Heat shock protein 90 (Hsp90) inhibition targets canonical TGF-β signalling to prevent fibrosis

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ABSTRACT

Objectives Targeted therapies for systemic sclerosis (SSc) and other fibrotic diseases are not yet available. We evaluated the efficacy of heat shock protein 90 (Hsp90) inhibition as a novel approach to inhibition of aberrant transforming growth factor (TGF)-β signalling and for the treatment of fibrosis in preclinical models of SSc.

Methods Expression of Hsp90 was quantified by quantitative PCR, western blot and immunohistochemistry. The effects of Hsp90 inhibition were analysed in cultured fibroblasts, in bleomycin-induced dermal fibrosis, in tight-skin (Tsk-1) mice and in mice overexpressing a constitutively active TGF-β receptor I (TβRI).

Results Expression of Hsp90β was increased in SSc skin and in murine models of SSc in a TGF-β-dependent manner. Inhibition of Hsp90 by 17-demethoxy-geldanamycin-17-demethoxy-geldanamycin (17-DMAG) inhibited canonical TGF-β signalling and completely prevented the stimulatory effects of TGF-β on collagen synthesis and myofibroblast differentiation. Treatment with 17-DMAG decreased the activation of canonical TGF-β signalling in murine models of SSc and exerted potent anti-fibrotic effects in bleomycin-induced dermal fibrosis, in Tsk-1 mice and in mice overexpressing a constitutively active TβRI. Dermal thickness, number of myofibroblasts and hydroxyproline content were all significantly reduced on treatment with 17-DMAG. No toxic effects were observed with 17-DMAG at anti-fibrotic doses.

Conclusions Hsp90 is upregulated in SSc and is critical for TGF-β signalling. Pharmacological inhibition of Hsp90 effectively blocks the profibrotic effects of TGF-β in cultured fibroblasts and in different preclinical models of SSc. These results have translational implications, as several Hsp90 inhibitors are in clinical trials for other indications.

INTRODUCTION

The major hallmark of systemic sclerosis (SSc) is an excessive accumulation of extracellular matrix in affected organs.1 The accumulation of extracellular matrix is caused by an increased release of collagen and other components of the extracellular matrix by pathologically activated fibroblasts.2 Several lines of evidence suggest a central role of transforming growth factor β (TGF-β) in fibroblast activation and tissue fibrosis in SSc: TGF-β signalling is activated in SSc with nuclear accumulation of the downstream mediator Smad2/3 and increased transcription of TGF-β target genes.3 TGF-β potently activates fibroblasts and induces an expression profile in resting normal fibroblasts that is reminiscent of SSc fibroblasts.4 Moreover, activation of TGF-β signalling—for example, by fibroblast-specific overexpression of constitutively active TGF-β receptor type I (TβRI)—is sufficient to induce fibrosis in mice.5 However, the knowledge of the crucial role of TGF-β in the pathogenesis of SSc has not yet been successfully translated into molecular therapies, and effective targeted treatments for fibrosis in SSc are still not available for clinical use.6

Heat shock proteins (Hsps) are a family of molecular chaperones. They were discovered by virtue of their induction in response to high temperature, but they are also upregulated by other forms of cellular stress.7 Functionally, most Hsps selectively recognise and bind non-native proteins under physiological and stress conditions, thereby Hsps prevent irreversible aggregation of proteins.8 However, Hsps have additional functions beyond prevention of protein aggregation. They not only modulate antigen processing and presentation to regulate immune responses,9–11 but they also interact with a broad variety of different kinases and transcription factors to regulate the cell cycle, apoptosis and cytoskeletal rearrangement.12

In this study, we evaluated Hsp90 as a potential target for the treatment of fibrosis in SSc. Hsp90 was selected because of its crucial role in folding and conformational stabilisation of TGF-β receptors (TβRI and TβRII)13 and also of Src kinases, which are intracellular mediators of the profibrotic effects of TGF-β.14–16 Inhibition of Hsp90 accelerates ubiquitination and increases proteasomal degradation of TβR and Src.13 Thus, inhibition of Hsp90 may be a novel approach to targeting TGF-β signalling. Of note, selective Hsp90 inhibitors from more than 10 different companies are currently being evaluated in clinical trials for their efficacy and tolerability in different oncological indications. Results from already completed trials suggest that pharmacological inhibition of Hsp90 is effective and not limited by toxicity.17,18 Thus, inhibition of Hsp90 has high translational potential, and various Hsp90 inhibitors would be available for clinical trials in SSc.

MATERIALS AND METHODS

Patients and fibroblast cultures

Fibroblast cultures were obtained from lesional skin biopsy samples from 15 patients with SSc and 12 healthy volunteers matched for age and sex. All
patients fulfilled the criteria for SSc as suggested by LeRoy et al. All had diffuse cutaneous SSc. Additional information is provided in table 1. The study was approved by the ethics committee of the University of Erlangen-Nuremberg.

Fibroblast cultures from patients and healthy volunteers were cultured as described. In selected experiments, fibroblasts were stained with TGF-β at a concentration of 10 ng/ml (R&D Systems, Wiesbaden-Nordenstadt, Germany).

Quantitative real-time PCR
Gene expression was quantified by SYBR Green real-time PCR using the MxPro 3005P QPCR System (Agilent Technologies, Santa Clara, California, USA). The following primers were used:

- human Hsp90β, 5'-ACCGCCTGCTATCTTCTGGCTT-3' and 5'-GCATCCTCATCGCCCTCGAGAGTCTTGCAG-3';
- human RPL27, 5'-ATCGCCAAGAGATCAAAGATAA-3' and 5'-CTTGGAGACATCCTTATTTGACG-3'. Samples without enzyme in the reverse transcription reaction were used as controls. Human RPL27 was used to normalise for the amounts of cDNA within each sample.

Sodium dodecyl sulphate/polyacrylamide gel electrophoresis and western blotting
Proteins were separated by sodium dodecyl sulphate/polyacrylamide gel electrophoresis as described. Primary antibodies against Hsp90 (sc-13119; Santa Cruz Biotechnology, Heidelberg, Germany) and β-actin (Sigma-Aldrich, Hamburg, Germany) and secondary horseradish peroxidase-conjugated goat anti-mouse IgG or rabbit anti-human IgG were used.

Immunofluorescence staining
HSP-90β and phosphorylated (p)Smad2/3 in cultured fibroblasts were detected by incubation with monoclonal mouse anti-human Hsp-90β antibodies (Assaydesigns, Ann Arbor, Michigan, USA) or polyclonal goat anti-human pSmad2/3 antibodies (Santa Cruz Biotechnology). Polyclonal goat anti-mouse Alexa Fluor 488 antibodies (Invitrogen) were used as secondary antibodies. Stress fibres were visualised with rhodamine-conjugated phalloidin (Sigma-Aldrich), and the cell nuclei were stained using 4',6-diamidino-2-phenylindole (1:800; Santa Cruz Biotechnology). Images were captured at 200-fold magnification using a Nikon Eclipse 80i microscope (Nikon, Badhoevedorp, The Netherlands).

Immunohistochemistry staining
Formalin-fixed, paraﬁn-embedded skin sections were stained with mouse anti-human Hsp90β or mouse anti-human α-smooth muscle actin (1:500; clone 1A4; Sigma-Aldrich) antibodies. Peroxidase-labelled species-speciﬁc immunoglobulins (Dako, Glostrup, Denmark) were used as secondary antibodies, and isotype-matched antibodies were used as controls.

Quantification of soluble collagen in cell culture supernatants
Soluble collagen in cell culture supernatants was quantiﬁed using the SirCol collagen assay (Biocolor, Belfast, Northern Ireland) as described.

TβRI-induced dermal ﬁbrosis
Injection of replication-deﬁcient adeno-associated virus (AAVs) encoding constitutively active TβRI (AdTBR) into the skin of mice induces dermal ﬁbrosis. AdTBR or AdLacZ (6.7×107 infectious units (ifu)/ml) was injected intracutaneously into deﬁned areas of the upper back of 4-week-old FVB mice at day 1 and day 28. Treatment with 17-dimethylaminoethylamino-17-deoxy-geldanamycin (17-DMAG; 5 mg/ml in phosphate-buffered saline; InvivoGen, San Diego, California, USA) was started at day 1 and continued every third day for 8 weeks. After 8 weeks, mice were killed, and dermal thickness, number of myoﬁbroblasts and hydroxyproline content were analysed as described.

Bleomycin-induced dermal ﬁbrosis
Skin ﬁbrosis was induced in 6-week-old DBA/2J mice by local injection of bleomycin every other day for 4 weeks. One group of mice challenged with bleomycin was intraperitoneally injected with 100 μl 17-DMAG every third day for 4 weeks. The control NaCl- and bleomycin-treated groups were injected intraperitoneally with 100 μl phosphate-buffered saline every third day for 4 weeks.

Tsk-1 mouse model of dermal ﬁbrosis
The tight-skin (Tsk-1) phenotype is induced by a dominant mutation in the brillin-1 gene, which results in increased activation of latent TGF-β. Tsk-1 mice were interbred with pa/pa mice, in which a recessive mutation (pa) induces a light grey color of the fur and eyes. Because the brillin 1 gene is genetically linked to the pa gene, mice can be prescreened for the tsk1 mutation based on the color of their fur and eyes. All mice with black fur and eyes carry the dominant tsk1 mutation and are heterozygous for the mutated pale gene. In contrast, mice with light grey fur do not carry the tsk1 mutation but are homozygous for the mutated pale gene. Apart from the change in skin color, the pale mutation itself does not alter skin physiology or ﬁbrogenesis. Treatment with 17-DMAG was started at the age of 5 weeks, and mice were killed after 5 weeks of treatment.

Statistical analysis
All data are presented as median with IQR, and differences between the groups were tested for their statistical signiﬁcance by non-parametric Mann–Whitney U test. A p value of less than 0.05 was considered signiﬁcant.

Table 1
Clinical characteristics of patients with systemic sclerosis (SSc) at date of biopsy

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (F/M)</td>
<td>11/4</td>
</tr>
<tr>
<td>Age (years), median (range)</td>
<td>52 (35–78)</td>
</tr>
<tr>
<td>Disease duration (years), median (range)</td>
<td>5 (0.5–11)</td>
</tr>
<tr>
<td>ANA positive</td>
<td>15/15</td>
</tr>
<tr>
<td>Anti-topoisomerase positive</td>
<td>6/15</td>
</tr>
<tr>
<td>Pulmonary fibrosis</td>
<td>5/15</td>
</tr>
<tr>
<td>Myocardial involvement</td>
<td>2/15</td>
</tr>
<tr>
<td>Pulmonary arterial hypertension</td>
<td>3/15</td>
</tr>
<tr>
<td>Digital ulcers</td>
<td>8/15</td>
</tr>
<tr>
<td>Active disease</td>
<td>7/15</td>
</tr>
<tr>
<td>Medication</td>
<td>No DMARDs, corticosteroids or NSAIDs</td>
</tr>
</tbody>
</table>

All patients had diffuse cutaneous SSc. The disease subset was determined according to the criteria proposed by LeRoy et al. Disease duration was measured from the onset of the first non-Raynaud symptoms, attributable to SSc. Pulmonary arterial hypertension was diagnosed by right heart catheterisation. Disease activity was determined using the EULAR Systemic Sclerosis Activity Score. Patients with scores of ≥3 were classified as having active disease.

DMARD, disease-modifying antirheumatic drug; F, female; M, male; NSAID, non-steroidal anti-inflammatory drug.

RESULTS

Hsp90β is upregulated in SSc fibroblasts

Hsp90β was induced in the skin of patients with SSc compared with healthy individuals (figure 1A). In healthy volunteers, Hsp90β was either undetectable (3/8 individuals) or expressed at low levels with faint staining of the dermis (5/8 individuals). In contrast, prominent staining for Hsp90β was detected in fibroblasts of the upper dermal layer of all patients with SSc (figure 1A). Keratinocytes and endothelial cells also stained positive for Hsp90β in three out of eight and six out of eight patients, respectively. Of particular interest, expression of Hsp90β in fibroblasts colocalised with expression of α-smooth muscle actin (data not shown). Indeed, all myofibroblasts stained intensely for Hsp90β in SSc skin. Although not statistically significant, the mRNA levels of Hsp90β tended to be higher in patients with high disease activity as indicated by a European League Against Rheumatism (EULAR) Systemic Sclerosis Activity Score ≥3 (increase of 22%, p=0.10). No differences were observed between patients with and without antibodies to topoisomerase. However, further studies on larger patient collectives are needed to determine differences in Hsp90 expression between different subgroups of patients with diffuse cutaneous SSc.

Consistent with the findings in human SSc, Hsp90β was also induced in the mouse model of bleomycin-induced fibrosis and in Tsk-1 mice (figure 1B,C).

We next analysed whether the upregulation of Hsp90β in SSc is due to increased TGF-β. The basal mRNA and protein levels of Hsp90 in the absence of exogenous stimulation were increased in cultured SSc fibroblasts compared with fibroblasts from healthy individuals. Consistent with persistent activation of TGF-β signalling in cultured SSc fibroblasts, the stimulatory effects of TGF-β on Hsp90 mRNA and protein were more pronounced in fibroblasts from healthy individuals than in SSc fibroblasts (figure 1D,E). The stimulatory effects of TGF-β signalling on Hsp90β were confirmed in vivo. Activation of TGF-β signalling by overexpression of a constitutively active TβRI upregulated the expression of Hsp90β in murine skin (figure 1F).

Inhibition of Hsp90 abrogates the stimulatory effects of TGF-β on fibroblasts

We next evaluated whether Hsp90 regulates the profibrotic effects of TGF-β. We first analysed whether the selective Hsp90 inhibitor, 17-DMAG, prevents the nuclear accumulation of pSmad2/3 on stimulation with TGF-β. Indeed, treatment with...
17-DMAG abrogated the nuclear accumulation of pSmad2/3 in TGF-β-stimulated fibroblasts (p<0.01) (figure 2A). Formation of stress fibres is a hallmark of myofibroblasts and is induced by TGF-β. Preincubation with 17-DMAG prevented the TGF-β-induced formation of stress fibres (p<0.001) (figure 2B). Moreover, 17-DMAG effectively inhibited the stimulatory effects of TGF-β on the collagen synthesis in dermal fibroblasts. The TGF-β-induced increase in secreted collagen protein and mRNA levels of col1a1 and col1a2 was completely abrogated (p<0.001, p<0.01 and p<0.01, respectively) (figure 2C–E). In addition, the mRNA levels of fibronectin were decreased by 72% in 17-DMAG-treated cells compared with sham-treated fibroblasts stimulated with TGF-β (p<0.05) (figure 2F).

The antifibrotic effects of Hsp90 inhibition were accompanied by potent inhibition of canonical TGF-β. The nuclear accumulation of pSmad2/3 was reduced by 55% in 17-DMAG-treated AdTBR mice compared with sham-treated AdTBR mice (p<0.001) (figure 3E).

Inhibition of Hsp90 prevents fibrosis induced by overexpression of TβRI

After demonstrating that Hsp90 inhibition reduces the profibrotic effects of TGF-β in vitro, we next aimed to confirm these findings in vivo. We thus evaluated the effects of 17-DMAG in the mouse model of TβRI-driven fibrosis, in which fibrosis is induced by selective activation of TGF-β signalling.33 Inhibition of Hsp90 exerted potent antifibrotic effects in AdTBR mice (figure 3A–E). Treatment with 17-DMAG reduced dermal thickening by 81% compared with sham-treated AdTBR mice (p<0.001) (figure 3A,B). Hydroxyproline content and myofibroblast count were also significantly decreased by 88% (p<0.05) and 69% (p<0.01), respectively, on treatment with 17-DMAG (figure 3C,D).

Treatment with 17-DMAG protects from bleomycin-induced fibrosis

To further evaluate the antifibrotic potential of the Hsp90 inhibitor in vivo, we used the mouse model of bleomycin-induced dermal fibrosis, which serves as a model for early, inflammatory stages of SSC.33 Treatment with 17-DMAG reduced dermal thickening by 56% (p<0.001 compared with sham-treated, bleomycin-challenged mice) (figure 4A,B). Furthermore, the hydroxyproline content in lesional skin was reduced by 81% (p<0.05) (figure 4C). The differentiation of resting fibroblasts into myofibroblasts was reduced by 65% (p<0.001) compared with sham-treated, bleomycin-challenged mice (figure 4D). Hsp90 inhibition effectively reduced canonical TGF-β signalling with a 74% decrease in the number of cells positive for pSmad2/3 (p<0.001) (figure 4E).

Inhibition of Hsp90 ameliorates fibrosis in Tsk-1 mice

To confirm the antifibrotic effects of Hsp90 inhibition in another, less inflammatory mouse model that resembles later, non-inflammatory stages of fibrosis,33 34 Tsk-1 mice were treated with 17-DMAG. Inhibition of Hsp90 reduced hypodermal thickening in Tsk-1 mice by 76% compared with sham-treated Tsk-1 mice (p<0.001) (figure 5A,B). Hydroxyproline...
content and myofibroblast count were also reduced by 67% (p<0.05) and 85% (p<0.001), respectively, in Tsk-1 mice treated with 17-DMAG (figure 5C,D). Similar to previous models, 17-DMAG inhibited canonical TGF-β signalling and reduced the number of cells with nuclear pSmad2/3 by 49% (p<0.001) compared with sham-treated Tsk-1 mice (figure 5E). To exclude the possibility that the antifibrotic effects of 17-DMAG are accompanied by major toxic side effects, mice in all models were closely monitored during the treatment. No clinical signs of toxicity were observed, and the mean body weight, activity of the mice and texture of the fur were not altered in mice treated with antifibrotic doses of 17-DMAG for up to 8 weeks. Moreover, gross macroscopic evaluation of the mice on necropsy revealed no signs of toxicity (data not shown).

**DISCUSSION**

We demonstrate in this study that inhibition of Hsp90 abrogates the profibrotic effects of TGF-β signalling. Incubation of cultured fibroblasts with 17-DMAG prevented the nuclear accumulation of pSmad2/3, inhibited the differentiation of resting fibroblasts into myofibroblasts and completely abrogated the stimulatory effects of TGF-β on the release of extracellular matrix in vitro. Pharmacological inhibition of Hsp90 also effectively attenuated TGF-β signalling in vivo and significantly reduced skin fibrosis induced by overexpression of a constitutively active TβRI. Hsp90 is required for correct folding and conformational stabilization of TβRs and Src kinases.13 Although we did not directly measure the activity of Hsp90 in fibrotic skin, the potency of the effects of Hsp90 inhibition with complete abrogation of the profibrotic effects of TGF-β signalling in vitro and the efficacy of 17-DMAG in short-term experiments suggest that Hsp90 controls TGF-β signalling in fibroblasts also via other mechanisms in addition to conformational stabilization. Indeed, Hsps have been recently suggested to directly affect the activity of kinases. In this context, JNK1/2 and JAK2 kinases may be interesting candidates as mediators of the effects of Hsp90 on TGF-β signalling, because (i) they have been shown to be regulated by Hsp9035 36 and (ii) they are established downstream mediators of the profibrotic effects of TGF-β in fibroblasts.28 37 However, further studies on Hsp90 activity and on its effects on JNK and JAK kinases are needed to elucidate how Hsp90 regulates TGF-β signalling in fibroblasts.

Hsp90 is overexpressed in patients with SSc and in murine models of skin fibrosis. The upregulation of Hsp90 in AdTBR mice suggests that increased TGF-β signalling may be an important factor for the upregulation of Hsp90 in fibroblasts. TGF-β stimulates the transcription and increased the protein levels of Hsp90β in cultured fibroblasts. Increased TGF-β signalling also stimulated the expression of Hsp90β in fibroblasts in murine skin. As Hsp90 is required for effective TGF-β signalling, Hsp90 serves as an intracellular amplifier of the profibrotic effects of TGF-β in SSc. TGF-β increases the expression of Hsp90, which in turn renders fibroblasts more susceptible to TGF-β signalling by stabilising TGF-β cell surface receptors and intracellular downstream mediators such as Src, JNK and JAK kinases. The disruption of this endogenous amplification loop and the interference with TGF-β signalling...
Figure 4  Inhibition of heat shock protein 90 (Hsp90) reduced bleomycin-induced fibrosis. Treatment with 17-dimethylaminoethylamino-17-demethoxy-geldanamycin (17-DMAG) prevents dermal thickening (A, B), accumulation of hydroxyproline (C) and myofibroblast differentiation (D) and reduces the nuclear accumulation of phosphorylated (p)Smad2/3 (E) in bleomycin-challenged mice. Representative images of trichrome-stained sections are shown at 100-fold magnification. Number of mice used: NaCl, 12; bleomycin, 9; bleomycin+17-DMAG, 9. p values are expressed as follows: 0.05>p>0.01 as *; p<0.001 as **. All data are presented as median with interquartile range (IQR). DAPI, 4',6-diamidino-2-phenylindole. This figure is only reproduced in colour in the online version.

Figure 5  17-Dimethylaminoethylamino-17-demethoxy-geldanamycin (17-DMAG) reduces fibrosis in the Tsk-1 mouse model. Pharmacological inhibition of heat shock protein 90 (Hsp90) by 17-DMAG reduces hypodermal thickening (A, B), decreases the hydroxyproline content (C), inhibits myofibroblast differentiation (D) and prevents nuclear accumulation of phosphorylated (p)Smad2/3 (E) in Tsk-1 mice. Representative images of trichrome-stained sections are shown at 40-fold magnification. Number of mice used: pa/pa, 7; Tsk-1, 6; Tsk-1+17-DMAG, 6. p values are expressed as follows: 0.05>p>0.01 as *; p<0.001 as **. All data are presented as median with interquartile range (IQR). DAPI, 4',6-diamidino-2-phenylindole.
on multiple levels may explain the potent antifibrotic effects of Hsp90 inhibitors.

We demonstrated that pharmacological inhibition of Hsp90 strongly inhibits TGF-β signalling as a core pathway of fibrosis in vitro and in vivo. The inhibitory effects on TGF-β signalling translate into potent antifibrotic effects. Treatment with the Hsp90 inhibitor, 17-DMAg, significantly reduced histological features of fibrosis, decreased collagen content and prevented the differentiation of resting fibroblasts into myofibroblasts in three different mouse models in pharmacologically relevant doses. The mouse models used herein mimic different stages and aspects of SSc. The mouse model of bleomycin-induced dermal fibrosis resembles early, inflammatory stages of SSc, in which fibroblasts are predominantly activated by profibrotic mediators released from infiltrating leukocytes. The first-in-class Hsp90 inhibitor, 17-AAG (17-N-allylamino-17-demethoxygeldanamycin), entered the first clinical trials in 1999. The promising results with this inhibitor stimulated the development of second-generation Hsp90 inhibitors such as alvespimycin (KOS-1022, 17-DMAg) with improved pharmacokinetics. Hsp90 inhibitors were initially evaluated for the treatment of several solid tumours, such as breast, prostate and lung cancers, but have more recently also been tested in haematological malignancies, such as chronic myeloid leukaemia and multiple myeloma. Moreover, recent trials have also evaluated the effects of combination therapies of Hsp90 inhibitors with either other targeted therapies or conventional chemotherapy or radiotherapy. Currently, 13 different Hsp90 inhibitors from several pharmaceutical companies in various oncological indications are ongoing (http://www.clinicaltrials.gov). Together, these data indicate that an application in patients with fibrotic diseases might not be limited by toxicity.

In summary, we demonstrate that Hsp90 amplifies the profibrotic effects of TGF-β and that pharmacological inhibition of Hsp90 potently inhibits fibrosis in preclinical models of different stages of SSc in pharmacologically relevant doses without obvious toxicity. These findings might have direct translational implications. Hsp90 is currently considered a prime candidate for the treatment of various cancers, and its good druggability and encouraging results from clinical trials have stimulated multiple companies to develop Hsp90 inhibitors. Selective and potent inhibitors from about a dozen companies are currently in clinical trial and would be available for clinical trials in SSc and other fibrotic diseases.

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