Available online at www.sciencedirect.com

SCIENCE DIRECT®

Life Sciences 76 (2005) 2011–2026

Life Sciences

www.elsevier.com/locate/lifescie

Inhibitory effect of dicationic diphenylfurans on production of type I collagen by human fibroblasts and activated hepatic stellate cells

Lela Stefanovic[a], Chad E. Stephens[b], David Boykin[b], Branko Stefanovic[a,*]

[a]*Department of Biomedical Science, Florida State University College of Medicine, Tallahassee, FL 32306, United States*
[b]*Department of Chemistry, Georgia State University, Atlanta, GA, United States*

Received 26 July 2004; accepted 23 September 2004

Abstract

Excessive production of extracellular matrix is responsible for clinical manifestations of fibroproliferative disorders and drugs which can inhibit excessive synthesis of type I collagen are needed for the therapy. Several dicationic diphenylfurans were synthesized and were found to bind RNA. Two of these type compounds were able to reduce synthesis of type I collagen by human fibroblasts and human activated hepatic stellate cells (HSCs). Activated HSCs are responsible for collagen production in liver fibrosis. When added at 40 μM compound 588 reduced intracellular level and secretion of procollagen α1(I) by 50%, while compound 654 reduced these parameters by more than 80% at 20 μM. 654 also significantly reduced secretion of fibronectin. Toxic effects were observed at 80 μM for 588 and 40 μM for 654. 654 reduced expression of a reporter gene with collagen signal peptide, while expression of the same gene without signal peptide was unaffected. Also, expression of intracellular proteins tubulin and calnexin was unchanged. 654 accumulated inside the cell in the cytoplasm and did not change the steady-state level of collagen mRNAs. Treatment of cells with proteosome inhibitor MG132 did not change the inhibitory effect of 654, suggesting that 654 acts as suppressor of translation of proteins containing a signal peptide. Most secreted proteins of fibroblasts and activated HSCs are components of extracellular matrix. Therefore inhibition of their production, as shown here for procollagen α1(I) and fibronectin, may be a useful property of some of diphenylfurans, making these compounds a basis for development of antifibrotic drugs.

Keywords: Diphenylfurans; Collagen type I; Inhibition; Hepatic stellate cells

* Corresponding author. Tel.: +1 850 644 7600; fax: +1 850 644 8924.
E-mail address: branko.stefanovic@med.fsu.edu (B. Stefanovic).

0024-3205/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.lfs.2004.09.043

Introduction

Fibroproliferative disorders are characterized by excessive and uncontrolled production of extracellular matrix composed primarily of type I collagen which results in tissue fibrosis (Bitterman and Henke, 1991). Fibrosis can affect almost any organ and cause progressive loss of function, but fibrosis of the liver is the most common and is the 8th leading cause of death in the USA (2000). Fibroblasts and myofibroblasts are cell types which synthesize type I collagen. In quiescent state these cells synthesize small amounts of type I collagen necessary to support tissue remodeling and wound healing. After a profibrotic stimulus, they become activated and increase collagen synthesis up to hundred fold (Friedman, 1996; Friedman, 1999b; Stefanovic et al., 1997; Stefanovic et al., 2000). Increased collagen synthesis is initiated and maintained by profibrotic cytokines like TGF-beta1, TGF-beta2 and connective tissue growth factor (CTGF), which are produced in response to the profibrotic insult (Friedman, 1999a; Hellerbrand et al., 1999; Pinzani et al., 1998). Upon a prolonged insult the collagen production evades normal regulatory mechanisms and proceeds to fibrosis. The regulation of type I collagen is still poorly understood and this precluded development of antifibrotic drugs.

Type I collagen is the most abundant protein in human body and is a heterotrimeric protein, composed of two α1(I) chains and one α2(I) chain (procollagens), which are encoded by separate genes (Kivirikko, 1998). Its biosynthesis starts on the membrane of endoplasmic reticulum (ER), where translating ribosomes drive the insertion of individual chains into the lumen of ER (Beck et al., 1996; Sauk et al., 1994). In the ER collagen chains undergo hydroxylation of the selected proline and lysine residues, with subsequent glycosylation of hydroxylysines (Myllyharju and Kivirikko, 2004). After these posttranslational modifications, two α1 and one α2 chains fold into a heterotrimeric protein. Procollagen heterotrimers contain globular N and C terminal domains and central rod like triple helical region. Heterotrimers are secreted into extracellular space where the terminal globular domains are cleaved off, yielding the mature type I collagen, which polymerizes into fibrils (Birk et al., 1997; Lo et al., 2004). Collagen fibrils are stabilized by intermolecular crosslinks made by the enzyme lysyl oxidase (Kagan and Li, 2003). Deposition of the crosslinked fibrils, which are resistant to proteolytic degradation, is responsible for alteration of normal tissue extracellular matrix (fibrosis) and change in organ architecture.

Cirrhosis is the end stage of liver fibrosis and is characterized by the accumulation of extracellular matrix proteins in the liver, including type I collagen and fibronectin (Aycock and Seyer, 1989; Schuppan, 1990). Hepatic stellate cells (HSCs also named Ito cells, lipocytes, or fat-storing cells) are the major cell type responsible for collagen synthesis in the cirrhotic liver (De Leeuw et al., 1984; Maher and McGuire, 1990). In normal liver, quiescent HSCs store vitamin A, (Hendriks et al., 1985) but only express trace amounts of type I collagen (Stefanovic et al., 1997). Upon a fibrogenic stimulus, HSCs become activated, processes in which they lose retinoid droplets, proliferate, change morphologically into myofibroblasts, and increase their synthesis of extracellular matrix proteins (Friedman, 1999b; Knittel et al., 1999). Culturing quiescent HSCs on plastic causes activation similar to that seen in liver fibrosis in vivo; including the accumulation of collagen α1(I) mRNA and protein (Friedman et al., 1992; Knittel et al., 1999). In vitro activation of HSCs can thus serve as an excellent model system to study regulation of collagen type I (Stefanovic et al., 1999; Stefanovic et al., 1997).

One of the goals of therapy for fibroproliferative disorders is to inhibit excessive synthesis of extracellular matrix proteins. Inhibitors of prolyl (Sakaida et al., 1999; Wang et al., 2002) and lysyl hydroxylases (Saika et al., 1995), as well as inhibitors of lysyl oxidase (Nagan et al., 1998; Nagan and Kagan, 1994), were suggested as antifibrotic drugs. In addition, neutralizing antibodies against CTGF

(Minato et al., 2004) and soluble TGF-beta receptors (Ezquerro et al., 2003; Nakamura et al., 2000) were tried as blockers of fibrosis. We have described a molecular decoy which can decrease collagen type I synthesis in HSCs and fibroblasts by sequestering a regulatory RNA binding protein. (Stefanovic et al., 2002). However, for now, the treatment of fibroproliferative disorders remains symptomatic and pharmacological compounds are urgently needed. In this paper we describe two compounds which are able to significantly decrease intracellular and extracellular level of type I collagen and fibronectin in human HSCs and fibroblasts.

Material and methods

Compounds 588 and 654

The synthesis of DB654 has been reported previously (Lansiaux et al., 2002). The synthesis of DB588 is described below.

2,5-Bis(4-{3-[2-(1-pyrrolidino)ethyl]ureido}phenyl)furan Dihydrochloride (DB588). To a suspension of 2,5-bis[4-(chlorocarbonyl)phenyl]furan (Boykin et al., 1998) (1.73 g, 5.0 mmol) and tetrabutyl ammonium bromide (0.10 g) in dichloromethane (100 ml) was added a solution of sodium azide (0.86 g, 13.2 mmol) in water (20 ml) and the mixture was stirred vigorously at room temperature for 5 hours. A clear yellow solution was quickly achieved followed almost immediately by formation of a yellow precipitate. The dichloromethane was then removed in vacuo (temp < 30 °C), additional water was added, and the product was collected, rinsed with ether, and air dried overnight at 40 °C to give the bis(acyl azide) as a bright yellow fluffy solid (1.66 g, 93%), mp 136–37 °C dec (with loss of nitrogen). IR (KBr): v 2140 (N_3), 1681 (CO) cm^{-1}. The compound was used directly in the next step.

A solution of the above bis(acyl azide) (0.45 g, 1.25 mmol) in dry dioxane (30 ml) was heated at reflux under nitrogen for 30 min to give the bis(isocyanate) 3 (with loss of nitrogen). 1-(2-Aminoethyl)pyrrolidine (0.45 g, 3.9 mmol) was then added and the resulting suspension was further refluxed for 2 hrs. The mixture was then cooled and filtered to give, after rinsing with ether, the free base as a pink solid (0.53 g, 80%). ^{1}H-NMR (dimethyl-d_6 sulfoxide): δ 1.67–1.71 (m, 8H), 2.44–2.51 (m, 12H), 3.20 (m, 4H), 6.13 (t, 2NH), 6.81 (s, 2H), 7.43 (d, J = 8.8 Hz, 4H), 7.61 (d, J = 8.8 Hz, 4H), 8.75 (s, 2NH). The salt was prepared by dissolving the free base in hot EtOH containing anhydrous HCl, filtering to remove trace insolubles, and then concentrating the solution in vacuo to an oil. Drying in vacuo gave a tan/yellow solid (mp > 150 °C dec). ^{1}H-NMR (dimethyl-d_6 sulfoxide): δ 1.88 (br m, 4H), 2.00 (br m, 4H), 3.02 (br m, 4H), 3.22 (m, 4H), 3.46 (m, 4H), 3.59 (br m, 4H), 6.74 (t, 2NH), 6.83 (s, 2H), 7.48 (d, J = 8.8 Hz, 4H), 7.63 (d, J = 8.8 Hz, 4H), 9.16 (s, 2NH), 10.29 (br s, 2NH). ^{13}C-NMR (dimethyl-d_6 sulfoxide): δ 23.1, 38.2, 53.4, 55.3, 106.1, 117.6, 123.3, 123.9, 139.9, 152.1, 154.9. Analysis for $C_{30}H_{38}N_6O_3$-2HCl-0.66H_2O (615.46): Calcd: C, 58.54; H, 6.77; N, 13.66. Found: C, 58.59; H, 6.89; N, 13.45.

Cell culture

Immortalized cell line of activated human HSCs (hHSC) was described before and was a kind gift of Dr. B. Schnabl (Schnabl et al., 2002). hHCS and primary human skin fibroblasts were grown in 6-well dishes in DMEM supplemented with 10% calf serum until confluent. 588 and 654 were added to the cells in concentrations indicated (between 5 and 40 μM) and incubated for 21 h or 45 h. MG132 (Sigma) was

added at 100 μM, where indicated. The medium was then changed to 0.5 ml of serum free DMEM and incubation continued for additional three hours. The medium, which contains newly accumulated collagen within 3 h, as well as the cell layer, were collected and analyzed for collagen protein and mRNA.

To check the toxicity of the drugs, images of cells were taken at various time points after addition of the drugs and cell viability was estimated by staining with propidium iodide and YO-PRO-1 nucleic acid stain (Molecular Probes).

To assess the subcellular accumulation of the drugs, 654 was added to hHSCs at 5 μM and after 30 min the image of the cells was taken under visible and UV light using blue filter with cutoff of nm.

Transient transfections were done into NIH3T3 fibroblasts seeded at subconfluent density in p100 plates and using 3 μg of plasmid DNA/plate and *Trans*IT-LT1 reagent (Mirus). 24 h after transfections the cells were trypsinized and equal number was seeded into 6-well plates where they were grown for additional 24 h. 654 was added at 5 μM and 20 μM and incubated with cells for 24 h. The cells were then lysed in 100 μL of luciferase lysis buffer (Promega) and luciferase activity (RLU) was measured in a 10 μL aliquot. RLU was normalized to total protein concentration in the samples and the results were expressed as percentage of RLU/μg relative to the control cells (without drug). Two independent transfection experiments were performed, each done in duplicate.

Plasmid constructs

Two hybrid mouse collagen α1(I)/luciferase gene without the signal peptide (COLL/LUC) was constructed by ligating a ds-oligonucleotide with sequence of mouse collagen α1(I) gene (nt + 85 to nt + 143 where + 1 is the start of transcription, contains internal XbaI site) and HindIII and NcoI overhangs into HindIII and NcoI sites of pEGFP-1 vector (construct I) and pGL3 vector (construct II). Then, XbaI – BamHI (blunt) fragment of the construct II (containing the entire luciferase gene) was recloned into XbaI-BsrGI (blunt) of construct I, yielding construct III. Finally, SpeI-XbaI fragment of mouse collagen α1(I) minigene, containing 220 nt of the promoter was cloned into SpeI-XbaI of construct III yielding COLL/LUC. COLL/LUC encodes for luciferase protein which has only 9 amino acids of mouse collagen α1(I) at the N-terminus. For the hybrid mouse collagen α1(I)/luciferase gene with the signal peptide, a ds-oligonucleotide with sequence of mouse collagen α1(I) gene (nt + 120 to nt + 188) and XbaI and NarI

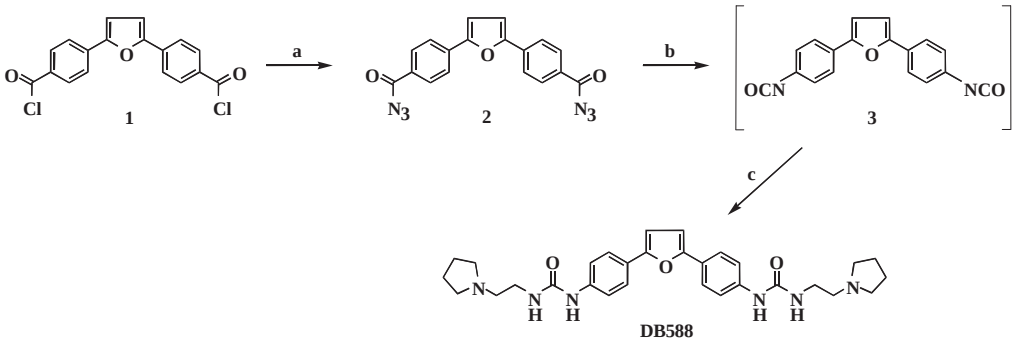


Fig. 1. Schematic representation of synthesis of 588. 588 was synthesized as inMaterial and methods. Reagents and conditions: a) NaN3, TBAB, CH2Cl2, H2O; b) 1,4-Dioxane, Δ; c) 1-(Aminoethyl)pyrrolidine, Δ. Synthesis of 654 has been described before (Lansiaux et al., 2002).

overhangs was cloned into XbaI and NarI sites of COLL/LUC. This plasmid encodes for luciferase protein which has all 22 amino-acids of the signal peptide of mouse collagen α1(I) protein at the N-terminus and was named SP-COLL/LUC.

Western blots

50 μg of cellular proteins were run on 7.5% SDS-PAGE gels under reducing conditions. After transfer, the blots were probed with 1:1000 dilution of anti-collagen type I antibody (600-401-103, Rockland). We have shown previously that this antibody specifically recognizes collagen α1(I) chain (Stefanovic and Brenner, 2003). Anti-fibronectin antibody (BD Bioscience), anti-tubulin antibody (Zymed) and anti-calnexin antibody (BD Bioscience) were also used at 1:1000 dilution. The signal was developed using ACL system (Amersham). 40 μL of cellular medium (corresponding to equal # of cells) was directly analyzed for expression of collagen and fibronectin, as above.

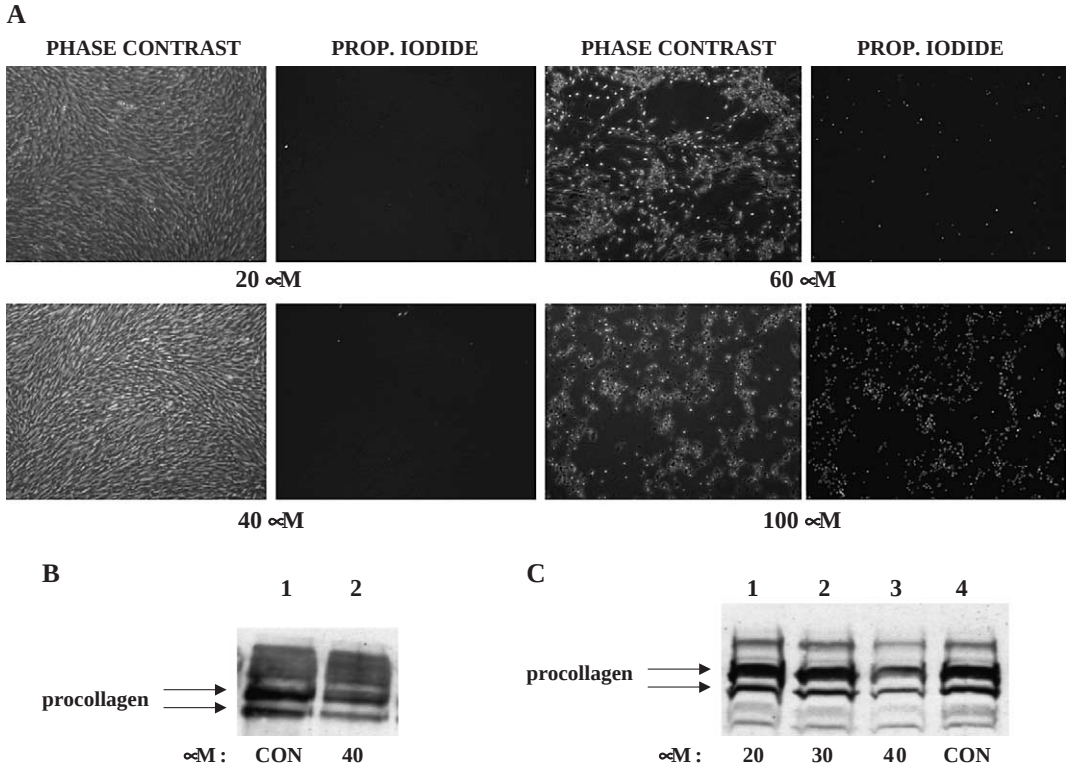


Fig. 2. 588 decreases procollagen in human fibroblasts. A. Morphology and apoptosis of human fibroblasts after treatment with 588. Compound 588 was added at the indicated concentrations to confluent culture of human fibroblasts and incubated for 24 h. Image of cells was taken under phase contrast and after propidium iodide (PI) staining under UV light. B. 588 decreases intracellular level of procollagen α1(I) protein. Western blot was done with 50 μg of cellular proteins from control cells (lane 1, CON) and cells treated with 40 μM of 588 for 24 h (lane 2, 40). Migration of procollagen α1(I) is indicated by arrows. C. 588 decreases accumulation of procollagen α1(I) in cellular medium. Cells were treated with 20 μM (lane 1), 30 μM (lane 2) or 40 μM (lane 3) of 588 for 21 h, cellular medium was changed and incubation continued for additional 3 h, when an aliquot of the medium was analyzed for procollagen α1(I) as in B. CON are control cells without 588.

RT-PCR

Total RNA was extracted according to the standard procedure (Chomczynski and Sacchi, 1987). RT-PCR reactions were done with 100 ng of total RNA using rTth reverse transcriptase RNA PCR kit (Perkin Elmer) in presence of 10 μCi of ^{32}P dCTP, according to the previously used protocol (Stefanovic et al., 1997). The primers specific for human collagen α1(I) mRNA were: 5′ ATCCCACCAATCACCTGCGTA and 3′ ACAGATCACGTCATCGCACAA, α2(I) mRNA were: 5′ CAGCAGGAGGTTTCGGCTAA and 3′ CAACAAAGTCCGCGTATCCA, α1(III) mRNA were: 5′ ATCTTGGTCAGTCCTATGCGG and 3′ GCAGTCTAATTCTTGATCGTCA. GAPDH specific primers, as an internal control, were described before. (Stefanovic et al., 1997)Reverse transcription (at 50 °C, 15 min) and amplification was done in a single reaction using only gene specific primers. The PCR step consisted of 20 cycles (1 min at 94 °C, 2 min at 50 °C), which was in the linear range of the reaction.

Results

Compounds

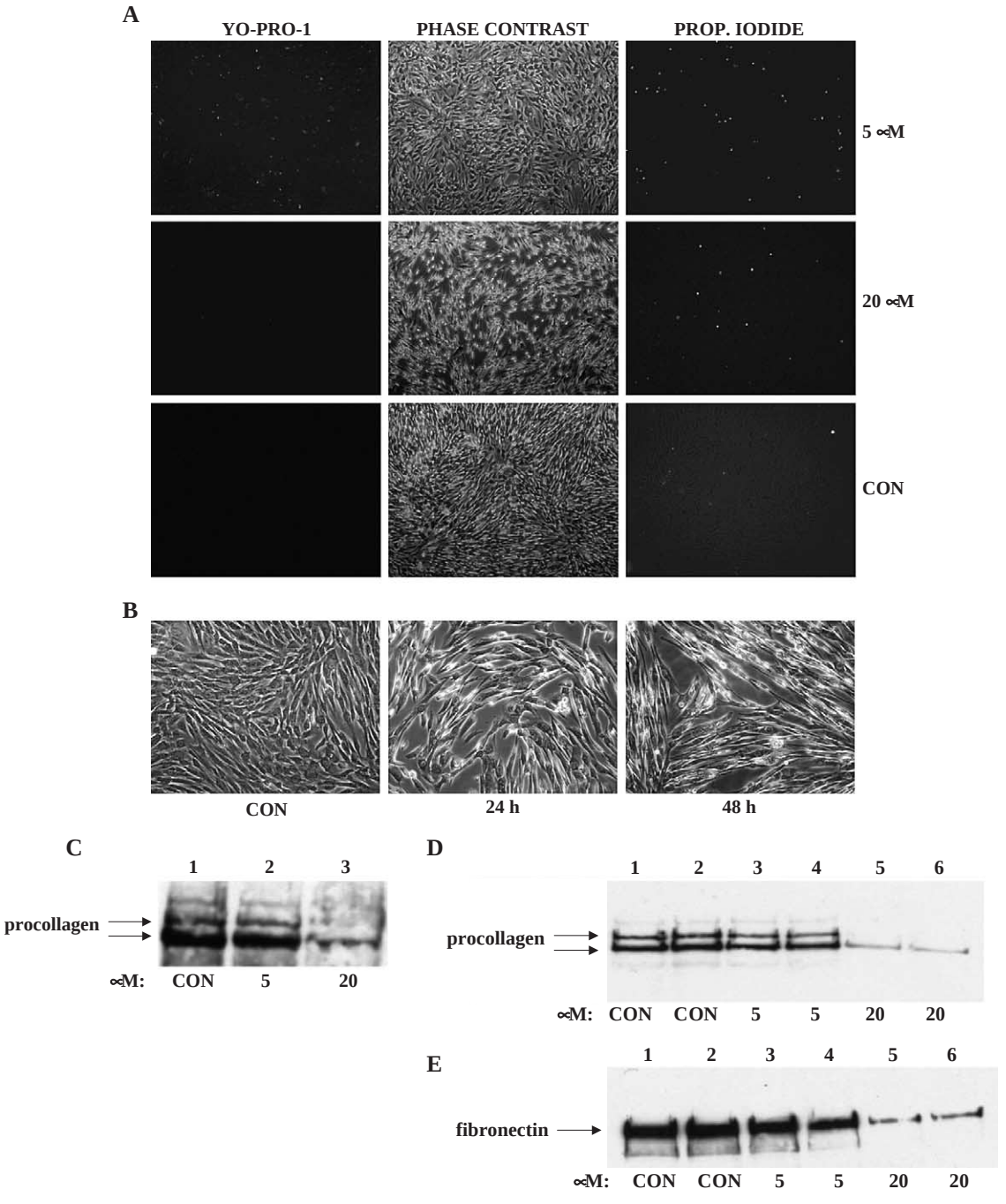
The synthesis of DB654 has been previously reported (Lansiaux et al., 2002). The synthesis used to prepare DB588 is straightforward and is outlined in Fig. 1. DB588 was obtained in an overall 74% yield from the starting diacid chloride.

Inhibition of collagen synthesis by compounds 588 and 654 in human fibroblasts

Based on preliminary experiments toxic effects of 588 were observed at 80 μM. Therefore, compound 588 was tested at concentrations from 20 to 40 μM. At 40 μM with human fibroblasts 588 did not cause a change in cell morphology or cell numbers when incubated for 24 h. No apoptosis or cell death was observed by staining of cells with propidium iodide at 40 μM of 588 (Fig. 2A). The number of apoptotic cells increases at 60 μM and at 100 μM all cells are apoptotic (Fig. 2A). Intracellular steady state level of procollagen α1(I) protein was determined by western blot after 24 h incubation with various concentrations of 588 (Fig. 2B). Two bands representing procollagen α1(I) are probably due to differences in posttranslational modifications. Procollagen α1(I) protein was decreased to 50% (lane 2) of the level seen in control cells, but only with 40 μM of 588 (lane 1). Lower concentrations were

Fig. 3. 654 decreases procollagen in human fibroblasts. A. Morphology and apoptosis of human fibroblasts after treatment with 654. Compound 654 was added at the indicated concentrations to confluent culture of human fibroblasts and incubated for 24 h. Image of cells was taken under phase contrast and, after YO-PRO-1 staining or propidium iodide staining, under UV light. CON are nontreated cells. B. Morphological change of the cells with 20 μM of 654. Cells were treated with 20 μM of 654 for 24 h (middle panel), 48 h (right panel) or left untreated (CON, left panel). Phase contrast image is shown. C. 654 decreases intracellular level of procollagen α1(I) protein. Cells were treated with 5 μM (lane 2) or 20 μM (lane 3) of 654 for 24 h and cellular proteins analyzed as in Fig. 2B. CON (lane 1) are control untreated cells. D. 654 decreases accumulation of procollagen α1(I) in cellular medium. Cells were treated with 5 μM (lanes 3 and 4) or 20 μM (lanes 5 and 6) or without 654 (CON, lanes 1 and 2) and cellular medium analyzed as in Fig. 2C. E. 654 decreases accumulation of fibronectin in cellular medium. The samples from C were probed for expression of fibronectin by western blotting.

without effect. We also estimated secretion of procollagen into cellular medium by incubating the cells for 21 h with 40 μM 588. The cellular medium was then changed and incubation continued for additional 3 h. By this approach we determined the amount of procollagen accumulated within the last 3



h (Fig. 2C). As shown in Fig. 2C, 588 was able to inhibit the rate of procollagen α1(I) secretion into cellular medium by about 50% at 40 μM (lane 3), while lower doses were ineffective (lanes 1 and 2).

Compound 654 was tested at concentrations of 5 μM and 20 μM. At these concentrations there was no increase in number of apoptotic cells after 24 h, as judged by staining with YO-PRO-1 and propidium iodide (Fig. 3A). However, at 20 μM we observed morphological changes of human fibroblasts (Fig. 3A), which was evident within 30 min of addition of 654. Under higher magnification the cells showed more elongated shape, which remained unchanged during 24 h and 48 h of incubation (Fig. 3B). At 5 μM, 654 did not have any effect on cell morphology, while it became toxic at 40 μM (not shown). The effect of 654 on procollagen synthesis was analyzed by western blot. At 20 μM, 654 caused reduction of cellular level of procollagen α1(I) level by about 80% (Fig. 3C, lane 3), while it had a minimal effect at 5 μM (lane 2). Likewise, 20 μM of 654 decreased accumulation of procollagen α1(I) into cellular medium to almost undetectable level (Fig. 3D, lanes 5 and 6), while at 5 μM this decrease was about 20–30% (lanes 3 and 4). We also measured accumulation of another extracellular matrix protein, fibronectin. 654 inhibited secretion of fibronectin to a similar extent as that of procollagen (Fig. 3E).

From these experiments we concluded that both compounds, 588 and 654 can inhibit synthesis of procollagen and fibronectin by human fibroblasts, however 654 seemed to be more potent with almost complete inhibition seen at 20 μM. Therefore, we decided to study the effects of 654 on other collagen producing cells, activated human HSCs.

Effects of 654 on human HSCs

Immortal human HSC line with activated phenotype was described by Schnabl et al. (Schnabl et al., 2002). HSCs were incubated with 5 μM and 20 μM of 654 for 48 h. At 20 μM, 654 caused morphological changes of activated HSCs, with rounding of cells and appearance of thin cytoplasmic protrusions (Fig. 4A). At this dose we did not see a significant cell toxicity, as assessed by propidium iodide staining (Fig. 4A, bottom panels). At 20 μM, 654 decreased intracellular level of procollagen α1(I) by 50–80%, depending on the experiment (Fig. 4B, lanes 2, 3 and 7). With 5 μM in one experiment we saw a decrease of 20–30% (lane 1), however this was not always reproduced (lane 6). The levels of α-tubulin and calnexin were unchanged with addition of 654 (Fig. 4B). Similar to fibroblasts, secretion of procollagen α1(I) from HSCs was completely abolished by 20 μM of 654 after 48 h (Fig. 4C, lane 3). 5 μM of 654 was ineffective to inhibit procollagen secretion (lane 2). Secretion of fibronectin was inhibited by 20 μM of 654 to about 50% (Fig. 4D, lane 3).

654 does not change the steady-state level of collagen α1(I) mRNA

To provide insight into a mechanism by which 654 may act, we first analyzed subcellular distribution of 654 employing its autofluorescence. 654 was added to HSCs at 5 μM and after 30 min the image of the cells was taken under UV light. Autofluorescence of 654 was seen throughout the cytoplasm, while the nuclei were mostly excluded (Fig. 5A). Control cells showed no autofluorescence. This indicates that the compound is rapidly taken up by the cells and that it accumulates in the cytoplasm.

Next, we wanted to see if 654 affects the steady state level of collagen mRNAs. Fibroblasts were treated as in Fig. 3 and total RNA extracted and analyzed by RT-PCR for expression of: 1. collagen α1(I) mRNA and α2(I) mRNA, which encode for type I collagen, 2. α1(III) mRNA, which encodes for

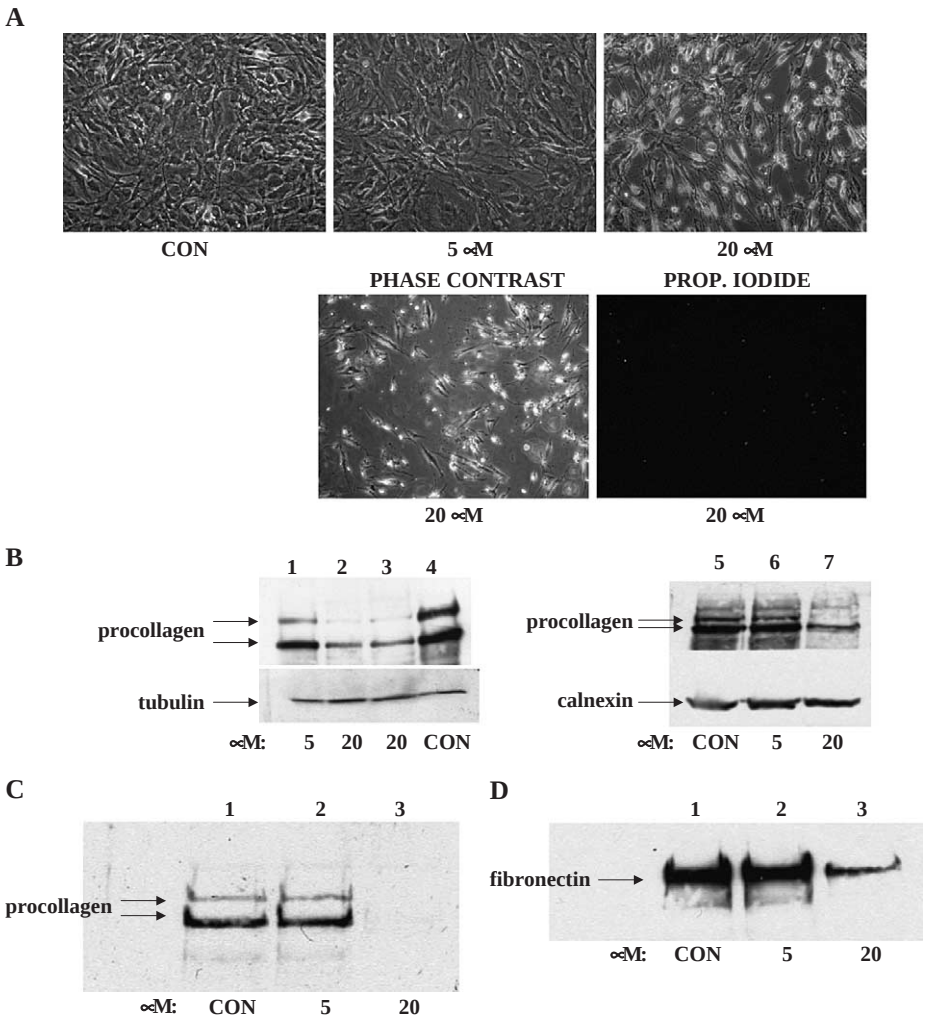


Fig. 4. 654 decreases procollagen in activated human HSCs. A. Change in morphology of HSCs. Cells were treated with 5 μM (top middle panel) or 20 μM (top right panel) or without 654 (CON, top left panel) for 48 h. The images were taken under phase contrast Bottom left panel shows cells treated with 20 μM of 654 for 48 h and bottom right panel the same cells after staining with propidium iodide. The latter image was taken under UV light. B. 654 decreases intracellular level of procollagen α1(I) protein. Cells were treated in two independent experiments with 5 μM (lanes 1 and 6) or 20 μM (lanes 2, 3 and 7) of 654 for 48 h and cellular proteins analyzed as in Fig. 2B. CON (lane 4 and 5) are control untreated cells. The samples were reprobed for expression of α-tubulin in experiment 1 (left panel) and for calnexin in experiment 2 (right panel). C. 654 decreases accumulation of procollagen α1(I) in cellular medium. Cells were treated with 5 μM (lane 2) or 20 μM (lane 3) or without 654 (CON, lane 1) and cellular medium analyzed as in Fig. 2C. D. 654 decreases accumulation of fibronectin in cellular medium. The samples from C were probed for expression of fibronectin by western blotting.

another fibrillar collagen, type III, and 3. GAPDH mRNA, as control. As shown in Fig. 5B, the steady-state level of all three collagen mRNAs was unchanged with addition of 654 for 24 h. The level of GAPDH mRNA was also unchanged. This suggests that the decrease in procollagen protein synthesis by 654 was not due to inhibition of transcription of collagen genes or decrease in stability of collagen

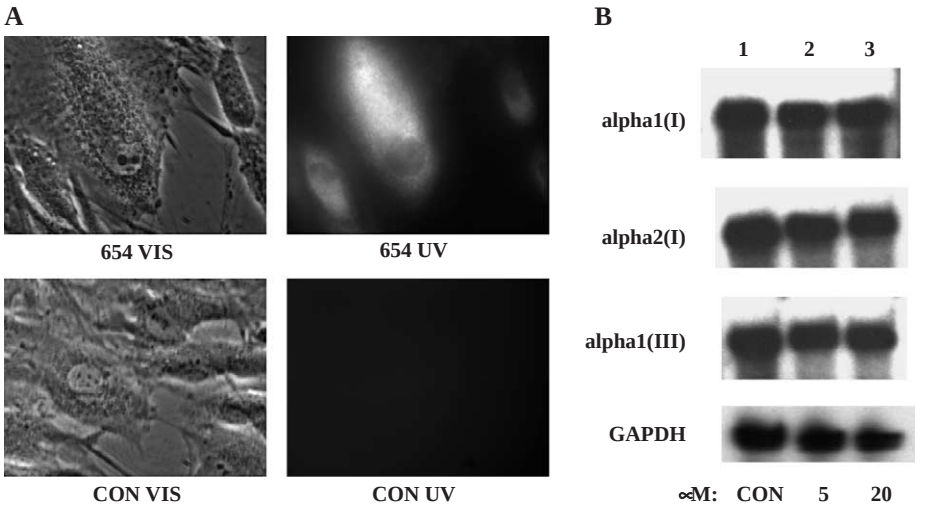


Fig. 5. 654 accumulates in cytoplasm but does not change the steady-state level of collagen mRNAs. A. Subcellular accumulation of 654. HSCs were incubated with 5 μM of 654 for 30 min and images taken under phase contrast (654 VIS, upper left panel) or under UV light (654 UV, upper right panel). CON are control untreated cells. B. Steady-state level of three collagen mRNAs remains unchanged with 654. Cells were treated with 654 as in Fig. 4 and total RNA was analyzed by RT-PCR for expression of collagen α1(I) mRNA, α2(I) mRNA, α1(III) mRNA and GAPDH mRNA, as control. Migration of the relevant bands is indicated to the left.

mRNAs. Since this compound does not accumulate in the nucleus an effect on transcription was not unexpected, but we concluded that cytoplasmic regulation of collagen mRNA stability (Stefanovic et al., 1997) was not affected by 654.

654 inhibits translation of a protein with collagen signal peptide

To investigate a possibility that 654 inhibits translation of collagen mRNA we designed two reporter genes (Fig. 6A). One reporter gene contained 220 nt of mouse collagen α1(I) promoter, the α1(I) 5′ UTR followed by the start codon and first 8 codons of collagen α1(I) open reading frame. This was ligated in frame to the second codon of luciferase mRNA. This gene encodes for protein luciferase activity which has 9 amino-acids of mouse collagen α1(I) at its N-terminus and was named COLL/LUC. Because only 9 amino-acids were derived from collagen, such short sequence is insufficient to serve as a signal peptide (Nothwehr and Gordon, 1989). Therefore this mRNA is translated by cytosolic ribosomes and serves as a representative of mRNAs encoding cellular proteins. The other gene is identical, except the sequence encoding the first 22 amino-acids of collagen α1(I), representing the complete signal peptide, was ligated in frame to luciferase gene. This mRNA encodes for functional signal peptide and is targeted for translation to the ribosomes associated with the membrane of ER (Hegde and Lingappa, 1999). It was named SP-COLL/LUC and is a representative of mRNAs encoding secreted proteins. The genes were transiently transfected into human fibroblasts, the cells were divided into two halves and one half was treated with 20 μM of 654, while the other half was left untreated (control). The luciferase activity was measured, normalized to total protein in the samples and expressed as percentage of control. As shown in Fig.

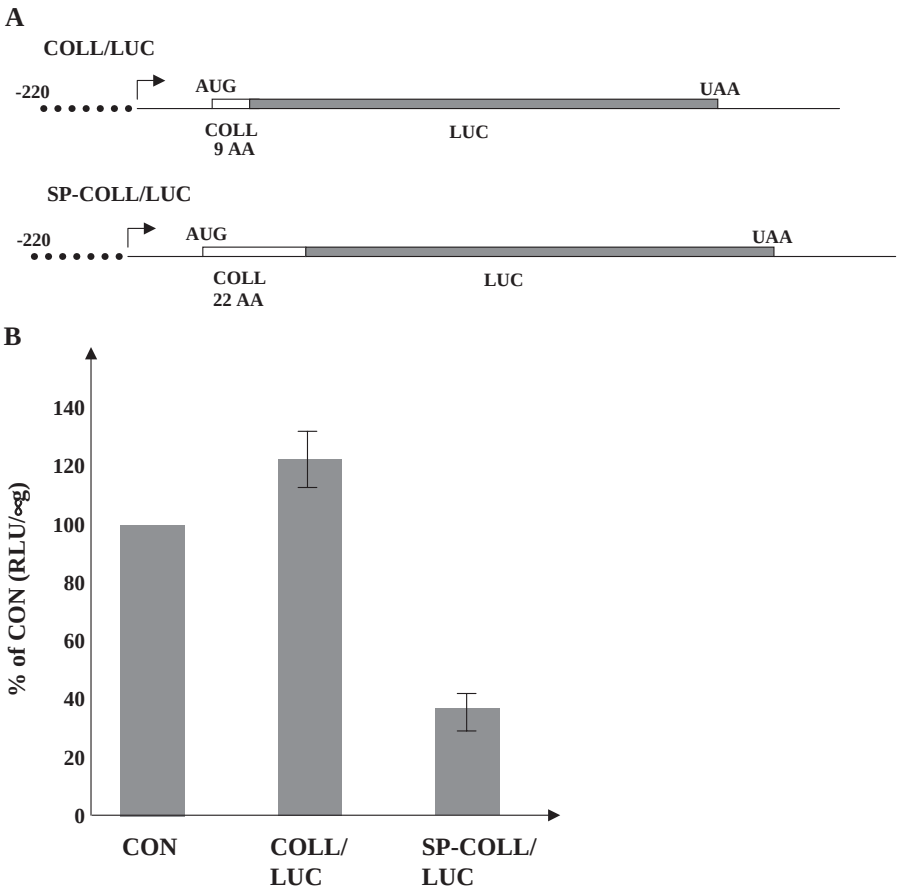


Fig. 6. 654 decreases expression of a reporter gene containing collagen signal peptide. A. Schematic representation of the constructs. Reporter genes were driven by 220 nt of mouse collagen α1(I) promoter (thick dotted line). Transcription start site is indicated by an arrow, 5′ UTR from collagen α1(I) is shown as thin solid line and amino-acids encoded by collagen α1(I) gene are shown as open box. Luciferase open reading frame was fused in frame with the collagen sequence and is shown as gray box. 3' UTR was from pGL3 vector and is shown as thin dotted line. Start and stop codons are indicated. COLL/LUC gene encodes for only 9 amino-acids of collagen signal peptide and SP-COLL/LUC encodes for all 22 amino-acids of collagen signal peptide. B. Expression of reporter genes after treatment with 654. COLL/LUC and SP-COLL/LUC were transiently transfected into human fibroblasts and luciferase activity was determined after 24 h treatment with 20 μM of 654. Results were calculated as relative luciferase units (RLU)/μg of total protein and shown as percentage, relative to the activity in control, untreated cells. Error bars represent ± 1 SD as calculated from two independent experiments performed in duplicate.

6B, 654 slightly increased the expression of COLL/LUC gene, however, expression of SP-COLL/LUC was decreased to 40% of control. From this result we concluded that 654 selectively inhibits synthesis of proteins containing a signal peptide and does not act as a general inhibitor of translation.

To corroborate that 654 acts by decreasing translation of proteins with signal peptide, like collagen and fibronectin, and not by increasing their degradation, we treated fibroblasts with proteosome inhibitor MG132 either alone or in combination with 654. Procollagen which can not be secreted out of the cell is degraded by a proteosome mediated mechanism (Fitzgerald et al., 1999; Stefanovic et al., 2004). As

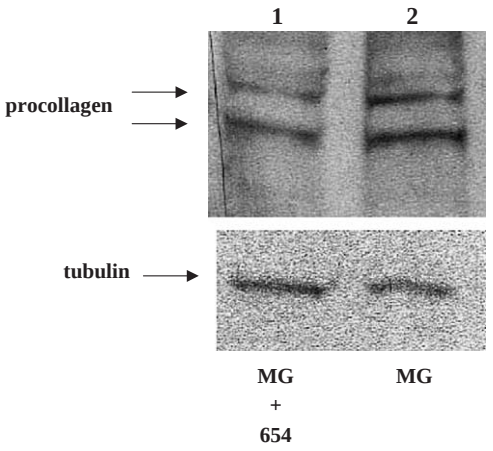


Fig. 7. 654 decreases procollagen in presence of an inhibitor of protein degradation. Human fibroblasts were treated with 100 μM of proteosome inhibitor MG132 (lane 2) or 100 μM of MG132 plus 20 μM of 654 (lane 1) for 24 h. Intracellular procollagen was measured by western blot as in Fig. 2B.

shown in Fig. 7, in the presence of MG132 654 was still able to reduce the cellular level of procollagen α1(I) (lane 1), as compared to MG132 alone (lane 2). The level of α-tubulin remained the same in both samples (bottom panel). This suggests that 654 decreases procollagen steady-state level by inhibiting its synthesis and not by stimulating protein degradation.

Discussion

Fibroproliferative disorders are progressive and, once initiated, uncontrolled production of extracellular matrix composed of type I collagen and fibronectin disrupts normal tissue architecture (Bitterman and Henke, 1991). Treatment of fibroproliferative disorders has been so far only symptomatic and drugs with ability to suppress synthesis of fibrous extracellular matrix are needed. The progression of most fibroproliferative disorders is slow (Chatziantoniou et al., 2004; McCaughan and George, 2004; Thannickal et al., 2004), therefore, inhibition of production of extracellular matrix even to a small degree would significantly delay the onset of organ failure. A series of diphenylfuran cations as potential RNA binding drugs and inhibitors of the Rev-Rev responsive element (RRE) complex have been designed and synthesized (Gelus et al., 1999; Li et al., 2001; Xiao et al., 2001). Analysis of the interaction of the diphenylfurans with RRE, as well as TAR RNA, indicated that they bind RNA in sequence and structure-dependent manner. Stability and translation of collagen α1(I) mRNA are dependent on binding of an RNA binding protein to the 5′ stem-loop located in the 5′ UTR (Stefanovic and Brenner, 2003; Stefanovic et al., 1999; Stefanovic et al., 2000; Stefanovic et al., 2002). Based on the ability of diphenylfurans to bind structured RNA we surmised that some compounds may interfere with binding of the 5′ stem-loop binding protein to the collagen α1(I) mRNA. Therefore, we have tested 21 related diphenylfurans for their ability to inhibit collagen synthesis by human HSCs and fibroblasts. Only two compounds, 588 and 654, showed significant reduction of collagen synthesis. Both compounds present quite similar spatial arrays for the two cationic centers. In each case the cationic centers are six atoms

removed from the diphenyl furan core. The major difference in the two structures is that 588 has an additional potential hydrogen bond donor in the cationic side chain. 588 was able to reduce collagen content by 50% when used at 40 μM (Fig. 2) and 654 reduced collagen by more than 80% at 20 μM (Figs. 3 and 4). However, since 654 also inhibited synthesis of fibronectin, the mRNA of which does not have a 5′ stem-loop, the mode of action is probably not by the inhibiting interaction of the 5′ stem-loop with its cognate RNA binding protein. More likely 654 inhibits translation of secreted proteins, because it decreased expression of luciferase protein when we made it as a fusion protein with the collagen signal peptide (Fig. 6). Without the signal peptide expression of luciferase protein was slightly increased (Fig. 6). Also, intracellular proteins, like α-tubulin and calnexin were not affected (Fig. 4). For activated HSCs and fibroblasts, most of the secreted proteins are components of extracellular matrix, so inhibition of synthesis of some or all of them is a desirable property.

How 654 selectively inhibits translation of secreted proteins is not known. Secreted proteins have a stretch of 20–25 hydrophobic amino-acids at the N-terminus called signal peptide (Nothwehr and Gordon, 1989). Translation of these proteins starts on cytosolic ribosomes and pauses after the signal peptide has been synthesized. Signal peptide is then recognized by signal recognition particle (SRP) which targets the ribosome/mRNA/signal peptide complex to the membrane of ER. Protein synthesis resumes by the membrane associated ribosomes and the protein is cotranslationaly inserted into the lumen of ER (Hegde and Lingappa, 1999). SRP is a ribonucleoprotein complex assembled from 6 proteins and 300 nt long 7SL RNA (Halic et al., 2004). It is possible that 654 interacts with 7SL RNA and interferes with the assembly or function of SRP. Alternatively, membrane bound ribosomes may have a different conformation from free cytosolic ribosomes (Lerner et al., 2003), which may allow binding of 654 to the ribosomal RNA of this ribosomal subset. Binding of 654 to ribosomal RNA may interfere with its function either as the catalyst of peptide bond formation (Green et al., 1998) or as a backbone for ribosomal proteins assembly (Ban et al., 1999). Cytoplasmic accumulation of 654 (Fig. 5) is consistent with the compound being an inhibitor of translation. It did not affect the steady state of collagen mRNAs (Fig. 5) nor increased procollagen protein degradation (Fig. 7).

Conclusions

654 and 588 were able to reduce collagen synthesis by 50 to 80% in two collagen producing cells, activated HSCs and fibroblasts. The effect was seen after 24 h to 48 h and at 20 μM and 40 μM, respectively, while no effect was seen at 5 μM for 654 and 30 μM for 588. Both compounds exhibited a toxic effect at a dose which was twice the effective dose. However, it is possible that a significant inhibition can be achieved with lower doses of 654 after prolonged administration in vivo. Activation of HSCs in vitro provides the strongest stimulus for collagen synthesis and usually the levels of collagen mRNA and protein are higher in culture activated HSCs than HSCs activated by animal models of liver injury (De Leeuw et al., 1984; Friedman, 1999b; Friedman et al., 1992; Geerts et al., 1989; Hellerbrand et al., 1999; Lindquist et al., 2000; Rojkind and Greenwel, 1993). Therefore, testing of 654 and related compounds in prolonged administration using animal models is needed. However, this study indicates a possibility that antifibrotic drugs can be developed based on the structure of 654 and 588 compounds.

References

Aycock, R.S., Seyer, J.M., 1989. Collagens of normal and cirrhotic human liver. Connect Tissue Res 23 (1), 19–31.

Ban, N., Nissen, P., Hansen, J., Capel, M., Moore, P.B., Steitz, T.A., 1999. Placement of protein and RNA structures into a 5 A-resolution map of the 50S ribosomal subunit. Nature 400 (6747), 841–847.

Beck, K., Boswell, B.A., Ridgway, C.C., Bachinger, H.P., 1996. Triple helix formation of procollagen type I can occur at the rough endoplasmic reticulum membrane. J Biol Chem 271 (35), 21566–21573.

Birk, D.E., Zycband, E.I., Woodruff, S., Winkelmann, D.A., Trelstad, R.L., 1997. Collagen fibrillogenesis in situ: fibril segments become long fibrils as the developing tendon matures. Dev Dyn 208 (3), 291–298.

Bitterman, P.B., Henke, C.A., 1991. Fibroproliferative disorders. Chest 99 (3 Suppl), 81S–84S.

Boykin, D.W., Kumar, A., Xiao, G., Wilson, W.D., Bender, B.C., McCurdy, D.R., Hall, J.E., Tidwell, R.R., 1998. 2,5-bis[4-(N-alkylamidino)phenyl]furans as anti-Pneumocystis carinii agents. J Med Chem 41 (1), 124–129.

Chatziantoniou, C., Boffa, J.J., Tharaux, P.L., Flamant, M., Ronco, P., Dussaule, J.C., 2004. Progression and regression in renal vascular and glomerular fibrosis. Int J Exp Pathol 85 (1), 1–11.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162 (1), 156–159.

de Leeuw, A.M., McCarthy, S.P., Geerts, A., Knook, D.L., 1984. Purified rat liver fat-storing cells in culture divide and contain collagen. Hepatology 4 (3), 392–403.

Ezquerro, I.J., Lasarte, J.J., Dotor, J., Castilla-Cortazar, I., Bustos, M., Penuelas, I., Blanco, G., Rodriguez, C., Lechuga Mdel, C., Greenwel, P., Rojkind, M., Prieto, J., Borras-Cuesta, F., 2003. A synthetic peptide from transforming growth factor beta type III receptor inhibits liver fibrogenesis in rats with carbon tetrachloride liver injury. Cytokine 22 (1–2), 12–20.

Fitzgerald, J., Lamande, S.R., Bateman, J.F., 1999. Proteasomal degradation of unassembled mutant type I collagen pro-alpha1(I) chains. J Biol Chem 274 (39), 27392–27398.

Friedman, S.L., 1996. Hepatic stellate cells. Prog Liver Dis 14, 101–130.

Friedman, S.L., 1999a. Cytokines and fibrogenesis. Semin Liver Dis 19 (2), 129–140.

Friedman, S.L., 1999b. Stellate cell activation in alcoholic fibrosis-an overview. Alcohol Clin Exp Res 23 (5), 904–910.

Friedman, S.L., Rockey, D.C., McGuire, R.F., Maher, J.J., Boyles, J.K., Yamasaki, G., 1992. Isolated hepatic lipocytes and Kupffer cells from normal human liver: morphological and functional characteristics in primary culture. Hepatology 15 (2), 234–243.

Geerts, A., Vrijsen, R., Rauterberg, J., Burt, A., Schellinck, P., Wisse, E., 1989. In vitro differentiation of fat-storing cells parallels marked increase of collagen synthesis and secretion. J Hepatol 9 (1), 59–68.

Gelus, N., Bailly, C., Hamy, F., Klimkait, T., Wilson, W.D., Boykin, D.W., 1999. Inhibition of HIV-1 Tat-TAR interaction by diphenylfuran derivatives: effects of the terminal basic side chains. Bioorg Med Chem 7 (6), 1089–1096.

Green, R., Switzer, C., Noller, H.F., 1998. Ribosome-catalyzed peptide-bond formation with an A-site substrate covalently linked to 23S ribosomal RNA. Science 280 (5361), 286–289.

Halic, M., Becker, T., Pool, M.R., Spahn, C.M., Grassucci, R.A., Frank, J., Beckmann, R., 2004. Structure of the signal recognition particle interacting with the elongation-arrested ribosome. Nature 427 (6977), 808–814.

Hegde, R.S., Lingappa, V.R., 1999. Regulation of protein biogenesis at the endoplasmic reticulum membrane. Trends Cell Biol 9 (4), 132–137.

Hellerbrand, C., Stefanovic, B., Giordano, F., Burchardt, E.R., Brenner, D.A., 1999. The role of TGFbeta1 in initiating hepatic stellate cell activation in vivo. J Hepatol 30 (1), 77–87.

Hendriks, H.F., Verhoofstad, W.A., Brouwer, A., de Leeuw, A.M., Knook, D.L., 1985. Perisinusoidal fat-storing cells are the main vitamin A storage sites in rat liver. Exp Cell Res 160 (1), 138–149.

Kagan, H.M., Li, W., 2003. Lysyl oxidase: properties, specificity, and biological roles inside and outside of the cell. J Cell Biochem 88 (4), 660–672.

Kivirikko, K.I., 1998. Collagen biosynthesis: a mini-review cluster. Matrix Biol 16 (7), 355–356.

Knittel, T., Kobold, D., Saile, B., Grundmann, A., Neubauer, K., Piscaglia, F., Ramadori, G., 1999. Rat liver myofibroblasts and hepatic stellate cells: different cell populations of the fibroblast lineage with fibrogenic potential [see comments]. Gastroenterology 117 (5), 1205–1221.

Lansiaux, A., Dassonneville, L., Facompre, M., Kumar, A., Stephens, C.E., Bajic, M., Tanious, F., Wilson, W.D., Boykin, D.W., Bailly, C., 2002. Distribution of furamidine analogues in tumor cells: influence of the number of positive charges. J Med Chem 45 (10), 1994–2002.

Lerner, R.S., Seiser, R.M., Zheng, T., Lager, P.J., Reedy, M.C., Keene, J.D., Nicchitta, C.V., 2003. Partitioning and translation of mRNAs encoding soluble proteins on membrane-bound ribosomes. Rna 9 (9), 1123–1137.

Li, K., Davis, T.M., Bailly, C., Kumar, A., Boykin, D.W., Wilson, W.D., 2001. A heterocyclic inhibitor of the REV-RRE complex binds to RRE as a dimer. Biochemistry 40 (5), 1150–1158.

Lindquist, J.N., Marzluff, W.F., Stefanovic, B., 2000. Fibrogenesis: III. Posttranscriptional regulation of type I collagen. Am J Physiol Gastrointest Liver Physiol 279 (3), G471–G476.

Lo, I.K., Marchuk, L.L., Leatherbarrow, K.E., Frank, C.B., Hart, D.A., 2004. Collagen fibrillogenesis and mRNA levels in the maturing rabbit medial collateral ligament and patellar tendon. Connect Tissue Res 45 (1), 11–22.

Maher, J.J., McGuire, R.F., 1990. Extracellular matrix gene expression increases preferentially in rat lipocytes and sinusoidal endothelial cells during hepatic fibrosis in vivo. J Clin Invest 86 (5), 1641–1648.

McCaughan, G.W., George, J., 2004. Fibrosis progression in chronic hepatitis C virus infection. Gut 53 (3), 318–321.

Minato, M., Kubota, S., Kawaki, H., Nishida, T., Miyauchi, A., Hanagata, H., Nakanishi, T., Takano-Yamamoto, T., Takigawa, M., 2004. Module-specific antibodies against human connective tissue growth factor: utility for structural and functional analysis of the factor as related to chondrocytes. J Biochem (Tokyo) 135 (3), 347–354.

Myllyharju, J., Kivirikko, K.I., 2004. Collagens, modifying enzymes and their mutations in humans, flies and worms. Trends Genet 20 (1), 33–43.

Nagan, N., Callery, P.S., Kagan, H.M., 1998. Aminoalkylaziridines as substrates and inhibitors of lysyl oxidase: specific inactivation of the enzyme by N-(5-aminopentyl)aziridine. Front Biosci 3, A23–A26.

Nagan, N., Kagan, H.M., 1994. Modulation of lysyl oxidase activity toward peptidyl lysine by vicinal dicarboxylic amino acid residues. Implications for collagen cross-linking. J Biol Chem 269 (35), 22366–22371.

Nakamura, T., Sakata, R., Ueno, T., Sata, M., Ueno, H., 2000. Inhibition of transforming growth factor beta prevents progression of liver fibrosis and enhances hepatocyte regeneration in dimethylnitrosamine-treated rats. Hepatology 32 (2), 247–255.

Nothwehr, S.F., Gordon, J.I., 1989. Eukaryotic signal peptide structure/function relationships. Identification of conformational features which influence the site and efficiency of co-translational proteolytic processing by site-directed mutagenesis of human pre(delta pro)apolipoprotein A-II. J Biol Chem 264 (7), 3979–3987.

Pinzani, M., Marra, F., Carloni, V., 1998. Signal transduction in hepatic stellate cells. Liver 18 (1), 2–13.

Rojkind, M., Greenwel, P., 1993. Animal models of liver fibrosis. Adv Vet Sci Comp Med 37, 333–355.

Saika, S., Ooshima, A., Hashizume, N., Yamanaka, O., Tanaka, S., Okada, Y., Kobata, S., 1995. Effect of lysyl hydroxylase inhibitor, minoxidil, on ultrastructure and behavior of cultured rabbit subconjunctival fibroblasts. Graefes Arch Clin Exp Ophthalmol 233 (6), 347–353.

Sakaida, I., Uchida, K., Hironaka, K., Okita, K., 1999. Prolyl 4-hydroxylase inhibitor (HOE 077) prevents TIMP-1 gene expression in rat liver fibrosis. J Gastroenterol 34 (3), 376–377.

Sauk, J.J., Smith, T., Norris, K., Ferreira, L., 1994. Hsp47 and the translation-translocation machinery cooperate in the production of alpha 1(I) chains of type I procollagen. J Biol Chem 269 (6), 3941–3946.

Schnabl, B., Choi, Y.H., Olsen, J.C., Hagedorn, C.H., Brenner, D.A., 2002. Immortal activated human hepatic stellate cells generated by ectopic telomerase expression. Lab Invest 82 (3), 323–333.

Schuppan, D., 1990. Structure of the extracellular matrix in normal and fibrotic liver: collagens and glycoproteins. Semin Liver Dis 10 (1), 1–10.

Stefanovic, B., Brenner, D.A., 2003. 5′ stem-loop of collagen alpha 1(I) mRNA inhibits translation in vitro but is required for triple helical collagen synthesis in vivo. J Biol Chem 278 (2), 927–933.

Stefanovic, B., Hellerbrand, C., Brenner, D.A., 1999. Regulatory role of the conserved stem-loop structure at the 5′ end of collagen alpha1(I) mRNA. Mol Cell Biol 19 (6), 4334–4342.

Stefanovic, B., Hellerbrand, C., Holcik, M., Briendl, M., Aliebhaber, S., Brenner, D.A., 1997. Posttranscriptional regulation of collagen alpha1(I) mRNA in hepatic stellate cells. Mol Cell Biol 17 (9), 5201–5209.

Stefanovic, B., Lindquist, J., Brenner, D.A., 2000. The 5' stem-loop regulates expression of collagen alpha1(I) mRNA in mouse fibroblasts cultured in a three-dimensional matrix. Nucleic Acids Res 28 (2), 641–647.

Stefanovic, B., Schnabl, B., Brenner, D.A., 2002. Inhibition of collagen alpha 1(I) expression by the 5′ stem-loop as a molecular decoy. J Biol Chem 277 (20), 18229–18237.

Stefanovic, B., Stefanovic, L., Schnabl, B., Bataller, R., Brenner, D.A., 2004. TRAM2 protein interacts with endoplasmic reticulum Ca2+ pump Serca2b and is necessary for collagen type I synthesis. Mol Cell Biol 24 (4), 1758–1768.

Thannickal, V.J., Toews, G.B., White, E.S., Lynch III, J.P., Martinez, F.J., 2004. Mechanisms of pulmonary fibrosis. Annu Rev Med 55, 395–417.

Wang, J., Buss, J.L., Chen, G., Ponka, P., Pantopoulos, K., 2002. The prolyl 4-hydroxylase inhibitor ethyl-3,4-dihydroxybenzoate generates effective iron deficiency in cultured cells. FEBS Lett 529 (2–3), 309–312.

Xiao, G., Kumar, A., Li, K., Rigl, C.T., Bajic, M., Davis, T.M., Boykin, D.W., Wilson, W.D., 2001. Inhibition of the HIV-1 rev-RRE complex formation by unfused aromatic cations. Bioorg Med Chem 9 (5), 1097–1113.