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Mithramycin Selectively Inhibits Collagen-α1(I) Gene Expression in Human Fibroblast

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Abstract

The products of the collagen-α1(I) and -α2(I) genes form the triple helical molecule collagen type I, which constitutes the major ECM protein in tissue fibrosis. The collagen-α1(I) gene is mainly transcriptionally regulated, and its promoter activity depends on the interaction of the transcription factors NF-1 and Sp1 with a tandem repeat of evolutionary conserved NF-1/Sp1 switch elements. An increased affinity of Sp1 to these elements has been observed in experimental liver fibrosis. Here, we demonstrate that the DNA binding drug mithramycin displays a high affinity binding to the GC-rich elements in the collagen-α1(I) promoter as measured by DNase I protection and gel retardation assays.

Mithramycin interferes with Sp1 but not with NF-I binding to these sites. At a concentration of 100 nM, mithramycin efficiently reduces basal and TGF-β-stimulated α1(I) gene expression in human primary fibroblasts. The transcriptional activity and mRNA steady state levels of other genes, including the collagenase gene, as well as the growth rate of fibroblasts remained unchanged on exposure to this drug. Taken together, our results indicate that the transcriptional activity of the type I collagen gene highly depends on its GC-rich regulatory elements, and further, that these elements can be differentially blocked, thereby changing the balance between ECM structural and degrading gene activities in human fibroblasts. (J. Clin. Invest. 1993. 92:2916–2921.) Key words: mithramycin • collagen α1(I) • regulation • gene expression • Sp1

Introduction

The synthesis and deposition of ECM proteins is highly regulated and plays a critical role in tissue development, homeostasis, wound healing, and inflammation (1–3). Overexpression of ECM proteins may lead to tissue fibrosis with severe impairment of organ function. A hallmark of fibrotic diseases such as pulmonary fibrosis, hepatic cirrhosis, progressive systemic sclerosis, and osteomyelofibrosis, is the pathological accumulation of the ECM proteins collagen type I and fibronectin (4–7).

Type I procollagen mRNA levels are increased in experimental and clinical fibrotic states (6, 8, 9), which implies a regulation at the pretranslational level. Colocalization of members of the TGF-β family of growth and differentiation factors with ECM proteins in affected tissues (3, 8) and the stimulatory effect of TGF-β on collagen and fibronectin steady state mRNA levels (3, 10) suggest an important role of these growth factors in tissue fibrosis. Collagen type I is a triple helical molecule composed of two α1(I) and one α2(I) collagen gene products. Recent progress has led to the identification of important regulatory elements of the murine and human α1(I) gene. Several binding sites for the GC box binding transcription factor Sp1 were located in the first intron of the human gene (11), to which enhancer-like activities were ascribed (12). In addition, two highly conserved GC box elements were functionally characterized in the promoter of the murine α1(I) gene (13). Both elements also contain an overlapping CCAAT box motif and function individually as mutually exclusive binding sites for the transcription factors NF-1 (CCAAT box factor) and Sp1 (GC box factor) thereby creating a direct repeat of NF-I/Sp1 switch elements (14). The two elements control the high basal activity of the murine α1(I) gene in fibroblasts (15), which contain both high NF-I and Sp1 binding activity. In contrast, only very low amounts of Sp1 binding activities can be detected in a normal liver where only minimal amounts of α1(I) transcripts can be measured. Interestingly, this weak binding affinity of Sp1 to the α1(I) promoter was substantially increased and correlated with induced α1(I) mRNA levels in different models of experimental liver fibrosis (16).

Under the assumption that GC-rich elements play a crucial role in determining the transcriptional activity of the human α1(I) gene, we have examined the effect of the GC-binding antibiotic mithramycin (16, 18–20) on collagen gene regulation in human primary fibroblasts. Our results reveal that mithramycin inhibits Sp1 but not NF-I binding to the NF-I/Sp1 switch elements in vitro.

At nontoxic doses, mithramycin selectively decreases basal α1(I) collagen gene transcription and inhibits TGF-β1 induction of α1(I) mRNA levels in human primary fibroblasts. This indicates that although the activity of the collagen-α1(I) gene is highly dependent on GC-rich regulatory elements, the activity of other genes, including the collagenase gene, is not.

Methods

Materials. Mithramycin was obtained from Sigma Chemical Co. (St. Louis, MO) as a powder that was resuspended in sterile water and was further diluted in culture medium before use. PMA (Sigma Chemical Co.) was dissolved in 100% ethanol and diluted in culture medium to a final concentration of 100 nM. Lyophilized human TGF-β1 was ob-
tained from Gibco BRL (Gaithersburg, MD) and was prepared as recommended by the manufacturer. All radiochemicals were obtained from Amersham International (Amersham, United Kingdom).

**Cell culture.** Primary human embryonic lung fibroblasts (strain FH109) were maintained at subconfluence in RPMI medium supplemented with 10% fetal calf serum and antibiotics in a 5% CO2 atmosphere in air. For the toxicity testing cells were plated out at low density on 35-mm tissue culture plates (Falcon Plastics, Cockeysville, MD) for 24 h. Next, media was replaced by fresh supplemented media containing either no or the indicated concentrations of mithramycin. Cell numbers were counted after trypsinization and trypan blue dye exclusion immediately, and 1, 5, and 8 h after addition of mithramycin. Average numbers of three independent experiments were calculated.

**Isolation and fractionation of RNA.** FH109 cells were scrapped off the culture plates and washed twice in PBS. RNA was extracted and purified according to the protocol by Huang and High (21). Total RNA (10 μg/lane) was fractionated on 1% agarose gels containing formaldehyde and transferred to nylon membrane filters (Schleicher & Schuell, Dassel, Germany) by standard procedure. Preparation and hybridization was carried out as described (22).

**Nuclear run-on assay.** For nuclear run on transcription assays, subconfluent FH109 cells (10^6 cells/experiment) were treated with the indicated concentrations of mithramycin for 3 h, scraped off the plates, washed several times in ice-cold PBS, and lysed by vortexing in 10 ml RSB (10 mM Tris-HCl, pH 7.5, 5 mM KCl, and 3 mM MgCl2) containing 0.5% Nonidet-P40 on ice for 5 min. Nuclei were collected by centrifugation at 500 g at 4°C for 5 min and washed once in TNM buffer (100 mM Tris-HCl, pH 7.5, 10 mM NaCl, and 3 mM MgCl2). After centrifugation at 500 g at 4°C for 5 min. nuclei were resuspended in 500 μl 1X reaction buffer (3X stock contains 60 mM Tris, pH 8.0, 30 mM MgCl2, 3 mM MnCl2, 420 mM KCl, 42 mM β-mercaptoethanol, and 60% glycerol), transferred to an Eppendorf tube, and spun again at 3,000 rpm/min at 4°C for 5 min to finally reduce the reaction volume to 150 μl. The nuclear run reaction was started by adding 125 μl reaction mix (250 μl 3X reaction buffer, 15 μl 100 mM ATP, 7.5 μl 100 mM GTP and CTP, 5 μl RNasin, and 340 μl DEPC H2O), and 25 μl [α-32P]UTP (10 μCi/μl, 3,000 Ci/mmoll) to the nuclei. After incubating the reaction at 26°C for 10 min, 1.5 μl of 0.2 M CaCl2 and 2.4 μl of RNase free DNase I (10 μg/μl) was added and incubated at 37°C for 15 min. The nuclear RNA was extracted by following the protocol of Huang and High (21). Generally 4-8 × 10^5 cpm radiolabeled RNA/reaction were extracted and hybridized to filter bound cDNA fragments (1 μg/spot) as described (22). After hybridization at 42°C for 72 h, filters were washed, air dried and exposed at -70°C to a X-O-Mat film (Eastman Kodak, Rochester, NY) with an intensifying screen (22). Autoradiograms were quantitated by a scanning laser densitometer interfaced with an integrator.

**cDNA and promoter probes.** The plasmid pHF677 (23) containing cDNA for the human α1(I) collagen and the plasmid pWS-2.5/CAT (24) containing the human α1(I) collagen promoter were kindly provided by Dr. F. Ramirez (Brookdale Center for Molecular Biology, Brookdale, NY). The plasmid pWS-2.5/CAT was restricted with PvuII (−252 bp) and BamHI (+123 bp) and the isolated promoter fragment was subcloned into pUC19 restricted with BamHI and SmaI yielding pUC19-α1(I). The plasmid pCIIase (25) containing the cDNA for human fibroblast collagenase was kindly provided by Dr. P. Herrlich (Kernforschungszentrum Karlsruhe, Karlsruhe, Germany). The plasmid pDT151-7 (26) for the human collagen-α1(III) cDNA was a gift of Dr. B. de Crombrugghe (M. D. Anderson Hospital and Tumor Institute, Houston, TX). The plasmid pHcGAP for human glyceraldehyde-3-phosphate dehydrogenase was purchased from American Type Culture Collection (Rockville, MD). The cDNA encoding c-jun was kindly provided by M. Kawasaki (University of California, San Diego) (27), and the cDNA of rat fibronectin was provided by R. O. Hynes (Massachusetts Institute of Technology, Cambridge, MA) (28).

**Nuclear protein extraction.** Nuclear proteins were extracted from cultured FH109 embryonic lung fibroblasts as previously described (15).

**DNAse I protection assay.** The plasmid pUC19-α1(I) was digested with EcoRI (multiple cloning site 5' of basepair position −252) and Smal (−17 bp) and the resulting fragment containing the α1(I) promoter was gel purified and end labeled by a fill in reaction at the EcoRI site with Klenow fragment of DNA polymerase I (Boehringer Mannheim GmbH, Mannheim, Germany) using [α-32P]dATP (3,000 Ci/mmoll). Either nuclear FH109 protein extracts, BSA, or mithramycin were incubated with 30 kcpm/reaction of probe for 20 min on ice. DNAse I protection assays were further carried out essentially as described (15).

**Gel retardation assay.** The FP2 oligonucleotide spanning the promoter sequences from −131 to −110 bp (15) was end labeled with T4 kinase (Boehringer Mannheim GmbH) using [α-32P]dATP (3,000 Ci/mmoll). The probe (20 kcpm/reaction) was either incubated alone or with 20 μg nuclear FH109 protein extracts containing no or increasing concentrations of mithramycin ranging from 10 nM to 100 μM. The standard binding reaction was carried out at 20°C for 20 min as described previously (29).

**Results**

**Collagen (I) and collagenase mRNA levels.** Human FH109 primary fibroblast cells were exposed to the antibiotic mithramycin. As measures of toxicity, we assessed cell growth, morphology and viability of FH109 cells cultured in the presence of different concentrations of mithramycin over a period of 8 d. At concentrations of mithramycin ranging from 10 nM to 100 nM, cell growth and morphology were not altered as compared to untreated controls. A fivefold reduction in cell growth was observed when a mithramycin concentration of 1 μM was used, while cell morphology remained unchanged (data not shown). However, concentrations as high as 10 μM resulted in complete cell death after 4 d (Fig. 1).

In the next set of experiments, total RNA was extracted

![Figure 1. Toxicity testing of mithramycin on cultured primary human FH109 fibroblast cells. FH109 cells were cultured as described in Methods. After 24 h, the medium was changed containing either no control) or the indicated concentrations of mithramycin. Cell morphology was examined daily by light microscopy. At the indicated time points, the FH109 cells were trypsinized, and viable cells were counted after trypan blue exclusion. The experiments were carried out in triplicate and the average numbers are shown. * , Control; ** , 10 nM; , 100 nM; ** , 1 μM; ** , 10 μM. 

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from control and mithramycin-treated FH109 cells after 24 and 48 h for Northern blot analysis. The results after hybridization with a human collagen-α1(I) and the human collagenase cDNA are shown in Fig. 2. Hybridization with the human α1(I) collagen gene probe revealed two transcripts of 4.7 and 5.7 kb in size, which result from the different usage of two polyadenylation signals ~1 kb apart (30). After 24 h, the α1(I) 4.7-kb transcript declined severalfold at all concentrations of mithramycin ranging from 10 nM to 10 μM (Fig. 2). A further decrease of α1(I) transcripts was observed after 48 h of incubation. In contrast, the steady state collagenase mRNA levels were reduced only at the toxic concentration of 10 μM but remained unaffected at concentrations ranging from 10 nM to 1 μM (Fig. 2).

**Protein kinase C (PKC) activation and mithramycin.** PKC activation by phorbol esters like PMA reduces steady state collagen-α1(I) mRNA levels (31) and stimulates collagenase transcription (32) in human fibroblasts. To study the interaction of mithramycin and PMA with respect to collagen-α1(I) mRNA expression, FH109 cells were incubated with both drugs for 1, 3, 6, and 24 h (Fig. 3). A concentration of 100 nM PMA (P) reduced α1(I) mRNA levels after 1 h with a maximal effect after 6 h when compared to controls (medium treatment only), while incubation of FH109 cells with mithramycin downregulated α1(I) mRNA levels after 3 h with still decreasing levels after 24 h, suggesting different kinetics of regulation. When both drugs were combined, the α1(I) transcript became even more reduced than with either drug alone, suggesting an additive effect that was best demonstrated after 24 h of treatment. Interestingly, while the strong increase of collagenase mRNA levels by PMA after 3, 6, and 24 h were reduced by the coinoculation with mithramycin, mithramycin treatment alone had no significant effect on steady state collagenase levels indicating that mithramycin directly or indirectly interferes with the enhanced collagenase induction by PMA.

The induction of the collagenase gene by PMA is mediated through an AP1 binding site in the collagenase promoter (reviewed in reference 33). We tested steady state mRNA levels of the proto-oncogene c-jun as one member of the AP1 complex (33) under the influence of both drugs. As shown in Fig. 3 both basal and early PMA-induced c-jun mRNA levels were unaffected after 1 and 3 h of coinoculation with mithramycin, but decreased below their respective control levels 6 and 24 h after mithramycin treatment. These data suggest that inhibition of the collagenase induction might be associated with a reduction of c-jun transcripts.

Preincubation of FH109 cells with a 100 nM concentration of mithramycin for 24 h resulted in a complete inhibition of PMA-induced transcription of the collagenase gene after 1 and 8 h of treatment (Fig. 4) when compared to the corresponding controls. In this experiment, it was also shown that incubation with human TGF-β1 at a 100 pM final concentration lead to a slight increase of α1(I) mRNA levels after 8 h in the absence of mithramycin, while a 24 h preincubation with mithramycin almost completely abolished this induction.

The mRNA levels of another important ECM protein in tissue fibrosis, fibronectin, were also tested (Fig. 3). While PMA alone shows a weak stimulatory effect after 24 h over control level, mithramycin decreased both basal and PMA-induced fibronectin mRNA levels (Fig. 3, lanes 13 and 15, respectively). Mithramycin had, however, no significant effect on the mRNA levels of the GAPDH housekeeping gene.

**Mithramycin interferes with transcription factor binding.** The first 500-bp upstream of the start site of transcription of the human α1(I) collagen gene is highly GC-rich (> 60%), as compared to the human collagenase promoter (GC-bp content = 45%). Since mithramycin has a high affinity to GC-rich stretches of DNA, we determined mithramycin binding sites in

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**Figure 2.** Northern blot analysis of total RNA (10 μg per lane) from mithramycin-treated FH109 fibroblasts. Cells were incubated for the indicated time (24 and 48 h) in medium containing either no mithramycin or mithramycin at a final concentrations of 10 μM, 1 μM, 100 nM, and 10 nM. The blot was sequentially probed with radiolabeled human collagen-α1(I), collagenase, and GAPDH cDNAs. Shown are densitometric plots after normalization of collagen-α1(I) and collagenase signals for GAPDH. Signal intensities from unstimulated cells were arbitrarily set 100%. –– , 24 h; --- , 48 h.

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**Figure 3.** Northern blot analysis of total RNA from mithramycin and PMA-treated FH109 fibroblasts. Cells were cultured at 70–80% confluence for the indicated time (1, 3, 6, and 24 h) in control medium or in medium containing either a 10 nM concentration of mithramycin, a 100 nM concentration of PMA, or both drugs combined. The same filter was probed, stripped, and reprobed with the indicated radiolabeled cDNAs including a GAPDH cDNA. Shown are densitometric plots after normalization of fibronectin, collagen-α1(I), c-jun, and collagenase signals for GAPDH. Signal intensities from unstimulated and uncultured cells were arbitrarily set 100%. --, Medium; ○, Mithramycin; ●, PMA; □, mithramycin/PMA.
w/o Mithramycin

![Graphs showing Northern blot analysis of total RNA from FH109 cells pre-treated with mithramycin.](image)

Figure 4. Northern blot analysis of total RNA from FH109 cells pre-treated with mithramycin. FH109 fibroblasts were cultured in medium containing either no or a 100 nM concentration of mithramycin for 24 h. Cells were then cultured in media supplemented with 1% fetal calf serum in the presence of human TGF-β1 at a 5 pM or PMA at a 100 nM final concentration for the indicated time period. The blot was hybridized with the human collagen-α1(I) oligonucleotide and GAPDH cDNA radiolabeled probes. Shown are densitometric plots after normalization of collagen-α1(I) and collagenase signals for GADPH. Signal intensities from unstimulated and uncultured cells were arbitrarily set 100%. α, PMA; δ, TGF.

the human α1(I) promoter by DNase I protection assays, alongside the footprints mediated by nuclear proteins from untreated FH109 cells (Fig. 5). This assay reveals a concentration-dependent binding of mithramycin (Fig. 5, lanes 2–4) to all GC-rich regions of the α1(I) promoter that are also protected from DNase I digest by the nuclear fibroblast extracts (Fig. 5, lanes 5 and 6, and schematic drawing). This result suggested the possibility that mithramycin might interfere with transcription factor binding to the collagen-α1(I) promoter.

Additional information on this possible interference of mithramycin with transcription factor binding was sought in gel retardation assays (Fig. 6). For this purpose, the FP2 (footprint 2) oligonucleotide, which contains an complete NF-I/ Sp1 switch element and spans from basepairs −130 to −110 of the α1(I) promoter, was prepared as a radiolabeled probe. While no FP2-probe was retarded in the absence of nuclear extract (Fig. 6, lane 1), both Sp1 and NF-I transcription factor binding activities were present in the FH109 protein extracts. The identity of both factors was tested in competition experiments using oligonucleotides with high affinity consensus binding sites for either factor ([15], and data not shown). Additionally, the nuclear extracts were coincubated with mithramycin ranging from 10 nM to 10 μM final concentration (Fig. 6, lanes 3–7, respectively). At 10 μM, mithramycin competed with the binding of Sp1 and increased NF-I binding to the FP2 probe (Fig. 6, lane 7).

![DNAse I protection assay of the human collagen-α1(I) promoter mediated by mithramycin or nuclear FH109 proteins.](image)

Figure 5. DNAse I protection of the human collagen-α1(I) promoter mediated by mithramycin or nuclear FH109 proteins. A DNA fragment containing the collagen-α1(I) promoter region (−252−17 bp) was radiolabeled at the −252 end. The G-specific Maxam and Gilbert sequencing reaction of the α1(I) promoter is shown in lane 1. The probe was incubated with binding buffer containing no or the indicated molar concentrations of mithramycin. The protection pattern with the two indicated amounts in milligrams of FH109 nuclear protein extracts is shown beside the control reaction containing 30 μg BSA. A schematic footprinting pattern of the DNAse I protections mediated by the nuclear extracts is also shown.

Mithramycin inhibits α1(I) collagen gene transcription. To determine the potential role of mithramycin on α1(I) gene activity, its rate of transcription was directly analyzed by nuclear run on transcription assays. Nuclei from controls and
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activities of the retarded

bands and the free

probe are shown.

FH109 cells that were incubated with mithramycin at 1 nM, 100 nM, and 10 μM final concentrations were harvested after 3 h. Radiolabeled run on transcripts were prepared and hybridized to filter-bound cDNA probes. Fig. 7 summarizes the individual transcription rates that were calculated as the transcriptional activity relative to the controls, which were set as 100%. A final concentration of 1 nM, mithramycin had no effect on the activity of the different genes tested. At 100 nM, mithramycin inhibits fibronectin and c-jun transcription and reduces α1(1) gene activity by 60%. In contrast, the transcription of the collagenase, α1(III) collagen, and GAPDH genes remained unaltered. Only at 10 μM were all genes affected, which again indicates a toxic effect of mithramycin at this concentration (see Fig. 1).

Discussion

The promoter of the α1(1) collagen gene is highly active in collagen-producing fibroblasts (13) and mainly regulated by the interaction of the transcription factors NF-I and Sp1 with a tandem repeat of NF-I/Sp1 switch elements (14, 15). Overexpression of Sp1 downregulated both unstimulated and NF-I-induced α1(1) collagen promoter/reporter gene activities in fibroblast cells (15). In contrast, elevated Sp1 binding activities were detected in models of experimentally induced liver fibrosis during periods of enhanced α1(1) gene transcription (16), indicating the potential of Sp1 to stimulate the α1(1) promoter within the context of the intact gene.

Our in vitro analysis demonstrates that the GC-binding antibotic mithramycin binds to regulatory elements of the human α1(1) collagen promoter, which inhibits Sp1 and increases NF-I binding to the NF-I/Sp1 switch elements. Recent functional studies on the isolated NF-I/Sp1 switch elements demonstrated that the ratio of the binding activities rather than the absolute concentrations of the two transcription factors determine α1(1) promoter activity (14). The present results suggest a similar function of these elements in the intact α1(1) gene. Competition with Sp1 and increased NF-I binding by mithramycin leads to a substantial decrease of α1(1) gene transcription and reduced levels of α1(1) mRNA in human fibroblasts. This implies further that Sp1 might be crucial for the maintenance of the high transcriptional activity of the collagen gene in human fibroblasts. These conclusions substantiate a possible significant role of increased Sp1 binding activities during the state of enhanced collagen production in experimental fibrogenesis (16). Previous studies have demonstrated that an enhanced activity of Sp1 can result from protein–protein interactions of Sp1 bound to the promoter with Sp1 bound to more distal sites (34). Additionally, high transcriptional activity of the SV40 enhancer required the binding of Sp1 to the SV40 early promoter (35). Although a collagen-α1(1) enhancer element has not been unequivocally identified, enhancer-like activities were ascribed to the first intron of the α1(1) gene (12), which contains functional Sp1 binding sites (11, 36). Interestingly, synergistic interactions between collagen-α1(1) intron and promoter sequences were reported recently (37, 38) and could explain the different activities of Sp1 bound to the isolated promoter or to the promoter in the intact gene. The potential of mithramycin to disrupt protein/protein interactions of distal factors by interfering with the binding of Sp1 to GC-rich regulatory elements of the α1(1) promoter could be a valuable tool in disclosing the mechanisms of experimental fibrogenesis.

Mithramycin reduces fibronectin mRNA levels, which are also upregulated in tissue fibrogenesis. This effect could only be explained when further insight into the regulation of this gene has been obtained. Similarly, the mechanisms how TGF-β upregulates the α1(1) gene are not well understood but are required to understand how mithramycin blocks this induction. Since TGF-β is thought to be involved in the development of many forms of tissue fibrosis, it will be interesting to examine in more detail how mithramycin interferes with its activity.

Figure 6. Mithramycin interferes with transcription factor binding to the NF-I/Sp1 switch element. In a gel retardation assay, a FP2 oligonucleotide spanning the -131--110 segment of the collagen-α1(1) promoter was radiolabeled and incubated with no additions (lane I) or 20 μg of nuclear FH109 fibroblast protein extract (lanes 2–7). The binding reaction contained either no (lanes 1 and 2) or increasing concentrations of mithramycin ranging from 10^{-4} to 10^{-4} M (lanes 3–7, respectively). The binding activities of the retarded bands and the free probe are shown.

Figure 7. Mithramycin differentially interferes with intracellular gene transcription. In a nuclear run-on transcription assay, nuclei from cultured FH109 cells were isolated after treatment with no or the indicated concentrations of mithramycin for 3 h. The mRNA transcription was allowed to complete in the presence of a [32P]UTP and the labeled transcript was extracted and hybridized to filter bound cDNA probes as described in Methods. Autoradiograms were laser scanned and the transcription rates calculated as the relative gene transcription of the respective control cDNAs (=100%). ○, 1 nM; □, 100 nM; ●, 10 μM.
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