Identification of Small Molecule Chemical Inhibitors of the Collagen-Specific Chaperone Hsp47

Christy A. Thomson,[†,§] Helen M. Atkinson,[§] and Vettai S. Ananthanarayanan*

Department of Biochemistry and Biomedical Sciences, McMaster University, 1200 Main St. W., Hamilton, Ontario, Canada, L8N 3Z5

Received October 25, 2004

Hsp47 is a collagen-specific molecular chaperone whose activity has been implicated in the pathogenesis of fibrotic diseases. Here, we describe the development of an assay for screening libraries of chemical compounds for inhibitors of Hsp47. A preliminary screen of 2080 compounds identified four that demonstrated inhibitory activity against Hsp47 in vitro, with IC_{50} values ranging from 3 to 27 μM. Compounds identified through this method may provide the basis for development of novel antifibrotic therapeutics.

Introduction

Collagen is the most abundant protein in the human body and is the major protein component of the extracellular matrix (ECM), which provides a structural framework for cells and tissues in vertebrates. Type I collagen is synthesized as a precursor molecule, termed procollagen, which undergoes extensive posttranslational processing before the formation of mature triple-helical collagen.[1] The interaction with molecular chaperones and modifying enzymes (e.g. prolyl hydroxylase) during its biosynthesis allows the polypeptide chains of procollagen to assemble into a triple helical structure and to be secreted out of the cell.[2] Once in the extracellular space, procollagen undergoes further modification, such as cleavage of the C- and N-terminal propeptides[3] and deamination of lysines[4] before incorporation into stable, cross-linked fibrils.

In healthy tissues, there exists a balance between the deposition of collagen into the ECM and its subsequent degradation.[5] During the process of fibrosis/sclerosis, this balance is tipped in favor of increased collagen deposition and decreased clearance. In conditions such as pulmonary fibrosis, the fibrotic lesions formed often result in basement membrane damage and alveolar collapse.[6] For patients suffering from pulmonary fibrosis, currently available therapies show only marginal effects with poor prognosis. These therapies have often centered on controlling the initial inflammatory response that prompted the disease state.[6] However, it has become increasingly evident that the later stages of disease progression, such as collagen production or deposition, may be a more relevant target for therapeutics.[7]

Recent evidence has implicated the ER-resident protein, Hsp47, as an appropriate target for combating diseases resulting from excess collagen deposition.[8] Hsp47 is a member of the heat shock family of proteins, which were initially discovered based on their enhanced expression when cells were subjected to sudden, slight increases in temperature and based on their ability to provide thermotolerance to subsequent lethal heat treatment. Although heat shock proteins can perform a variety of functions, their roles in cells can be generalized under two broad categories.[9] The first is as molecular chaperones, which are required during the protein folding process to prevent off-pathway interactions and aggregation. The second is in recognition of aberrant proteins and their subsequent turnover. Hsp47 is a collagen-specific molecular chaperone whose synthesis parallels that of collagen in normal and disease states.[10] TGF-β and IL-1β, the principal fibrosis-causing cytokines, have been shown to enhance Hsp47 expression.[8] Hsp47 binds to nascent procollagen chains in the ER and remains associated with them during triple-helix formation and subsequent transfer to the Golgi apparatus.[11,12] Although the exact role of Hsp47 in collagen biosynthesis is unknown, Hsp47 knockout mice were embryonic lethal before 11.5 dpc.[13] These mice demonstrated aberrant formation of triple-helical type I collagen molecules as well as defects in type IV collagen production and basement membrane formation. This underscores the importance of Hsp47 in collagen biosynthesis.

In this study, we report the development of an assay to screen for small molecules from a large chemical library that would inhibit Hsp47's collagen chaperone activity. Inhibition of Hsp47 gene expression, using both anti-sense oligonucleotides[14,15] and a chemical compound,[16] has been shown to repress collagen production in fibrotic model systems. However, to our knowledge, there are no known molecules which directly affect Hsp47's protein function. Since proper functioning of Hsp47 is essential for in vivo collagen biosynthesis, ours represents a novel approach for identifying potential therapeutics to combat collagen-related diseases such as fibrosis. Initial screens of 2080 compounds in duplicate identified four inhibitors of Hsp47 activity. The IC_{50} values of the inhibitors ranged from 3 μM to 27 μM. In future, these and structurally related compounds with lower IC_{50} values will be tested in cell culture and whole animals for their effect on collagen synthesis and deposition. Not only does this study pave the way to

* To whom correspondence should be addressed. Phone: (905) 525-9140 x22783. Fax: (905) 522-9033. E-mail: ananth@mcmaster.ca.

† Current address: The Biomedical Research Centre, 2222 Health Sciences Mall, University of British Columbia, Vancouver, BC, Canada, V6T 1Z3. Phone: (604) 822-7842. E-mail: christy@brc.ubc.ca.

§ These authors contributed equally to the work.

10.1021/jm049148+ CCC: $30.25
Published on Web 02/11/2005

development of therapeutics for modulating collagen synthesis in disease conditions, but it will also improve our understanding of Hsp47's function in collagen biosynthesis.

Results

Assay for Hsp47 Inhibition. Hsp47's collagen chaperone activity does not require any cofactors or coenzymes, nor does its activity cause the chemical modification of its substrate, collagen. As a result, conventional assays that monitor the transfer of electrons, atoms, or functional groups cannot be used to determine Hsp47 activity. We have previously shown that Hsp47 inhibits collagen fibril formation in vitro.[17] This fibril-inhibition activity supports a proposed role for Hsp47 in preventing the premature association of collagen triple helices in the ER.[18] The assay for this activity is based on the fact that a solution of mature triple-helical collagen monomers incubated at 34 °C and pH 7.4 will spontaneously associate to form fibrils, causing an increase in the turbidity of the solution which can be monitored using a spectrophotometer. The changes in turbidity of the solution over time produce a curve characterized by an initial lag phase with no change in turbidity, followed by a growth phase during which the turbidity rapidly increases, and ending with a plateau phase.[19] Addition of Hsp47 to the collagen solution can significantly increase the lag time of collagen fibril formation, from about 30 min in its absence to over 4 h when it is present at a 2:1 molar ratio (Figure 1A). Changes in Hsp47 activity can therefore be quantified indirectly, by monitoring the lag time of collagen fibril formation in its presence.[17]

The turbidity assay of Hsp47's chaperone activity was originally conducted in 1 cm quartz cuvettes.[17] To facilitate the screening of a large number of compounds for inhibitors of Hsp47, we have converted the assay into a 96-well format. Figure 1 shows examples of the fibrillogenesis assay in both formats, and the effect Hsp47 has on the lag time of fibril formation. The data shown in Figure 1B resulted in a Z′ score of 0.83 at 75 min, which indicated a good quality assay for use in screening.[20]

Screening. The screens for inhibitors of Hsp47 activity were carried out in duplicate. Note that because active Hsp47 attenuates collagen fibril formation for more than 2 h, a strong inhibitor of Hsp47, or positive hit in this assay, will cause a restoration of collagen fibril formation before this time, whereas with a noninhibitor no fibril formation will be observed. The screens were carried out using a chemical library (Maybridge Chemical Co., Cornwall, U.K) that was compiled based on Lipinski's rules.[21]

Figure 2 shows replicate plots of two separate screens. The Z and Z′ for the first screen (Figure 2A) were 0.56 and 0.59 respectively, and the Z and Z′ for the second screen (Figure 2B) were 0.58 and 0.64 respectively (see Experimental Section for calculations). These values indicate that the assay conditions were optimized sufficiently to have confidence that the data generated was accurate.[20] As shown in Figure 2, the majority of the 2080 compounds screened had no activity against Hsp47, as seen by the large fraction of replicates clustered around 0% fibril formation. Some of the test

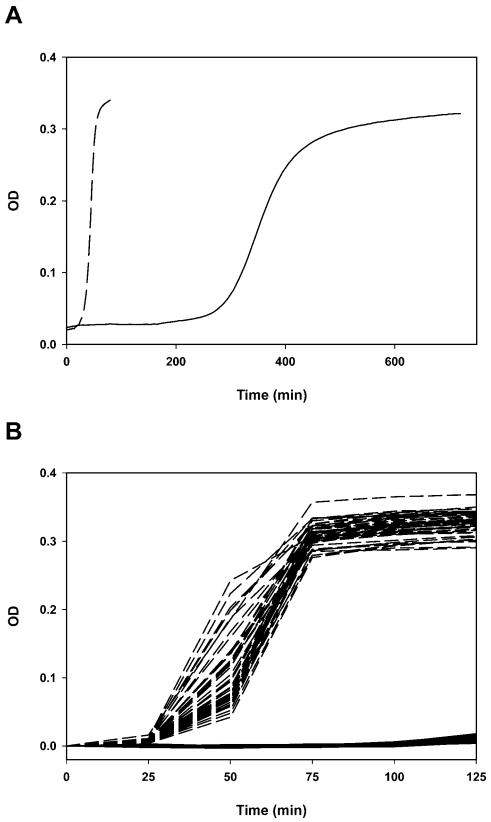


Figure 1. Collagen fibril formation was measured in phosphate buffer, pH 7.4, at 34 °C, in the presence (solid lines) and absence (dashed lines) of Hsp47. The concentrations of collagen and Hsp47 were, respectively, 0.7 μM and 1.2 μM. Fibril formation was monitored using either 1 cm cuvettes at 450 nm in a Cary 300 spectrophotometer (A), or 96-well plates at 313 nm in a SpectraMax Plus plate reader (B). In B, 48 reactions of each type were monitored to determine reproducibility of the assay in the 96-well format.

reactions displayed a net decrease in absorbance at 313 nm over time, which was subsequently calculated as negative fibril formation. This was likely due to degradation of the test compounds, or interference in the initial absorbance measurement caused by small air bubbles in the wells.

Eight compounds, shown lying in the "hit zones", caused the appearance of optical turbidity in the presence of Hsp47, to a degree three standard deviations above that of the negative controls. To rule out false positives caused, for example, by precipitation of the compound, these eight compounds were retested as described in the Experimental Section. Upon retesting, four of the compounds showed inhibitory activity against Hsp47, allowing fibril formation to occur after a reduced lag time. These four compounds were subsequently selected for secondary analysis. With 4 out of 2080 compounds being shown to be inhibitors of Hsp47, a primary hit rate of 0.2% was observed.

Secondary Analysis. The four positive hit compounds identified from screening were further examined for their ability to inhibit Hsp47 activity. Hsp47 activity was defined as the degree to which Hsp47 could extend the lag time of fibril formation. Figure 3 shows the effect of increasing concentration on the ability of a representative compound to inhibit Hsp47 activity, thus decreasing the lag time of collagen fibril formation. IC_{50} values for each of the four hit compounds were determined as

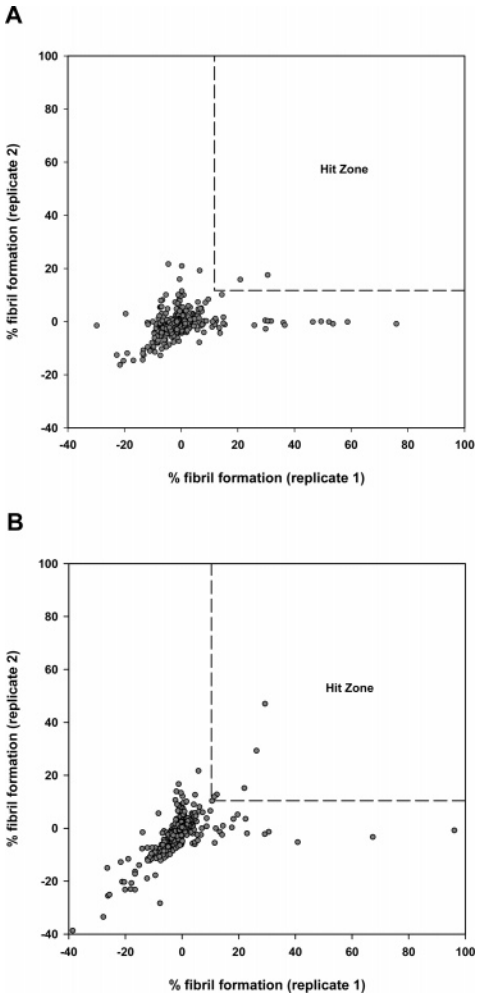


Figure 2. Identification of Hsp47 inhibitors through compound library screening. Schown are replicate plots of screen 1 (A) and screen 2 (B) of a combined total of 2080 small molecules against Hsp47. Compounds that restored fibril formation (as measured by optical turbidity) to three standard deviations above the negative control mean in both replicates (the "Hit Zone") were deemed active against Hsp47 and selected for retesting.

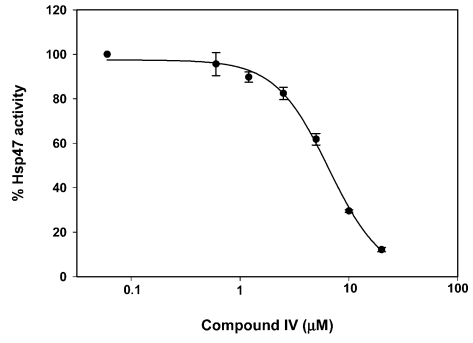


Figure 3. IC_{50} analysis of inhibition of Hsp47 by a hit compound. Fibril formation assays were carried out at 34 °C, in phosphate buffer at pH 7.4. Final collagen and Hsp47 concentrations were 0.7 μM and 1.2 μM, respectively. Nonlinear regression analysis was used to fit the data to a sigmoidal dose−response curve using SigmaPlot 8.0 software (SPSS Science, Chicago, IL). The concentration of compound which caused 50% inhibition of activity was extracted from the calculated curve. Error bars represent standard error of the mean ($n = 3$).

explained in the Experimental Section. The IC_{50}'s ranged in value from 3 μM to 27 μM indicating that the primary screen generated good preliminary inhibitors worthy of further study and development.

Table 1. Chemical Structures and IC_{50} Values of Hsp47 Inhibitors Identified from Screening

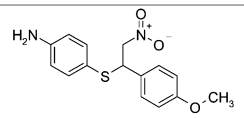
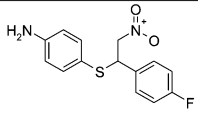
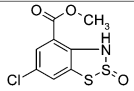
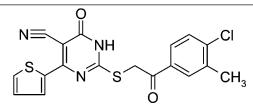
Compound	Structure	IC_{50} (μM)
I		26.6
II		26.6
III		3.1
IV		6.3

Table 1 shows the chemical structures of the Hsp47 inhibitors, along with their IC_{50} values. The four compounds represent three different structural categories. Compounds I and II have identical IC_{50} values, indicating that the minor differences in structure between them have little effect on their inhibitory mode of action. Further screening of a small library of structurally related compounds will help to identify the elements in these compounds important for inhibitory activity. Additionally, homology modeling of Hsp47 along the lines of earlier studies[22] and docking of inhibitors in the protein's putative collagen-binding site may provide interesting data on the topography of the binding site, and assist in designing inhibitors with high affinities for Hsp47.

Discussion

Low-molecular weight compounds that interfere with Hsp47's chaperone activity may act similarly to inhibitors of other heat shock protein chaperones, by affecting the ability of the chaperone's substrate to fold and assemble into an active conformation. For instance, Hsp90 family members are ATP-dependent molecular chaperones whose role in the cell is to assist in the proper folding of a wide variety of proteins.[23] Inhibitors of the Hsp90 chaperones are successful in halting the proliferation of cells because they inhibit the chaperone activity necessary for the proper folding, assembly and function of key cellular signaling proteins such as tyrosine kinases and steroid receptors.[24] 17-*N*-allylamino-17-demethoxygeldanamycin, a small-molecule inhibitor of Hsp90, has reached phase II clinical trials as a novel anticancer agent.[25]

Two important considerations make the design of Hsp47 inhibitors an attractive proposition for therapeutic intervention in collagen diseases. First, Hsp47 has a single substrate, collagen. Therefore, inhibition of Hsp47 function should result in the destabilization

or improper folding of collagen only. Second, parallel regulation of Hsp47 and collagen expression is apparent in many disease states in which changes in collagen expression are observed, including fibrotic diseases of the liver,[26] kidney,[27] lung[28] and dermis,[29] as well as atherosclerosis[30] and cancer.[31] Although antisense oligodeoxynucleotides against Hsp47 have demonstrated their effectiveness in attenuating collagen deposition in a glomerulonephritis rat model[14] and in a wound model,[15] the potential for these compounds as pharmacological candidates for antifibrotic therapies is limited.[32] The purpose of this work, therefore, was the development of a screen for small molecule inhibitors of Hsp47 which, once identified, could be developed into potent antifibrotic therapies. The screen, based on the inhibition of collagen fibrillogenesis by Hsp47, resulted in reproducible high quality data. A preliminary screen of a chemical library generated four positive hit compounds with IC_{50} values ranging from 3 μM to 27 μM. While these values are sufficiently low to allow for testing the effects of the compounds on collagen deposition in cells and animal models, further screens may be necessary to expand the pool of lead compounds and develop more effective inhibitors. It should be noted, however, that the screening procedure is laborious and time-consuming. This is due to difficulties in isolating pure, active Hsp47,[17,33] and due to the fact that the assay itself is lengthy. That being said, with the marginal efficacy of treatments for fibrosis presently on the market,[7] and with few new viable options available, the importance of identifying novel treatments for fibrosis may outweigh the technical constraints of this approach.

In conclusion, the small molecule compounds identified in this study as inhibitors of Hsp47 may serve as lead compounds for developing effective drugs for the treatment of fibrosis and related diseases.

Experimental Section

Materials. All chemicals were purchased from commercial suppliers and used without further purification. The compounds used for screening were purchased from Maybridge (Tintagel, Cornwall, U.K.). Calf-skin collagen, Type I, was purchased from Worthington Biochemical Corporation (Lakewood, NJ).

Production of Hsp47. Recombinant Hsp47 was purified from 10 to 20 L worth of BL21Star(DE3) cells using the protocol previously described.[33] Lysis and wash buffer volumes were increased approximately 5-fold from those described,[33] to account for the larger mass of pelleted cells used. Following the final hydroxyapatite column step, the isolated Hsp47 was dialyzed against fiber buffer (20 mM sodium phosphate pH 7.4, 50 mM NaCl) for ~15 h. Experiments using Hsp47 were conducted within 28 h of elution from the chitin column to avoid loss of activity of the protein. Hsp47 used in secondary testing of putative hit compounds was purified from 2 to 4 L worth of BL21Star(DE3) cells as previously described.[33]

Screening. Hsp47 activity was assayed using a modification of the procedure described by Thomson and Ananthanarayanan.[17] An initial pilot screen of 1040 compounds (screen 1) was carried out to determine the feasibility of screening under the conditions outlined below. A further 1040 compounds were then screened. Data from the two screens were not combined because certain conditions (collagen concentration, lag time of fiber formation) differed slightly due to the use of two different collagen stocks. Both concentration and the kinetics of fibril formation (lag time, height of plateau phase) can vary slightly between different lots of purchased type I collagen; therefore, screening conditions were adjusted accordingly to keep the signal-to-noise and signal-to-background ratios high. Both screens were carried out in duplicate. The assays were carried out in a final volume of 200 μL in 96-well plates (Nalge Nunc International). To each well were added 190 μL of 1.2 μM Hsp47, 4 μL of 1mM stock solution of the test compound in DMSO, and 6 μL of collagen stock, to yield a final test compound concentration of 20 μM, and final collagen concentration of 0.67 μM in screen 1 and 0.8 μM in screen 2. Two types of controls were present on each plate, alternating in columns 1 and 12. Positive control wells (positive for fibril formation, no Hsp47) contained fiber buffer instead of Hsp47, and DMSO in place of the test compound. Negative control wells (no fibril formation, fully active Hsp47) contained DMSO instead of the test compound. Reagent additions were done either by hand using 12-channel multipipets (Biohit), or with a Biomek FX liquid handler. Turbidity in each well was monitored at 313 nm using a SpectraMax Plus plate reader (Molecular Devices, Sunnyvale, CA). Readings were taken immediately after collagen addition, and at 75 min (screen 1) or 135 min (screen 2). The plates were incubated at 34 °C between readings. Putative hits were subsequently confirmed in a separate procedure, by measuring the turbidity at 313 nm every 2 min interval and looking for a sigmoidal increase over time which is characteristic of fibril formation.

Calculations. The turbidity value in each well of a given plate was correlated to the extent of fibril formation using the formula:

$$\text{percent fibril formation} = [(\Delta A - \mu_-)/(\mu_+ - \mu_-)] \times 100$$

where ΔA is the change in turbidity from time zero to the final measurement time, μ_- is the average ΔA of the negative controls on the same plate, and μ_+ is the average ΔA of the positive controls on that plate. A compound in a well that displayed a value for percent fibril formation greater than 3 standard deviations above the average value for the negative controls of the entire screen was deemed a putative hit and subjected to further testing. Z and Z′ scores were calculated as in Zhang et al.,[20] using the following formula:

$$Z = 1 - [(3\sigma_s + 3\sigma_+)/|\mu_s - \mu_+|]$$

where σ_s is the standard deviation of the sample ΔA values and σ_+ is the standard deviation of the positive controls. μ_s and μ_+ are the mean ΔA values of the samples and positive controls, respectively. To calculate the Z′ score, μ_- and σ_- were substituted, respectively, for μ_s and σ_s.

Secondary Testing. IC_{50} determinations were performed for compounds identified from primary screening. Fibril formation was monitored in quartz cuvettes, in the presence of Hsp47 and varying hit compound concentrations, using the procedure described by Thomson and Ananthanarayanan.[17] Turbidity was monitored at 450 nm, since the compounds themselves showed varying degrees of absorption at 313 nm. The lag time of fibril formation was determined at each compound concentration by finding the intersection point of the tangent to the horizontal portion of the curve, before fibril formation, and the tangent to the increasing portion of the curve during fibrillogenesis. These values for lag time were then used to calculate Hsp47 activity at each compound concentration, relative to the lag time in the absence of compound. The lag time of fibril formation in the presence of the test compound and in the absence of Hsp47, was measured and used in the calculations to control for any slight differences in fibril formation lag time caused by the test compounds. These differences were negligible, if not entirely absent for most compounds.

Acknowledgment. We thank the McMaster High Throughput Screening Facility for technical assistance and use of equipment. We also thank the Heart and Stroke Foundation of Canada for financial support.

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JM049148+