INHIBITION OF BASAL AND TRANSFORMING GROWTH FACTOR β-INDUCED
STIMULATION OF COL1A1 TRANSCRIPTION BY THE DNA INTERCALATORS,
MITHOXANTRONE AND WP631,
IN CULTURED HUMAN DERMAL FIBROBLASTS

Svetlana Gaidarova and Sergio A. Jimenez
Division of Rheumatology, Department of Medicine,
Thomas Jefferson University, Philadelphia, PA 19107 USA

Address all correspondence to:
Sergio A. Jimenez, M.D.
Thomas Jefferson University
Division of Rheumatology
233 S. 10th Street, Room 509 BLSB
Philadelphia, PA 19107-5541
Phone: 215-503-5042
Phone: 215-923-4649
E-mail: Sergio.Jimenez@mail.tju.edu
SUMMARY

The Sp1 transcription factor plays a crucial role in COL1A1 transcriptional regulation of COL1A1 under normal and pathologic conditions and under the effects of transforming growth factor β (TGFβ). Sp1 activity is elevated in numerous diseases characterized by tissue fibrosis. Therefore, inhibition of Sp1 binding to COL1A1 regulatory elements may represent an effective treatment for these diseases. Here, we examined the effect of two DNA intercalators that prevent Sp1 binding on the expression of COL1A1 in human dermal fibroblasts.

Cultured human adult dermal fibroblasts were treated with WP631 (50 pM/ml to 500 nM/ml) or mithoxantrone (5-500 nM/ml). Cytotoxicity, cellular apoptosis and collagen deposition were examined by fluorescence microscopy. Collagen production was examined by ELISA and metabolic labeling, COL1A1 steady-state mRNA levels and stability were assessed by Northern hybridizations, and COL1A1 transcription by *in vitro* nuclear transcription assays and transient transfections. Competition of the drugs for Sp1 binding and their effect on TGFβ-induced stimulation of COL1A1 transcription was also examined.

Both drugs caused a dose-related inhibition of COL1A1 production and mRNA levels without cytotoxicity or apoptosis. COL1A1 transcriptional activity showed a profound reduction mediated by a short proximal promoter region containing an Sp1 binding element at −87 to −82 bp. Furthermore, both drugs inhibited Sp1 DNA complex formation and abrogated the stimulation of COL1A1 transcription induced by TGFβ. WP361 showed 10-fold higher potency than mithoxantrone.

These data indicate that mithoxantrone and WP631 are very potent inhibitors of basal and TGFβ-stimulated COL1A1 expression and suggest that Sp1-DNA intercalators may be an
COL1A1 transcriptional inhibition by mithoxantrone and WP631

effective and novel approach for the treatment of fibrotic diseases and modulation of profibrogenic effects of TGFβ.

INTRODUCTION

There are numerous human diseases including systemic sclerosis (SSc), pulmonary fibrosis, liver fibrosis, and kidney glomerulosclerosis which are characterized by exaggerated deposition of collagen and other connective tissue macromolecules in the affected organs (1-4). These disorders have been collectively termed fibrosing diseases. Studies of fibroblasts from affected tissues of these patients in culture display an activated phenotype and overproduce type I and type III collagens relative to normal fibroblasts (5-7).

Despite the recent advances in the understanding of the regulation of collagen gene expression under normal conditions or under the effects of various cytokines and growth factors, very little has been learned regarding the intimate mechanisms responsible for the pathologic increase in the expression of collagen genes in fibrotic diseases. The exaggerated extracellular matrix production by fibroblasts from these diseases largely results from increased transcription of the corresponding genes (8-11) although in some instances modulation of transcript stability may also be involved (12). The most important molecule in these fibrotic processes is type I collagen, the prototype of the interstitial collagens. Type I collagen is a heterotrimeric molecule comprised of two α1 chains and one α2 chain encoded by separate genes, COL1A1 and COL1A2, respectively. The transcriptional activities of these two genes are coordinately regulated so that under normal conditions and during fibrotic processes, such as those responsible for the fibrosing diseases, their expression is increased in parallel. It has been demonstrated that COL1A1 and COL1A2 transcriptional regulation involves interactions
between transcription factors and regulatory elements contained within their promoters and first introns. However, the precise elements involved in their abnormal transcriptional regulation and the transcription factors that participate in these interactions are not entirely known (reviewed in 7, 13-15).

The transcriptional regulation of COL1A1 expression has been examined in numerous studies and many regulatory elements of the gene and the transcription factors interacting with these elements have been identified (16-20). These studies have shown that some of these transcription factors are members of the Sp1 family of DNA binding proteins and it has been suggested that Sp1 plays an important role in the pathogenesis of fibrosis (21-30). Indeed, it was demonstrated that Sp1 is involved in the transcriptional activation of the COL1A1 promoter by the profibrogenic growth factor, TGFβ (25, 31-33), that activated stellate hepatic cells, the cells responsible for increased collagen production in liver fibrosis, contain higher levels of Sp1 binding activity than non-activated cells (34), that there is greater than a 3-fold increase in DNA-binding activity to Sp1 binding elements of the COL1A1 promoter in SSc fibroblast nuclear extracts in comparison to nuclear extracts from normal cells (35), and that there is increased phosphorylation of Sp1 in SSc cells (36).

The crucial role that Sp1 plays in the regulation of collagen gene expression suggests that agents capable of interfering with its binding or its activity may be effective in controlling the exaggerated fibrotic process in the fibrotic diseases. Recently, several studies demonstrated that certain DNA binding drugs which resemble transcription factors in their preference for specific DNA sequences and DNA groove orientation are capable of causing a potent inhibition of the binding of transcription factors to their cognate DNA elements (37-40). This inhibition is caused either by direct competition for DNA binding or by induction of DNA conformational
changes. The results of these studies demonstrated that mithramycin, mithoxantrone, daunorubicin, and the bisanthracycline WP631 are potent inhibitors of the formation of Sp1-DNA complexes. Given the importance of Sp1 on the regulation of collagen gene expression, it is expected that these agents may cause potent and selective inhibition of the expression of these genes. Indeed, one study demonstrated that mithramycin selectively inhibited COL1A1 gene expression in human fibroblasts (41). The objective of the present study was to examine the effects of newly discovered and more potent DNA-binding drugs than mithramycin on collagen gene expression and transcriptional activity. We chose to focus on mithoxantrone and WP631, two potent drugs shown previously to exert specific effects on Sp1 binding at very low concentrations. We found that WP631 and mithoxantrone are very potent inhibitors of fibroblast COL1A1 expression. WP631 showed greater than 10-fold higher potency than mithoxantrone. The inhibitory effects of the drugs on COL1A1 expression were shown to be due to inhibition of transcriptional activity of the gene which was exerted through the competition with Sp1 binding to the short COL1A1 promoter segment containing the most 3' Sp1 binding element located at –87 to –82 bp. Both drugs were also shown to completely abrogate the stimulation of COL1A1 transcriptional activity induced by TGFβ, a process that involves the participation of Sp1. These results provide the basis for the potential use of Sp1-DNA intercalators as a novel and effective approach for the treatment of the fibrotic manifestations of various fibrotic diseases and for the modulation of the profibrogenic effects of TGFβ.

**EXPERIMENTAL PROCEDURES**

*Fibroblasts cultures.* Human dermal fibroblasts were obtained from skin samples as described previously (42). All studies were approved by the Institutional Review Board of
Thomas Jefferson University. Fibroblast cultures were established and maintained in Eagle’s MEM supplemented with 10% fetal bovine serum (FBS), 1% vitamins, 2mM glutamine and antibiotics (Cellgro, Mediatech, Inc., Herndon, VA) and incubated at 37°C in 5% CO₂ in a humidified atmosphere. When the cultures reached confluency, 50 µg/ml ascorbic acid was added for 24 h prior to initiation of the experiments in order to optimize their level of collagen production.

Mithoxantrone (Sigma, St. Louis, MO) was dissolved in dimethyl sulfoxide and WP631 (Calbiochem, San Diego, CA) was dissolved in distilled water. Both solutions were stored at -20°C. All other chemicals were of reagent grade (Fischer Scientific, Pittsburgh, PA). Four different fibroblast strains were studied. The cells were plated in 35-mm plastic culture dishes at a density of 100,000 cells per dish and cultured until they reached confluency. For dose-response studies, the cells were pre-incubated for 24 h and then incubated for 48 h with 500 pM to 100 nM mithoxantrone or with 50 pM to 50 nM WP631, dose ranges found to lack significant cytotoxicity.

**Assessment of apoptosis and cell viability.** Induction of apoptosis by the drugs was examined employing the Annexin V-FITC conjugate Apoptosis Detection Kit (R and D Systems, Minneapolis, MN). Annexin V binding to phosphatidyl serine exposed in apoptotic cells after treatment of fibroblasts with various drug concentrations was analyzed by fluorescence microscopy (43,44). Propidium iodide (PI), a marker for late apoptotic or necrotic processes was also included. For assessment of cytotoxicity, cells in exponential phase of growth were exposed to various concentrations of the drugs. After 48 h the media containing the drugs were removed and the cells were maintained in fresh media to allow proliferation for two-three doubling times (48 h) in order to distinguish between cells that remain viable and capable of
proliferation and those that remain viable but can not proliferate. The number of surviving cells was determined the MTT dye reduction assay (45). Briefly, the tetrazolium compound MTT (3-[4,5dimethylthiazol-yl]-2,5-diphenyltetrazolium bromide) was added to the wells (20 µl per well) and the cells were incubated for 3h. In this assay, MTT is reduced by metabolically active cells (viable) to insoluble purple formazan dye crystals. At the end of the incubation period, 100 µl of detergent was added to the wells to solubilize the crystals and allow determination of absorbance at 560 nm using a spectrophotometer. Samples were read directly in the wells. The data were analyzed by plotting cell number versus absorbance, allowing quantitation of changes in cell viability as the rate of tetrazolium reduction is proportional to the number of viable cells.

Effect of mithoxantrone and WP631 on type I collagen production and accumulation in the cell layers. In parallel with the cytotoxicity studies, type I collagen production was assessed. For this purpose, media recovered after drug treatment was subjected to indirect ELISA and production of type I collagen was quantitated using an affinity purified human collagen type I polyclonal antibody (Rockland, Gilbertsville, PA) as described previously (46,47). The effect of the drugs on collagen accumulation in the cell layers was analyzed by fluorescence immunomicroscopy essentially as described previously (47). Briefly, fibroblasts were plated on coverslips at a density of 40,000 cell/coverslip and when the cells reached confluency, 5 nM WP631 or 50 nM mithoxantrone was added for 48 h. After treatment, the cells were washed in PBS and fixed in 3.7% paraformaldehyde. After washing off the fixing solution with PBS, the cells were permeabilized with 0.05% Triton X-100 (TX-100). Non-specific binding was blocked with 1.5% normal goat serum (NGS) in PBS-Tween. The cells were incubated for 30 min with the affinity purified anti-type I collagen antibody in blocking solution. After removing and washing the unbound antibody, fluorescein-conjugated goat-anti-rabbit IgG secondary antibody
in 4% NGS with 0.05 TX-100 was added to the coverslips. Following the last staining step, the coverslips were mounted on glass slides using Slow Fade–Light Antifade Kit (Molecular Probes Inc., Eugene, Oregon). All steps of fixation were carried out at room temperature and staining was carried out at 37°C. Fluorescence was visualized using excitation filters (450-490 nM) and emission filters (500-550nM) with a 495 LP dichroic filter (Chrome Technology, Brattleborough, VT). Scientific imaging software from IP Laboratories (Scanalytics, Fairfax, VA) was used for image acquisition and analysis.

**Fibroblast collagen biosynthesis.** Confluent fibroblast cultures were employed to examine the effect of WP631 and mithoxantrone on fibroblast collagen biosynthesis as described previously (42,48). Briefly, the medium was removed and 2 ml of fresh DMEM containing various concentrations of WP631 or mithoxantrone, 50 µg/ml ascorbate, 100 µg/ml β-aminoproprionitrile and 5 μCi/ml [14C]–proline (ICN Biomed, sp. activity: 247mCi/mM) was added. After 48 h of incubation, the media and cell layers were harvested and processed as described previously (42,48). The amount of radiolabeled, newly synthesized collagen was measured by a collagenase digestion assay (49) employing pure bacterial collagenase (BRL Laboratories, Bethesda, MD) in the presence of protease inhibitors. For gel electrophoresis, separate aliquots of media and cell layer lysates were electrophoresed on 7.5% SDS-polyacrylamide gels under reducing conditions as described previously (49). Samples were electrophoresed for 5 h at 100V constant voltage. In a separate time-course experiment cell cultures were incubated for 1-24 h with either 0.5 nM WP631 or 5 nM mithoxantrone. After electrophoresis the gels were processed for fluorography and exposed to X-Omat AR film (Eastman Kodak, Rochester, NY).
Fibroblasts were grown to confluency and following incubation either under control conditions or with mithoxantrone or WP631 for 48 h total RNA was isolated (50) employing the Qiagen Easy RNA extraction method (Qiagen, Valencia, CA). For Northern hybridizations 5µg/µl aliquots of total RNA were electrophoresed on formaldehyde 1% agarose gels. The RNA was then transferred to Nylon+ membranes (Ambion, Austin, TX) and the filters hybridized to $^{32}$P-radiolabeled human cDNA for COL1A1, COL1A2 and GAPDH, as described previously (47). Equivalent amounts of RNA loading and transfer were evaluated by probing with a ribosomal 18S probe (Ambion). The filters were scanned by densitometry (Storm 840, Molecular Dynamics, Sunnyvale, CA) and the results were quantified to determine the relative amounts of mRNA (Image-Quant V5.1 software; Molecular Dynamics). For determination of mRNA stability, confluent fibroblasts were cultured in T-75 flasks in the presence or absence of mithoxantrone (5 nM) or WP631 (0.5 nM) as described above. The cultures received α-amanitin (1 µg/ml) 4 h after addition of the drugs. Pairs of treated and untreated cultures were harvested at 4, 8, 12, 18 and 24 h after the addition of α-amanitin and total RNA was extracted and analyzed by Northern hybridizations as described previously (51). Autoradiographs were obtained and sets of blots prepared with the same concentration of total RNA from all the samples were scanned in a laser densitometer as described previously (51).

**In vitro nuclear transcription assay.** The effect of the drugs on COL1A1 transcription was determined by an *in vitro* nuclear run-off assay. Fibroblasts were cultured in T-162 flasks for 24 hr in the presence or absence of 5 nM mithoxantrone or 0.5 nM WP631 as described above. At the end of the treatment period, the cell layers were trypsinized, nuclei were isolated and transcription reactions were carried out as described previously (51). Filters were exposed and
then scanned and quantified using storage phosphorimager (Image-Quant V5.1 software, Molecular Dynamics).

**Transient transfections with COL1A1 promoter – CAT constructs.** Transient transfections were performed employing the FuGene transfection method (Roche Molecular Biochemicals, Indianapolis, IN), as described previously (52). The fibroblasts were plated at 70% confluence in 35 mm dishes. The media were changed the following day and 2 h later the cells were transfected with a total of 1μg of various plasmids. The constructs tested are progressive 5´deletions of the human COL1A1 promoter each cloned upstream of the chloramphenicol acetyl transferase (CAT) reporter gene. All constructs end at nucleotide +42 bp to assure a proper reading frame and their 5´end was at the following positions: -4 kb, -2.3 kb, -675 bp, -174 bp, and -90 bp. The detailed procedures for the preparation of these constructs have been described previously (19). Following transfections, fresh medium with or without drugs was added, and cells were harvested 48 h after transfection and fractured by sonication. Total protein content of the cytoplasmic extracts was measured by the Bradford procedure (53) and CAT activity was determined by thin layer chromatography (54). The efficiency of transfection was normalized by cotransfecting the vector containing E. coli β-galactosidase followed by assays of β-galactosidase enzymatic activity (pCMV β-galactosidase, Clontech, Palo Alto, CA).

**Electrophoretic mobility shift analyses.** The ability of the drugs to bind to the COL1A1 promoter and compete with Sp1 for its DNA binding site was examined by electrophoretic mobility shift assays. Fibroblasts were grown to confluence in T-162 flasks and nuclear extracts were prepared from cells exactly as described previously (47,52). In a typical experiment a confluent 162-cm² flask yielded ~100 μg crude protein. Protein concentrations were determined by a dye-binding assay, and the nuclear extracts were stored in small aliquots at −70°C until
used. Nuclear extracts were incubated with a synthetic 26 bp double-stranded oligonucleotide probe corresponding to the −90 to −64 bp region of the COL1A1 promoter containing the putative proximal Sp1 binding site (5′ CAC GGG CGG CCG GCT CCC CCT CTC CG 3′). The oligonucleotide was end-labeled with γ-32P-ATP by using polynucleotide kinase (Boehringer-Mannheim, Mannheim, Germany). Each binding reaction contained 5-10 µg of nuclear extract, 3 µg of double-stranded poly (d[I-C]) (Pharmacia/LKB Biotechnology, Piscataway, NJ), and ~5 ng radiolabeled oligonucleotide (~50,000 cpm). Competition studies were performed with 100-fold molar excess of an unlabeled Sp1 consensus oligonucleotide or a mutated oligonucleotide in which nucleotides required for Sp1 binding were changed. For supershift studies fibroblast nuclear extracts were preincubated with 5 µg of anti-Sp1 antibody (Santa Cruz, Santa Cruz, CA) before incubation with the probe. The reaction mixtures were incubated for 30 min at room temperature in a buffer containing 40mM KCl, 10mM HEPES (pH7.9), 1mM DTT, 1mM EDTA, and 5% glycerol in a total volume of 20 µl. For the competition studies, the radiolabeled consensus oligonucleotide was preincubated for different time intervals with various concentrations of each drug prior to the addition of the nuclear extracts. After incubation, the DNA-protein complexes were resolved from the free probes in nondenaturing 5% polyacrylamide gels. Electrophoresis was performed in 1 x Tris-acetate buffer at 30mA for 120 min. The gels were dried under vacuum and exposed to X-ray film with intensifying screens at −70°C for 24 h. The autoradiographs were quantified by densitometry.

**Effects of mithoxantrone or WP631 on TGFβ-induced stimulation of COL1A1 transcription.** The effects of treatment of fibroblasts with the drugs on TGFβ-induced stimulation of COL1A1 transcription were analyzed by transient transfections. Subconfluent cultured fibroblasts were transfected with −90 bp and −174 bp COL1A1 promoter constructs as
described above. Following transfections, fresh serum-free medium (AIM5, Gibco, Rockville, MD) supplemented with ITS (Browhittaker, Walkersville, MD) and 50 µm ascorbic acid and containing either 5 mM mithoxantrone or 0.5 mM WP631 and 15 mM human recombinant TGFβ1 was added. After 48 h of culture, the cells were harvested and processed as described above.

STATISTICAL ANALYSIS

In experiments in which more than triplicate values were available, the statistical significance of differences observed between untreated (control) and treated cells was assessed employing a one-way ANOVA with the Graph Pad inStat program (GraphPad Software Inc., San Diego, CA). P values of less than 0.5 were considered significant.

RESULTS

*Effects of mithoxantrone and WP631 on fibroblast viability and apoptosis.* To determine a safe range of concentrations of the drugs for further study a dose response of mithoxantrone or WP631 on fibroblast viability and apoptosis was performed. Induction of apoptosis and necrosis was examined by Annexin V-FITC conjugate binding to phosphatidyl serine and by propidium iodide staining and was analyzed by fluorescence microscopy. Cells not undergoing either apoptosis or necrosis appeared unstained. Annexin V positive staining indicated early apoptosis events, whereas, both Annexin V and propidium iodide positive staining corresponded to late apoptotic or necrotic events (Fig. 1). Concentrations of 500 pM and higher for mithoxantrone (Fig. 1A) and 50 nM and higher for WP631 (Fig. 1B) caused cellular apoptosis and were, therefore, not utilized in subsequent studies.
Effects of mitoxantrone and WP631 on fibroblast viability and collagen production and biosynthesis. Cytotoxicity of the drugs was also quantified by a MTT based cell viability assay and it was found that both mitoxantrone and WP631 were not cytotoxic until concentrations greater than 80 nM of either drug were employed (Fig. 2A). Type I collagen production was assessed in aliquots of culture media following 48 h treatment with various drug concentrations employing an ELISA (Fig. 2B). All samples were analyzed in triplicate and the collagen concentration determined directly from a standard curve established relating the inhibition of color development to the concentration of added antigen. Treatment of fibroblasts with either drug caused a dose-related inhibitory effect on collagen Type I production without significant cytotoxicity, reaching a maximal collagen production inhibition of about 76% for mitoxantrone and 86% for WP361 (Fig. 2B).

Biosynthetic studies demonstrated that both drugs caused a profound reduction in the amounts of newly synthesized collagen in a concentration dependent manner so that 5 nM WP631 or 50 nM mitoxantrone caused about 85-90% inhibition (Fig. 3A). A time course experiment from 1 to 24 h period of culture with either 0.5 nM WP631 or 5 nM mitoxantrone showed that total collagen biosynthesis progressively decreased as a function of time, reaching a maximal inhibition of about 70% and 85% after 24 h of treatment with mitoxantrone or WP631, respectively (Fig 3B). Gel electrophoretic analysis of the newly synthesized proteins present in the culture media of the labeled fibroblasts confirmed a dose-dependent reduction of newly synthesized procollagen and α1(I) and α2(I) collagen chains by both drugs (Fig. 4A). Similar findings were obtained when newly synthesized collagen present in the cell layers was analyzed (Fig. 4B).
COL1A1 transcriptional inhibition by mithoxantrone and WP631

Analysis of the effect of the drugs on total collagen accumulated in the cell layers by immunofluorescence microscopy showed that the amount of type I collagen present in the cell layers of the cultures after 48 h of treatment with 5 nM WP631 or 50 nM mithoxantrone was substantially decreased without detectable changes in gross cellular morphology (Fig. 5). In our experience, the experimental conditions employed for these studies do not favor the accumulation of an abundant extracellular matrix owing to the short period of culture (48 h) and to the fact that most of the newly synthesized collagen escapes into the culture media. However, the data clearly show a profound reduction of intracellular collagen and of the small amounts of collagen assembled in the peri- and extracellular matrix. In contrast, the amounts of type III collagen and fibronectin were not affected (Fig. 5).

**Effects of mithoxantrone and WP631 on collagen mRNA levels and stability and on COL1A1 transcription.** Study of the effect of the drugs on the steady-state mRNA levels of type I collagen showed that mRNA for both type I collagen chains from fibroblasts cultured in the presence of the drugs was substantially decreased in comparison to that of untreated cells (Fig. 6A). Mithoxantrone at concentrations of 5 and 50 nM reduced COL1A1 mRNA levels by 61 and 68%, respectively (Fig. 6B) whereas WP631 at 0.5 and 5 nM concentrations reduced these levels by 56 and 78%, respectively (Fig. 6C). The levels of COL1A2 mRNA were reduced by the two drugs to approximately the same levels as those of COL1A1 mRNA. In contrast, the drugs did not affect the mRNA levels of the GAPDH gene used as control (Fig. 6). Studies of COL1A1 mRNA stability showed that mithoxantrone or WP361 did not affect transcript stability over a 24 h period (Fig. 7).

The effect of the drugs on COL1A1 transcription was examined by a nuclear run off assay. The results showed that the transcription of the gene was decreased by 45 to 50% with the
effective concentrations of each drug (Fig. 8), whereas the transcription of the genes encoding type III collagen, fibronectin and actin was not substantially changed.

**Identification of COL1A1 elements involved in mithoxantrone and WP631 effects.** To identify the region of the COL1A1 promoter responsive to the drugs, untreated and treated fibroblasts were transiently transfected with gene constructs containing various deletions of the COL1A1 promoter fused to the CAT reporter gene. The results demonstrated that a very short region of the proximal COL1A1 promoter encompassing only −90 to +42 bp and containing the most 3’ Sp1 binding site between −87 to −82 bp was involved. There was a 65% to 76% reduction in CAT activity driven by the −90 to +42 bp construct in response to either of the drugs (Fig. 9). The same pattern was observed with the longer constructs, indicating that both drugs exert their effect through this short region of the proximal COL1A1 promoter (Fig. 9).

The ability of the drugs to bind directly to the COL1A1 promoter and to compete with Sp1 for its DNA binding site was analyzed by EMSA. The results showed that preincubation of a −90 to −64 bp COL1A1 promoter fragment for various intervals and with various drug concentrations prior to the addition of nuclear extracts resulted in inhibition of up to 75% to 80% of DNA–Sp1 complex formation (Fig. 10). The results directly confirmed that this short sequence of the COL1A1 promoter contains the 3’ Sp1 binding site in COL1A1 that is affected by both drugs.

**Abrogation of TGFβ-induced stimulation of COL1A1 transcription by mithoxantrone and WP631.** The effects of the drugs on TGFβ stimulation of COL1A1 transcription were evaluated by transient transfection assays. For this study the −174 bp and −90 bp COL1A1-CAT promoter constructs were transfected into subconfluent fibroblasts followed by treatment of the cells with TGFβ in the presence and absence of the drugs. The results shown in Figure 11
demonstrate that, as expected, TGFβ induced a 75% increase in the transcriptional activity of the –90 bp COL1A1 construct and a 110% stimulation of the transcriptional activity driven by the –174 bp COL1A1 construct. Remarkably, this stimulation of transcriptional activity of both constructs induced by TGFβ was completely abolished by treatment with the effective concentration of either drug.

**DISCUSSION**

Whether drugs that target transcription factor regulatory binding sites within eukaryotic genes cause subsequent biological effects on transcription factor-regulated gene expression that can be useful for the treatment of human diseases has been the subject intense recent investigation (for reviews see refs. 55-57). In this study we employed a human COL1A1 promoter to examine whether two DNA-binding drugs, mithoxantrone and WP631, interfered with the formation of Sp1-DNA complexes and inhibited COL1A1 transcription. Both, mithoxantrone and WP631 are antineoplastic agents used in the treatment of breast and ovarian carcinomas and certain leukemias and lymphomas. Both drugs are DNA-intercalators inhibiting transcription initiation of the adenovirus major late promoter by linkage to GC-rich sequences (Sp1 sites) and basal RNA synthesis in a concentration dependent manner (58-60). The monointercalating anthraquinone, mithoxantrone, is structurally and chemically related to the anthracyclines. The bis-intercalating anthracycline, WP631, is a member of a new class of bis-intercalating drugs shown to have activity against multidrug resistant cancer cells. WP631 comprises two monomeric units of daunorubicine, symmetrically linked together via p-xylenyl 3-NH₂ sites and exhibits extremely high DNA binding affinity (59) while showing slightly less cytotoxicity than daunorubicine in the sensitive cell lines (58). Recent publications described a
remarkable ability of WP631 to displace Sp1 from its putative binding site, while showing less cytotoxicity compared to daunorubicine (60). Both, mithoxantrone and WP631, drugs with distinct DNA sequence preferences (GC-rich sites) and binding motifs (i.e. intercalators and minor groove binders), were examined for their ability to inhibit binding of Sp1 to its consensus binding site on the human COL1A1 promoter. The COL1A1 promoter is highly active in collagen producing fibroblasts and its transcriptional activity is regulated by Sp1 binding both under basal conditions (21,22) as well as under the stimulation of the profibrogenic growth factor, TGFβ. Furthermore, elevated Sp1 binding activity has been detected in fibrotic diseases such as SSc as well as in models of experimentally induced fibrosis during periods of enhanced COL1A1 transcription (34-36), indicating the important role of Sp1 in stimulation of COL1A1 promoter within the context of the intact gene.

An enhanced activity of Sp1 can result from protein-protein interactions of Sp1 bound to the promoter with Sp1 bound to more distal sites of the gene. The potential of WP631 and mithoxantrone to disrupt these interactions with distal factors by interfering with the binding of Sp1 to GC-rich regulatory elements of the COL1A1 proximal promoter could be a valuable tool in disclosing the mechanisms of transcriptional activation of the gene which ultimately result in pathologic fibrogenesis. Indeed, it has recently been shown that competition of Sp1 activity with an antisense expression vector resulted in a broad inhibition of expression of numerous genes encoding extracellular matrix molecules, including COL1A1 and COL1A2 (61). It should, therefore, not be unexpected that WP631 and mithoxantrone also cause downregulation of COL1A2 as the promoter of this gene also contains Sp1 sites that could be targeted by the drugs. In support of the results reported here, a recent publication described the utilization of WP631 to
demonstrate the participation of an Sp1 site in the stimulation of the transcriptional activity of the endoglin gene promoter by TGF-β (62).

The results of our study indicate that mithoxantrone and WP631 are very potent inhibitors of the expression of COL1A1 and cause a remarkable reduction in the transcriptional activity of this gene at concentrations well below those that cause cellular cytotoxicity or apoptosis. Furthermore, the effects of the drugs on COL1A1 expression were not due to a global inhibition of gene transcription as we failed to observe significant reduction of the expression of fibronectin and COL3A1 or of the two genes examined as controls, actin and GAPDH. The gel mobility shift data demonstrated that both drugs prevented Sp1 from binding to the proximal COL1A1 promoter which contains the typical Sp1 binding sequence 5′ GGGCGG 3′ at nt –87 to –82.

It is clear that some drugs that interfere with Sp1 binding to DNA cognate elements can affect the expression of many genes. These effects might explain their narrow therapeutic range when used clinically. However, new and improved Sp1-DNA intercalators such as WP631 have much lower effective concentrations while still achieving maximal therapeutic effects on a target genes. In our study both WP631 and mithoxantrone demonstrated great effectiveness and potency in inhibition of COL1A1 transcription although WP631 exhibited approximately 10-fold greater potency than mithoxantrone. One remarkable observation was that both drugs also caused a very potent inhibition of the TGFβ-induced stimulation of COL1A1 transcription.

The data presented provide sound experimental basis for the potential use of Sp1-DNA intercalators as a novel and effective approach for the treatment of various diseases accompanied by exaggerated fibrotic responses and for the modulation of the profibrogenic effects of TGFβ.
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FIGURE LEGENDS

Figure 1. *Effects of mithoxantrone and WP631 on fibroblast apoptosis*. Confluent cultures of dermal fibroblasts were cultured under control conditions or treated with various concentrations of either mithoxantrone (A) or WP631 (B) for 48 h as described in Materials and Methods. Necrosis and apoptosis were examined by fluorescence microscopy using the Annexin V-FITC conjugate apoptosis detection method and propidium iodide. Cells not undergoing either necrosis or apoptosis appear unstained. Annexin V-FITC positive staining (shown in green color) corresponds to early apoptotic processes. Both, propidium iodide (shown in red color) and Annexin V-FITC positive staining, correspond to the late apoptotic or necrotic processes.

Figure 2. *Effects of mithoxantrone and WP631 on fibroblast viability and collagen type I secretion onto the culture media*. Cytotoxicity of the drugs was tested by a MTT based cytotoxicity assay. Collagen type I production was examined by an indirect ELISA. (A) Fibroblast viability. Values shown represent the percentage of viable cells following 48h of treatment with a given drug concentration compared to untreated cells. (B) Collagen production. The values shown represent the percentage of collagen following treatment of fibroblasts with concentrations from 0.5 nM to 1000 nM for mithoxantrone and from 0.05 nM to 100 nM for WP631 compared to untreated cells. Mitoxanthrone: O—O; WP631: ●–●. The results shown are from three separate experiments each performed in triplicate. The bars indicate the standard deviations.

Figure 3. *Effect of mithoxantrone and WP631 on collagen biosynthesis*. Confluent fibroblast cultures were incubated under control conditions or with various drug concentrations for 24h
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(A), or for a 1 to 24 h time course in the presence of 0.5 nM WP631 or 5 nM mithoxantrone (B), and were labeled with $[14^C]$ proline as described in Materials and Methods. Following labeling the culture media were harvested and the amount of radiolabeled collagen representing newly synthesized protein was determined by a collagenase digestion assay. The values shown are the averages of triplicate samples.

Figure 4. Gel electrophoresis of newly synthesized collagen in the media of control or treated fibroblasts. Equal volume aliquots of culture media and cell layers from the cells labeled with $14^C$ proline in the presence of various drug concentrations (shown in 3A) were electrophoresed and analyzed by fluorography as described in Materials and Methods. A: Media; B: Cell layers. The migration of the procollagen and collagen chains are indicated.

Figure 5. Effect of the drugs on type I collagen deposition in cultured fibroblasts. The amounts of collagen type I and III and of fibronectin deposited in the cell layers of the cultures following treatment with either 5nM WP631 or 50 nM mithoxantrone were analyzed by fluorescence microscopy as described in Materials and Methods. The figure shows a substantial decrease in collagen type I amount compared to untreated cells following treatment of fibroblasts with WP631 or mithoxantrone for 48 h. Collagen type III and fibronectin amounts were not affected.

Figure 6. Effects of WP631 and mithoxantrone on type I collagen mRNA steady-state levels. Confluent fibroblast cultures were incubated for 48h with or without drugs and COL1A1 and COL1A2 mRNA levels determined by Northern hybridization analysis. (A) Representative autoradiograph of Northern blot. (B) Densitometric analysis of Northern blots shown in
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arbitrary densitomeric units following correction for values obtained with a ribosomal 18S probe. The results shown represent four independent experiments and values are expressed as a percentage relative to untreated fibroblasts. Hatched bars are the lower concentrations and dotted bars are the higher concentrations.

**Figure 7. Effects of mithoxantrone and WP631 on the stability of the COL1A1 transcripts.**
Confluent fibroblasts were incubated in T-75 flasks in MEM supplemented with 10% FCS and ascorbic acid (50 µg/ml) with or without 5 nM mithoxantrone or 0.5 nM WP631 for 4 h and then α-amanitin (1 µg/ml) was added to arrest further transcription. Cells were harvested at the intervals shown after the addition of α-amanitin and total RNA was isolated and equal amounts were analyzed by Northern hybridizations as described in Materials and Methods.

**Figure 8. Effects of mithoxantrone and WP631 on in vitro nuclear transcription.** Confluent fibroblast cultures were incubated in T-162 flasks either under control conditions or treated with the drugs. After 48h nuclei were isolated and processed as described in Materials and Methods. (A) Aliquots of [³²P] -labeled RNA containing equal cpm were hybridized to dot-blotted cDNAs for α1(1) collagen, fibronectin, and control pBR322 plasmid and following hybridization were analyzed using phosphor storage technology. The values obtained were corrected for the transcription of actin following subtraction of the background represented by pBR322. (B) Densitometric analysis shown in arbitrary densitometric units. The values are averages of results obtained in two independent experiments and the values are expressed as a percentage relative to untreated fibroblasts. Dotted bars: WP631; hatched bars: mithoxantrone.
Figure 9. **Identification of the COL1A1 promoter region responsive to the drugs.** CAT assays were performed with extracts from four cell lines that had been transfected with 1 µg of various COL1A1 promoter CAT constructs and incubated with either 0.5 nM WP631 or 50 nM mithoxantrone for 48h following transfection as described in Materials and Methods. (A) Autoradiograph of one illustrative experiment. (B) Densitometric analysis shown in arbitrary densitometric units. The values represent the average of four independent experiments and the values are expressed as a percentage relative to untreated fibroblasts. Dotted bars: WP631; Hatched bars: mithoxantrone.

Figure 10. **Competition of Sp1 binding to COL1A1 promoter by mithoxantrone and WP631 analyzed by gel mobility shift assays.** Nuclear extracts from untreated fibroblasts were incubated with a synthetic 26 bp double-stranded radiolabeled oligonucleotide encompassing –90 bp to –64 bp of the COL1A1 promoter containing the putative Sp1 binding site at –87 to –82 bp. (A) Fluorogram showing the formation of two DNA-protein complexes, C1 and C2 (Lane 1). Addition of specific anti-Sp1 antibody resulted in the appearance of supershifted bands, C3,4 (Lane 2) accompanied by a decrease in the C1 and C2 Sp1-oligonucleotide complexes. The C1 and C2 complexes were not detectable in the presence of excess (100-fold) unlabeled oligonucleotide competitor (Lane 3) and were retained in the presence of excess mutated competitor oligonucleotide in which nucleotide bases important for Sp1 binding were changed (Lane 4). (B) The same radiolabeled oligonucleotide was preincubated for various lengths of time with different concentrations of the drugs prior to the addition of nuclear extracts. Note the inhibition (up to 89%) of Sp1-DNA complex formation.
Figure 11. Effects of mithoxantrone and WP631 on TGFβ-induced stimulation of COL1A1 promoter construct transcriptional activity. Subconfluent cultures of dermal fibroblasts were transfected with the –174 bp or –90 bp COL1A1-CAT promoter constructs and then treated with 15 mM of TGFβ plus either 5 mM mithoxantrone or 0.5 mM WP631 in serum-free medium for 48 h as described in Materials and Methods. At the end of the experiment, the cells were harvested and cell extracts assayed for CAT activity. Two experiments were performed in triplicate and one in quadruplicate. (A) Illustrative autoradiograph of CAT assay. Lanes 1: untreated; 2: treated with 15 mM human recombinant TGFβ; 3: treated with 15 mM human recombinant TGFβ plus 5 mM mithoxantrone; 4: treated with 15 mM human recombinant TGFβ plus 0.5 mM WP631. (B) Densitometric analysis of CAT activity shown in arbitrary densitometric units. The values are averages of the three separate experiments and are expressed as a percentage relative to untreated fibroblasts.
Fig. 1

WP631

| nM | Annexin V | PI |
|----|-----------|----|
| 0  | ![Image](#) | ![Image](#) |
| 5  | ![Image](#) | ![Image](#) |
| 10 | ![Image](#) | ![Image](#) |
| 50 | ![Image](#) | ![Image](#) |
| 100| ![Image](#) | ![Image](#) |
Fig. 2

A

Cellular Viability (%)

Drug concentration (nM)

B

Collagen Production (%)

Drug concentration (nM)
Fig. 3

A

C\(^{14}\) collagen, dpm x 10\(^{-5}\)

Dose range, nM

WP631
Mithoxantrone

B

C\(^{14}\) collagen, dpm x 10\(^{-5}\)

Time, hr

control
WP631, 0.5 nM
Mithoxantrone, 5 nM
Fig. 4

**A**

|       | MTX (nM) | WP631 (nM) |
|-------|----------|-------------|
|       | 0.5      | 5           |
|       | 50       | 0.05        |
|       | 0.05     | 0.5         |
|       | 5        | 5           |

Proα(II+III)  
αs(II+III)  
αs(II)

**B**

|       | MTX (nM) | WP631 (nM) |
|-------|----------|-------------|
|       | 0.5      | 5           |
|       | 50       | 0.05        |
|       | 0.05     | 0.5         |
|       | 5        | 5           |

Proα(II+III)  
αs(II+III)  
αs(II)
Fig. 5

|        | Control | WP631  | Mithoxantrone |
|--------|---------|--------|---------------|
| Col Type I |
| Col Type III |
| Fibronectin |
Fig. 7

Control

Mithoxantrone (5nM)

WP631 (0.5nM)

4 8 12 18 24

Time (h)
Fig. 8

A

COL1A1
Fibronectin

B

ADU (％)

COL1A1  FN
Fig. 9

A

B

CAT Activity, ADU (%)

4kb  2.3kb  675bp  174bp  90bp
Fig. 10

A

1. Control nuclear extract (NE)
2. NE + anti-Sp1 antibody
3. NE + consensus oligo competitor
4. NE + mutant oligo competitor

B

WP631 Mithoxantrone

| Time | 0.5 nM | 5 nM | 5 nM | 50 nM |
|------|--------|------|------|-------|
| 20'  |       |      |      |       |
| 40'  |       |      |      |       |
| 60'  |       |      |      |       |

C1
C2
Inhibition of basal and transforming growth factor β-induced stimulation of COL1A1 transcription by the DNA intercalators, mithoxantrone and WP631, in cultured human dermal fibroblasts

Svetlana Gaidarova and Sergio A. Jimenez

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