determine cell viability, the morphology of the cells and the presence of nuclear condensation were evaluated, as described previously (13).

**Metabolic labeling for the estimation of collagen biosynthesis.** Systemic sclerosis fibroblasts were grown to confluency in Eagle’s MEM media supplemented with 10% FBS, 1% vitamins, 2 mM glutamine, antibiotics and 50 μg/ml ascorbic acid, and then they were treated with various concentrations of mithramycin. Following 5 h of incubation, 100 μg/ml β-aminopropionitrile and L[U-14C]-proline (ICN Biomed, specific activity: 247 mCi/mmol) were added and the incubations were continued for 24 h. β-aminopropionitrile was employed to prevent collagen crosslinking and to allow newly synthesized molecules to remain in the culture media. Aliquots of media were dialyzed to remove unincorporated radioactive proline. Total incorporation of L[U-14C]-proline into macromolecules was measured in a scintillation spectrometer. Aliquots of media and cell layers were pooled together and used for determination of collagenase-sensitive proteins employing a bacterial collagenase digestion assay as described previously (11). Two separate experiments each in duplicate were performed on two different cell lines. The labeled proteins in the media were also analyzed by SDS-polyacrylamide slab gel electrophoresis in 7.5% SDS-polyacrylamide gels under reducing conditions as described previously (12). Samples were electrophoresed for 5 h at 100V constant voltage. Following electrophoresis, the gels were processed for fluorography and exposed to X-Omat AR film (Eastman Kodak, Rochester, NY).

**Confocal imaging of collagen in control and mithramycin treated systemic sclerosis dermal fibroblasts.** Systemic sclerosis fibroblasts (four different cell lines in five separate experiments) were plated on poly-D-lysine-treated coverslips in 35-mm dishes at a density of 35,000 cells/dish and cultured for 24 h prior to mithramycin treatment. Various concentrations of mithramycin were added and the cells were cultured for 48 h and then prepared for analysis. The fibroblasts were fixed with 3% paraformaldehyde and then permeabilized with digitonin. Following blocking nonspecific binding with 1.5% goat serum and 1% bovine serum albumin for 1 h, the cells were incubated with a 1:200 dilution of an affinity-purified anti-type I collagen polyclonal antibody which exhibits no cross-reactivity to types II, III, IV, V and VI collagens (Rockland, PA). A fluorescein conjugated secondary antibody was applied along with rhodamine-labeled phalloidin to stain F-actin, the polymeric form of actin. The coverslips were mounted on slides using antifade reagent (SlowFade Light Antifade Kit, Molecular Probes, Eugene, OR). Fluorescence was visualized using a laser scanning confocal microscope system (BioRad Radiance 2000 confocal system coupled to an Olympus IX70 microscope, 40X oil objective,
1.35NA) equipped for dual excitation and emission (KrAr laser illumination at 488 nm for FITC and 568 nm for rhodamine). Fluorescence of the FITC-conjugated anti-rabbit IgG was visualized as green color and that of the rhodamine-labeled phalloidin as red color. To quantitate the collagen immunostaining, 10 fields (3-10 cells/field) were taken from each sample. Data analysis was performed using custom-made software. A mask was drawn over every whole cell area and the average fluorescent intensity was calculated for each cell. Then the mean fluorescence of the cells was calculated and was normalized to the control.

Analysis of steady-state mRNA levels by Northern blot hybridizations. Fibroblasts in confluent culture were either maintained under control conditions or were treated with various concentrations of mithramycin (5, 10, 50, and 100 nM) for 48 h and total RNA extracted as described previously (12). Equal aliquots of the isolated total RNA were electrophoresed on formaldehyde-1% agarose gels and then transferred onto nitrocellulose membranes. The filters were hybridized to 32P-radiolabeled human complementary DNA (cDNA) for COL1A1. Equivalent loading and transfer were evaluated by hybridizations with a GAPDH cDNA. Results were quantified to determine the relative amounts of mRNA in duplicate samples using densitometry.

Transient transfections of systemic sclerosis fibroblasts with COL1A1 promoter-chloramphenicol acetyl transferase (CAT) constructs. Fibroblasts were grown to 70-80% confluence in 35-mm dishes. The cells were transfected with a total of 2.5 µg of various COL1A1 promoter constructs fused to the chloramphenicol acetyl transferase (CAT) reporter gene and 0.2 µg of a vector containing E. coli β-galactosidase cDNA (-pCMV β-galactosidase) using Fugene-6 kit (Roche Molecular Biochemicals, Indianapolis, IN). Expression of β-galactosidase was used to correct for transfection efficiency. The constructs tested are progressive 5’ deletions of the human COL1A1 promoter each cloned upstream of the CAT reporter gene (14). All constructs end at nucleotide +42 bp to assure the proper reading frame and their 5’ ends were at -804 bp, -174 bp and -84 bp. Four h later the fibroblasts were either maintained in control conditions or treated with various concentrations of mithramycin for additional 48 h. Cytoplasmic extracts were examined to determine CAT activity using [14C] chloramphenicol as substrate. Acetylated and non-acetylated forms of radiolabeled chloramphenicol were separated by thin layer chromatography and visualized by autoradiography.

Determination of mRNA stability. Confluent fibroblasts were cultured in the presence or absence of 50 nM mithramycin. The cultures received 1 µg/ml of the specific RNA polymerase II inhibitor α-amanitin (Sigma) 4 h following the addition of mithramycin to arrest further gene transcription. Total RNA was then extracted at 0, 6, 18 and 24 h following the addition of α-amanitin and processed by Northern hybridizations. The experiment was performed in duplicate except for the 24 h sample.

RESULTS

Effects of mithramycin on cell viability. The results of experiments employing various concentrations of mithramycin (1 nM to 1 µM) applied for 48 h to confluent systemic sclerosis
fibroblast cultures on cell viability revealed that 1 µM mithramycin decreased the number of cells by over 80% (82.5±7.5%; n=4), whereas 10 nM, 50 nM or 100 nM did not cause any detectable change in cell viability (Figure 1). Concentrations of 50 nM and 100 nM did not cause any detectable cytotoxicity (data not shown). Phase contrast image analysis showed that treatment with 1 µM mithramycin altered the shape of the cells, which shrunk and lost their cytoplasm, although the nuclear staining with Hoechst did not indicate the occurrence of apoptosis. No change in cell shape was observed using 10 nM mithramycin (Figure 1) or 50 nM or 100 nM mithramycin (data not shown).

**Evaluation of type 1 collagen accumulation by confocal imaging following mithramycin treatment.** In immunocytochemistry experiments, evaluation of the mithramycin effect on type I collagen accumulation at the single cell level by confluent cultures of systemic sclerosis dermal fibroblasts revealed that 48 h of treatment with 3 nM, 10 nM and 100 nM of mithramycin decreased the type I collagen levels by about 21%, 33% and 50%, respectively (Figure 2A and B). The differences between cells treated with the three concentrations of mithramycin and untreated cells were statistically significant (Figure 2B). However, no significant change was obtained in the F actin level in the cells treated with mithramycin (not shown).

**Evaluation of type 1 collagen biosynthesis by metabolic labeling following mithramycin treatment.** Metabolic labeling with L[U-14C]-proline of confluent fibroblast cultures incubated under control conditions or in the presence of 1 nM, 10 nM or 100 nM of mithramycin revealed that newly synthesized collagen determined in pooled media plus cell lysates by a specific collagenase digestion assay decreased by 30% following 10 nM mithramycin treatment and by 53% following administration of 100 nM mithramycin compared to untreated control cells. These differences were statistically significant (Figure 3A). The inhibitory effects of mithramycin on collagen production were confirmed by SDS gel electrophoresis of media proteins which showed a dose-related decrease in newly synthesized collagenous proteins following treatment with the drug (Figure 3B).

**Analysis of steady-state mRNA levels by Northern blot hybridization.** Northern hybridization of total RNA from untreated and mithramycin-treated cultures was employed to determine whether the results observed at the collagen biosynthesis and confocal microscopy analyses were accompanied by parallel changes in the steady-state levels of the corresponding COL1A1 transcripts. Two different cell lines were examined. As described in Methods the intensity of bands corresponding to COL1A1 mRNA was corrected by the intensity of the band corresponding to GAPDH mRNA to normalize for differences in loading and transfer during the procedure. The inset in Figure 4 shows a Northern hybridization analysis of results obtained with one cell line and the bar graph shows the individual values and the averages of results obtained with both cell lines following correction for the levels of GAPDH mRNA. The mithramycin concentrations employed were 5, 10, 50 and 100 nM. A dose-related decrease in COL1A1 transcripts upon treatment with mithramycin was observed. Mithramycin (50 nM) decreased the steady-state COL1A1 mRNA levels by 40 and 60% in the two cell lines examined when normalized to the expression of GAPDH (Figure 4).

**COL1A1 promoter activity following mithramycin treatment.** Transient transfection of systemic sclerosis fibroblasts with COL1A1 promoter-chloramphenicol acetyl transferase (CAT)
constructs followed by 50-100 nM mithramycin treatment revealed that mithramycin had an inhibitory effect on the transcriptional activity of the three COL1A1 promoter constructs which was mediated primarily by sequences contained in the region spanning -84 bp (Figure 5). Administration of 50 nM mithramycin resulted in approximately 30% inhibition of the promoter activity (not shown), whereas 100 nM mithramycin caused a promoter activity decrease by about 60% in cells that were transfected with the –84 bp COL1A1 construct.

**Messenger RNA stability following mithramycin treatment.** Confluent cultures of systemic sclerosis dermal fibroblasts were maintained in culture medium containing 10% fetal bovine serum and ascorbic acid or were incubated for 4 h with 50 nM mithramycin before α-amanitin addition. Total RNA was prepared from cells at the time of α-amanitin addition (time 0) and at 6, 18 and 24 h following α-amanitin addition and the samples were examined by Northern analysis. COL1A1 mRNA stability experiments showed that the stability of the α1(I) collagen transcripts decreased by 40% at 18 h compared with untreated cells (Figure 6A and B).

**DISCUSSION**

Mithramycin, a DNA binding antitumor antibiotic, has been used in the treatment of certain malignant testicular tumors and of hypercalcemia and hypercalciuria associated with advanced neoplasms. It has also been utilized to prevent the fibrotic reaction following glaucoma surgery. It has been established that mithramycin has a high binding affinity to GC-rich DNA sequences that are commonly found in the Sp1 transcription factor binding site of the promoter region of numerous genes such as c-myc, H-ras, dihydrofolate reductase, c-Ki-ras, COL1A1, and COL1A2 (7, 15-19). In one study, mithramycin inhibited myointimal proliferation after balloon injury of the rat carotid artery in vivo. This action occurred through inhibition of transcription of the c-myc proto-oncogene (20). Another study found that mithramycin (100 nM) inhibited COL1A1 activity by 60% in embryonic lung fibroblasts in culture (21). However, the results of these studies were recently retracted (21). Our previous transfection experiments have shown that the proximal COL1A1 promoter region extending to nt –174 upstream from the transcription initiation point resulted in maximal fibroblast-specific gene expression in systemic sclerosis fibroblasts (5). The proximal promoter is relatively G+C rich and contains two potential binding sites for Sp1. Functional assays indicated that Sp1 interacts with specific elements within the proximal promoter of the human COL1A1 and this interaction results in increased transcription from the promoter (7). The transcriptional activity of Sp1 can be modulated at the post-translational level by glycosylation or phosphorylation (22). Modulation of the level or of the transcriptional activity of Sp1, may play an important role in the collagen gene expression regulation and very likely also in the overproduction of collagen.

Our experiments showed that 10-100 nM mithramycin in confluent cell culture did not cause cytotoxicity effects although higher mithramycin concentrations resulted in cell death which did not appear to occur via apoptosis. Type I collagen production decreased by 33-40% in cells cultured with 10 nM, and by 50-70% in fibroblasts treated with 100 nM mithramycin. Mithramycin at 50 nM also decreased the steady-state mRNA levels of α1(I) collagen by 40-60% without affecting the mRNA levels of a control gene (GAPDH). The results of the experiments on the promoter activity of the COL1A1 gene indicated that the mithramycin effects
on collagen gene expression were exerted through a region of the proximal COL1A1 promoter spanning only -84 bp upstream of the initiation of transcription site. This region contains a GC region which is likely a Sp1 binding site. Although mithramycin caused a reduction of COL1A1 promoter activity, its effects on collagen gene expression also appeared to be exerted at a post-transcriptional level. Indeed, we found that COL1A1 mRNA stability in cultures treated with 50 nM mithramycin was decreased by 40% at 18 h compared with untreated cells. The reduction of COL1A1 transcript stability induced by mithramycin in systemic sclerosis fibroblasts demonstrated here is of particular relevance since previous studies have shown increased COL1A1 mRNA stability in tridimensional lattice cultures of these cells (6).

In summary, mithramycin at concentrations that are achievable in vivo employing currently recommended doses of the drug, causes potent in vitro inhibition of collagen production by systemic sclerosis fibroblasts and these effects appear to be due to a reduction of COL1A1 gene transcription as well as to a decrease in COL1A1 mRNA stability. Given these effects on collagen gene transcription and mRNA stability, we believe that mithramycin could prove to be a very effective therapy for systemic sclerosis and suggest that clinical trials should be conducted to examine its efficacy in patients affected by the disease.

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FIGURE LEGENDS

Figure 1. Effects of mithramycin on cell viability. Cell viability was examined as described in Materials and Methods employing various concentrations of mithramycin (10 nM to 1 µM) applied for 48 h to confluent systemic sclerosis fibroblast cultures in three experiments using three different cell lines each in duplicate. The results revealed that 1 µM mithramycin decreased the number of cells by over 80%, whereas 10 nM (or 50 nM; not shown) did not cause any significant change in cell viability. The phase contrast image shows that treatment with 1 µM mithramycin altered the shape of the cells which shrunk and lost their cytoplasm, although the nuclear staining with Hoechst did not indicate an apoptotic process. No change in cell shape was observed using 10 nM mithramycin.

Figure 2. Evaluation of type 1 collagen production in systemic sclerosis dermal fibroblast monolayer cultures by confocal imaging following mithramycin treatment. Type I collagen present in individual cells of systemic sclerosis fibroblast cultures was examined by immunomicroscopy and confocal microscopy imaging as described in Materials and Methods in five separate experiments using four cell lines. A. Fluorescence of the FITC-conjugated anti-rabbit IgG labeling type 1 collagen is shown in green and that of rhodamine-labeled phalloidin labeling actin in red in one illustrative experiment. B. Quantitative evaluation of the mithramycin effect on type I collagen production at the individual cell level revealed that 48 h of treatment with 10 nM and 100 nM of mithramycin decreased the protein level by about 33% and 50%, respectively. The results show the averages ± S.D. from five separate experiments with four different cell lines.

Figure 3. Evaluation of type 1 collagen biosynthesis by metabolic labeling following mithramycin treatment. Confluent monolayer cultures of systemic sclerosis fibroblasts were labeled with [14C]-proline under control conditions or under treatment with various concentrations of mithramycin and collagen biosynthesis was determined employing a specific collagenase assay in pooled media plus cell lysate samples as described in Materials and Methods. A. Collagen biosynthesis was decreased by about 30% using 10 nM of mithramycin and by about 50% using 100 nM mithramycin. The results of one experiment are shown. B. SDS gel electrophoresis of labeled proteins from the media revealed similar results with a 30% decrease in [14C] collagen following 10 nM mithramycin treatment and about a 50% decrease after 100 nM mithramycin administration. Two separate experiments each in duplicate were performed with similar results.

Figure 4. Analysis of steady-state mRNA levels by Northern blot hybridization. Confluent monolayer cultures of two systemic sclerosis dermal fibroblast cell lines were treated with various concentrations of mithramycin and total RNA extracted from the cultures was examined by Northern hybridization with cDNA for COL1A1 and GAPDH as described in Materials and Methods. Inset: Northern analysis of cell line 1. Bar graph: open circles, cell line 1; closed circles, cell line 2. Mithramycin (50 nM) decreased the steady state mRNA level of α1(I) by 40-60%.

Figure 5. COL1A1 promoter activity following mithramycin treatment. Transient transfection of systemic sclerosis fibroblasts with COL1A1 promoter-chloramphenicol acetyl transferase
(CAT) constructs followed by 50-100 nM mithramycin was performed as described in Materials and Methods. A. The results with -804 bp, -174 bp and -84 bp constructs are shown. The efficiency of transfection was corrected by assays of β-galactosidase activity measured by an enzymatic assay. These experiments were repeated 3 times each in duplicate. B. Densitometric analysis of three separate experiments each performed in duplicate. P values obtained comparing the transcriptional activity of each of the constructs in cells treated with mithramycin compared with the transcriptional activity of the same constructs in the untreated cells were statistically significant (p < 0.001 for each of the constructs).

Figure 6. mRNA stability after mithramycin treatment. The effects of mithramycin on COL1A1 mRNA stability were examined as described in Materials and Methods. A. Confluent cultures of systemic sclerosis dermal fibroblasts were either kept in culture medium containing 10% fetal bovine serum and ascorbic acid (50 µg/ml) (lanes 1-7) or were incubated for 4 h with 50 nM mithramycin (lanes 8-14) before addition of α-amanitin (1 µg/ml). Total RNA was prepared from cells at various intervals following α-amanitin addition and examined by Northern analysis. Northern analysis was performed on samples prepared at the time of α-amanitin addition (‘0’ time point): lanes 1-2, 8-9; at 6 h after α-amanitin administration: lanes 3-4, 10-11; at 18 h after α-amanitin treatment: lanes 5-6, 12-13; and at 24 h following α-amanitin: lanes 7 and 14. B. COL1A1 mRNA stability following mithramycin treatment assessed by densitometric analysis. Values shown were corrected to the GAPDH and represent the average of duplicate cultures of different cell lines. The 24 h value was not included in the graph since it was not performed in duplicate.
REFERENCES


Figure 1.
Figure 2A

Control

actin

type I collagen

overlay

3nM

10nM

100nM

100 µm
Figure 2B
DMEM type I collagen

[\[^{14}\text{C}\] collagen cpm/1\mu l]

Figure 3A
Figure 3B

Type I and III pro-collagen
α₁(I) and α₁(III) collagen
α₂(I) collagen
Figure 4
<table>
<thead>
<tr>
<th>Construct</th>
<th>- 84 bp</th>
<th>-174 bp</th>
<th>- 804 bp</th>
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<tr>
<td>Mithramycin 100 nM</td>
<td>- +</td>
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**Figure 5**
Figure 6 A
Figure 6 B

- ○ Non-treated
- ● Treated
Inhibition of collagen gene expression in systemic sclerosis dermal fibroblasts by mithramycin

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