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High-Throughput Assay for Collagen Secretion Suggests an Unanticipated Role for Hsp90 in Collagen Production

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Abstract

Collagen overproduction is a feature of fibrosis and cancer, while insufficient deposition of functional collagen molecules and/or the secretion of malformed collagen are common in genetic disorders like osteogenesis imperfecta. Collagen secretion is an appealing therapeutic target in these and other diseases, as secretion directly connects intracellular biosynthesis to collagen deposition and biological function in the extracellular matrix. However, small molecule and biological methods to tune collagen secretion are severely lacking. Their discovery could prove useful not only in the treatment of disease, but also in providing tools to better elucidate mechanisms of collagen biosynthesis. We developed a cell-based, high-throughput luminescent assay of collagen type-I secretion, and used it to screen for small molecules that selectively enhance or inhibit that process. Among several validated hits, the Hsp90 inhibitor 17-allylaminogeldanamycin (17-AAG) robustly decreases collagen-I secretion by our model cell line and by human primary cells. In these systems, 17-AAG and other pan-isoform Hsp90 inhibitors reduce collagen-I secretion post-translationally, and are not global inhibitors of protein secretion. Surprisingly, the consequences of Hsp90 inhibitors cannot be attributed to inhibition of the endoplasmic reticulum's Hsp90 isoform, Grp94. Instead, collagen-I secretion likely depends on

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Author Contributions

M.Y.W. and M.D.S. conceived the project. M.Y.W., M.D.S., and J.D.H. designed experiments and analyzed data. M.Y.W., N.D.D., A.S.D., L.J.P., J.H.C., C.K.S., and N.W. generated reagents and performed experiments. M.Y.W. and M.D.S. drafted the paper, and all authors edited the manuscript. </author_notes>

The authors declare that they have no conflicts of interest with the contents of this article.

ASSOCIATED CONTENT

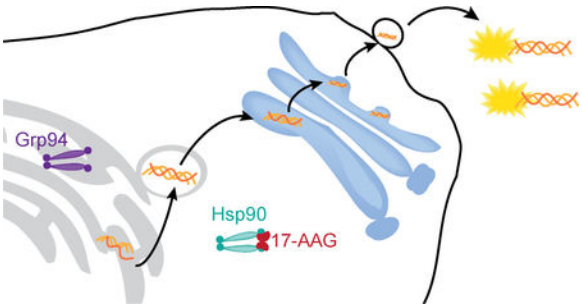
Supporting Information

Full screening and counter-screening results (Table S1.xlsx)

Supporting Information – extended experimental procedures, supporting tables, and supporting figures (.pdf)

the activity of cytosolic Hsp90 chaperones, even though such chaperones cannot directly engage nascent collagen molecules. Our results highlight the value of a cell-based high-throughput screen for selective modulators of collagen secretion, and point to an unanticipated role for cytosolic Hsp90 in collagen secretion.

Graphical Abstract



INTRODUCTION

By both function and sheer mass percentage, collagen constitutes the major component of animal tissue.[1] Twenty-eight distinct types of collagen play important roles in architecturally diverse extracellular matrices, ranging from skin and bone to cartilage and basement membranes.[2] In addition to providing the structural framework for these tissues, the collagens have dynamic functions in numerous biological processes.[3,4] For example, collagens engage integrins on cell surfaces, influence wound-healing responses and inflammation, and play critical roles in cell differentiation, organ development, and tissue maintenance.

Collagen biosynthesis is a complex process, encompassing extensive post-translational modifications, folding and assembly, propeptide cleavage, secretion, and extracellular fibril formation.[5] It is not surprising, then, that dysregulated collagen homeostasis is closely related to numerous pathologies.[6] Fibrosis is characterized by collagen overproduction and often leads to organ damage or failure.[7] Many cancers also feature high levels of collagen secretion and matrix remodeling, which promote metastasis.[8–10] Conversely, insufficient deposition of properly structured collagen and/or excessive accumulation of misfolded collagen molecules give rise to an array of phenotypically diverse pathologies.[11] These genetic disorders, ranging from osteogenesis imperfecta to Ehlers-Danlos syndrome, are typically caused by mutations in collagen genes or chaperones.[12]

Despite substantial work characterizing fibrosis[13] and the collagenopathies,[11, 14] many of the un-derlying disease mechanisms remain poorly defined, especially with respect to why and how collagen homeostasis fails. Biological and small molecule tools that selectively target the processes involved in the biosynthesis of collagen type-I, the most abundant collagen type,[15] could yield new mechanistic insights, in addition to providing leads for new therapeutic strategies. However, relatively few small molecules have been identified to date that can selectively alter either collagen-I folding or secretion.[16–18] Furthermore, high-throughput methods to facilitate the discovery and design of new compounds are limited, in

large part because assaying the folding and secretion of a protein that not only lacks enzymatic activity, but also requires a complex cellular folding environment,[5] is inherently challenging.

To address this need, we developed a cell-based, luminescent assay for modulators of collagen-I secretion. We screened 1228 known bioactive and FDA-approved compounds, validating a number of hit molecules that selectively affect collagen-I secretion but not the secretion of a control protein. Of particular interest, we find that the Hsp90 inhibitor 17-allylaminogeldanamycin (17-AAG) selectively reduces secretion of our collagen-I reporter. Follow-up studies confirmed the ability of numerous other pan-isoform Hsp90 inhibitors to reduce endogenous collagen-I secretion from human primary cells. Pan-isoform Hsp90 inhibitors have previously shown efficacy in fibrosis models,[19–24] but those effects have been attributed to disruption of inflammatory signaling pathways, such as those involving TGF-β. Our findings suggest a new relevant mechanism, as we identify direct effects on collagen secretion that are post-translational, thereby expanding the anti-fibrotic mechanism of Hsp90 inhibitors. Surprisingly, the effects of Hsp90 inhibitors cannot be attributed to reduced activity of the endoplasmic reticulum's (ER's) Hsp90 isoform Grp94, suggesting a previously unanticipated role for cytosolic Hsp90 in facilitating collagen-I secretion that may also prove relevant for other large or fibrillar secreted proteins.

MATERIALS AND METHODS

Cell Lines and Reagents

Saos-2-TREx cells[25] were grown in complete DMEM (Corning) supplemented with 15% heatinactivated fetal bovine serum (FBS), 100 IU penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine (Corning). Healthy dermal fibroblasts (GM05659, denoted "Line 1," or GM05294, denoted "Line 2;" Coriell Cell Repositories) were cultured in complete MEM supplemented with 15% FBS, 100 IU penicillin/100 μg/mL streptomycin, and 2 mM L-glutamine (Corning). Expression of eGLuc2 and eGLuc2.Colα2(I) was induced in stable Saos-2 lines by treating cells with 1 μg/mL doxycycline (Dox; Alfa Aesar). Secretion of endogenous collagen type-I was induced in Saos-2 cells or primary fibroblasts using 50 or 200 μM sodium ascorbate (Amresco), respectively. The ICCB Bioactives and FDA-Approved compound decks were purchased from Enzo as DMSO stock solutions in 96-well plate format and stored at –80 °C for < 2 years prior to use. For screening, the compounds were re-arrayed in V-bottom 96- and 384-well plates. Compound plates were thawed overnight at rt before pinning, and heat-sealed before re-freezing at –20 °C. The Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit with Alexa Fluor 488 Annexin V and propidium iodide was obtained from Life Technologies. Luciferase assays were performed either with homemade buffers[26] containing assay stabilizer from the commercially available Biolux kit (New England Biolabs) and 10 μg/mL coelenterazine (prepared from a 1 mg/mL stock in acidified methanol; Biotium) for high-throughput screening or using the Biolux kit (with commercial stabilizer) for dose-response curves. Immunoblots were probed with the following primary antibodies, as indicated: rabbit anti-GLuc (New England Biolabs E8023), mouse anti-Colα1(I) (DSHB SP1.D8), rabbit anti-Colα2(I) (Abcam ab96723), rabbit anti-Colα1(I) (NIH LF-68), rat anti-Hsp70/Hsp72 (Cell Signaling Technologies 4873), rabbit

anti-Akt (Cell Signaling Technologies 9272), mouse anti-β-actin (Sigma A1978), rat anti-FLAG (Agilent 200473), mouse anti-fibronectin (Sigma F7387), mouse anti-KDEL (Enzo 10C3 ADI-SPA-827; recognizes Grp94 and Grp78), mouse anti-Hsp90α (Santa Cruz sc515081), mouse anti-Hsp90β (Abcam ab53497), or TRAP1 (Santa Cruz sc13557).

Vector and Stable Cell Line Construction

The gene encoding eGLuc2 was PCR-amplified from an eGLuc2.pENTR1A plasmid[27] to remove the stop codon and then re-inserted into the pENTR1A plasmid. *COL1A2* (UniProt ID P08123) was cut from a previously developed PPT.FLAG.Colα2(I). pENTR1A plasmid[25] and inserted after eGLuc2 using the NotI and EcoRV sites to create the eGLuc2.Colα2(I).pENTR1A plasmid. eGLuc2 and eGLuc2.Colα2(I) were then recombined into pLenti.CMV.TO.DEST Gateway destination vectors[28] using LR clonase (Life Technologies). Lentiviruses were generated as described previously[29] (details provided in the Supporting Information) and used to transduce Saos-2-TREx cells. Stable cells were selected with 250 μg/mL hygromycin B and propagated in the same, with the addition of 10 μg/mL blasticidin to maintain the tetracycline repressor. Single colonies inducibly expressing moderate levels of eGLuc2.Colα2(I) (termed Saos-2$^{GLuc.Col}$ cells) were selected using immunoblotting.

High-Throughput Screening and Dose-Response Curves

Saos-2$^{GLuc.Col}$ cells were plated in flat-bottomed, white 384-well plates (Corning) at a density of 7,000 cells/well with an EL406 Washer Dispenser (BioTek) in a total volume of 50 μL of complete media. 5–6 h post-plating, 1 μL of a 50 μg/mL Dox stock solution was added and cells were treated with 50 nL of compound using a Freedom Evo 150 Liquid Handler (Tecan) fitted with a floating, slotted pin tool (V&P Scientific). Plates were incubated for 23 h at 37 °C, followed by equilibration for 1–1.5 h at rt in a single layer before reading. 10 μL of assay buffer was then dispensed into the wells using a plate washer, plates were mixed by orbital shaking for 5 sec, and luminescence signal was read using a Thermo Infinite M1000 Plate Reader (Tecan) with a 100 msec integration time, beginning 35 sec after buffer addition. Saos-2^{GLuc} cells were plated at a density of 3,000 cells/well prior to screening using an otherwise identical protocol. For cytotoxicity counter-screening, intracellular ATP was quantified after the same time courses of Dox and compound treatments using the CellTiter-Glo Assay (CTG; Promega). Briefly, 10 μL of CTG buffer was added to the cells, the plate was incubated for 10 min at rt, and then luminescence was read using an integration time of 100 msec. All screens were performed in biological duplicate. Screening results were filtered for cytotoxicity using 3 standard deviations below the average of DMSO-treated cells as a cutoff. A Z′ factor of 0.72 was approximated for the assay by calculating the separation between the highest and lowest compound-treated wells, according to Zhang et al.[30] The coefficient of variation (% CV) was calculated by taking the ratio of the SD to the average DMSO signal for each cell line. Complete raw screening results are presented in Supplementary Table 1.

Co-Immunoprecipitation Experiments

Saos-2$^{GLuc.Col}$ cells were plated in 10-cm dishes at a density of 1.25×10^6 cells per plate and allowed to adhere overnight. The next day, the media was changed and cells were

treated with ascorbate with or without 1 μg/mL Dox. After 24 h, cells received fresh media and were re-treated with fresh ascorbate and Dox. After another 24 h, media was removed, and cells were trypsinized, pelleted, washed with 1× phosphate-buffered saline (PBS), and then lysed in Triton-X lysis buffer (200 mM NaCl, 1% Triton-X 100, 50 mM Tris pH 7.5, 1 mM EDTA, and 1.5 mM $MgCl_2$) +1.5 mM phenylmethylsulfonyl fluoride (PMSF; Sigma) and protease inhibitor tablet (Thermo Fisher). Lysates were cleared by centrifugation at 21.1 × 10^3 ×g 4 °C for 15 min, total protein was quantified using the bicinchoninic acid assay, and 2 mg of lysate was incubated with 5 μL of α-GLuc antibody (NEB) in 500 μL volume end-over-end at 4 °C. After 4 h, 30 μL of Protein A/G PLUS-Agarose bead slurry (Santa Cruz) was added and samples were incubated end-over-end at 4 °C overnight. The next day, samples were washed 3× with Triton-X lysis buffer, dried with a 30.5G needle, and eluted by boiling in Laemmli buffer supplemented with 167 mM 1,4-dithiothreitol (DTT; Sigma). Samples were separated on 4/8/12% Tris-Gly SDS-PAGE gels, with 2.5% of unprecipitated lysate as input and 50% of the elution, before transferring to nitrocellulose membranes (Bio-Rad). Blots were probed using an anti-GLuc antibody or SP1.D8, a mouse monoclonal antibody against the N-terminal propeptide of Colα1(I) (DSHB).

Precipitation of Collagen-I from Conditioned Media

Saos-$2^{GLuc.Col}$ cells were plated in 10-cm dishes at a density of 1.25 × 10^6 cells per plate and allowed to adhere overnight. The next day, the media was changed and cells were treated with ascorbate either with or without 1 μg/mL Dox. After 24 h, cells were re-treated with fresh ascorbate and Dox. After another 24 h, conditioned media was transferred to falcon tubes and spun at 228 ×g for 5 min to pellet cell debris. The supernatants were then transferred to new tubes and collagen-I was precipitated according to Makareeva and co-workers.[31] Briefly, 1.5 mM PMSF, 20 μM EDTA (Aqua Solutions), and 100 mM Tris pH 7.4 were added. Samples were then gently mixed and cooled on ice before adding 176 mg/mL ammonium sulfate (Sigma) and incubating end-over-end at 4 °C overnight. The resulting samples were centrifuged at 2800 ×g at 4 °C for 25 min. Pellets were then resuspended in 1 mL of supernatant, transferred to 1.7 mL Eppendorf tubes, and centrifuged at 21.1 × 10^3 ×g at 4 °C for 20 min. The supernatant was removed and pellets were stored at −20 °C until use.

Disulfide-Dependent Assembly Assays

Precipitated collagen-I pellets were thawed on ice, resuspended in 80 μL of 0.1 M $NaHCO_3$ pH 8.3, and split into Eppendorf tubes (35 μL each). 3.5 μL of 200 mM of DTT in 0.1 M $NaHCO_3$ pH 8.3 was then added (to give a final concentration of 20 mM) and samples were heated at 60 °C for 15 min; non-reduced samples were treated with an equal volume of 0.1 M $NaHCO_3$ pH 8.3 before heating. After reduction, samples were cooled at rt for 15 min, and then 4.28 μL of 600 mM iodoacetamide (IAA; VWR) in $NaHCO_3$ buffer was added to yield a final concentration of 60 mM. Samples were incubated in the dark with IAA at rt for 1 h, mixed with 8.6 μL of Laemmli buffer, heated, and separated on 4/7% Tris-Gly SDS-PAGE gels. Proteins were then transferred to nitrocellulose membranes, which were developed with anti-GLuc and SP1.D8.

Western Blotting Analysis of Pan-Isoform Hsp90 Inhibitor Treatment

GM05659 or GM05294 primary fibroblasts were plated in 6-well plates at a density of 2×10^5 cells/well and allowed to adhere overnight. The next day, the media was changed to full MEM with 0.5× penicillin/streptomycin, collagen expression was induced with ascorbate, and cells were treated with DMSO or the indicated compound concentrations for 24 h. Media samples were denatured by boiling in Laemmli buffer (supplemented with 167 mM DTT), separated on 4/8% Tris-Gly SDS-PAGE gels, and transferred to nitrocellulose membranes. Due to cross-reactivity between the collagen-I antibodies, media samples for collagen-I blots were run on duplicate gels, transferred, and then probed separately for either rabbit anti-Colα2(I) or rabbit anti-Colα1(I).

For Western blots of cell lysates, cells were trypsinized, washed with PBS, and lysed in Triton-X lysis buffer +1.5 mM PMSF and protease inhibitor tablet. Total protein was quantified using the bicinchoninic acid assay, and 50 μg of total protein was analyzed for each sample. Each experiment was run in biological triplicate. Blots were imaged after incubating with appropriate primary and 800CW or 680LT secondary antibodies (LI-COR) by scanning on an Odyssey infrared imager (LI-COR), followed by quantification using ImageJ.

Fluorescence-Assisted Cell Sorting

GM05659 primary fibroblasts were plated at a density of 1×10^6 cells/plate in 6-cm dishes and allowed to adhere overnight. The next day, cells were treated as described for Western blotting. After 24 h of compound treatment, cells were harvested and labeled with an Annexin V-Alexa Fluor 488 conjugate and propidium iodide (Life Technologies) according to the manufacturer's instructions, and analyzed on a FACSAria sorter (BD Biosciences) using a flow rate of 2.7 mL/h with 20,000 events gated to exclude cell debris.

Pulse Labeling

Collagen translation was assessed by [5-^{3}H]proline-labeling. GM05659 primary fibroblasts were plated at a density of 1.2×10^5 cells/well in 12-well plates and allowed to adhere overnight. The next day, cells were treated as described for Western blotting. After 6 h of treatment, media was removed, cells were washed 3× with PBS, and then serum-starved in MEM + 15% dialyzed FBS and 2 mM L-glutamine. Media was changed to radiolabelled media (100 μCi [5-^{3}H]proline/mL; Perkin Elmer) for 30 min, after which cells were washed 3× with full, non-radiolabeled MEM and harvested. Both ascorbate (200 μM) and 17-AAG (250 nM) were included during the 30 min serum starve and 30 min pulse. Harvested samples were washed with PBS, lysed in standard RIPA buffer containing protease inhibitor tablets and 1.5 mM PMSF, and run on 4/6% Tris-Gly SDS-PAGE gels. Gels were dehydrated, incubated with scintillant solution (20% w/w 2,5-diphenyloxazole in DMSO; Sigma), washed, and then dried as previously described.[32] Results were quantified in ImageJ.

Metabolic Labeling of Secreted Proteins

GM05659 primary fibroblasts were plated at a density of 2×10^5 cells/well in 6-well plates and allowed to adhere overnight. The next day, cells received fresh media containing

ascorbate and DMSO or 17-AAG (250 nM) and were returned to the incubator for 2 h. After pre-treatment, media was then replaced with ^{35}S-Met and Cys-containing media: 100 μCi of EXPRESS Protein Labeling Mix [35S] (Sigma) per mL of Met- and Cys-free DMEM (Corning) supplemented with 15% dialyzed FBS and 2 mM L-glutamine. Cells were re-treated with ascorbate and DMSO / 17-AAG, and incubated for 6 h. Plates were then removed to ice, and then media samples were transferred to Eppendorf tubes, spun at 207 ×g at 4 °C for 10 min to pellet cell debris, and transferred to new tubes. Media samples were separated on 4/8% Tris-Gly SDS-PAGE gels and dried on a gel slab dryer for 75 min at 80 °C. Dried gels were developed with a GE Healthcare Phospho Screen and imaged the next day on a Typhoon FLA 7000.

Quantitative PCR

GM05659 primary fibroblasts were plated and treated as for Western blotting. After 24 h of compound treatment, cells were washed with PBS and RNA was extracted using the Omega E.Z.N.A. Total RNA extraction kit. cDNA was prepared from 500 ng RNA, normalized for all samples in each run, using an Applied Biosystems Reverse Transcriptase cDNA Kit in a BioRad Thermocycler. Samples were run on a Light Cycler 480 II Real Time PCR Instrument in the MIT BioMicro Center using previously described primers[25, 33] and analysis methods.[29] qPCR primer sequences used for detecting Grp94 (*HSP90B1*) transcript levels were: 5′-GGCCAGTTTGGTGTCGGT-3′; 5′-CGTTCCCCGTCCTAGAGTGTT-3′.

Transthyretin and Fibulin-3 Secretion

GM05659 primary fibroblasts were plated at a density of 2×10^5 cells/well in 6-well plates the day before transduction. After being allowed to adhere overnight, cells received fresh media and were transduced with adenoviruses encoding GFP, transthyretin (TTR), or fibulin-3. The next day, cells were split 1:2 into 12-well plates. Collagen-I expression was induced the following day as described for Western blotting, and cells were treated with either DMSO or 250 nM 17-AAG. After 24 h, media was collected, separated on 4/8% (collagen-I), 4/10% (fibulin-3), or 4/15% (TTR) Tris-Gly SDS-PAGE gels, and probed for collagen-I or FLAG. Details of adenoviral production are provided in the Supporting Information.

Grp94 shRNA Knockdown

GM05659 primary fibroblasts were plated at 2×10^5 cells/well in 6-well plates the day before transduction. After being allowed to adhere overnight, cells were given fresh media (+4 μg/mL polybrene) and 600 μL of the indicated lentivirus. The next day, cells received a media change and were incubated until 72 h post-transduction, when media was changed and collagen-I expression was induced with ascorbate. Conditioned media was collected 24 h later for Western blotting, as described above. Sequences for shRNA constructs (Table S3) and details of lentiviral production are provided in the Supporting Information. Transductions for co-treatment experiments with Grp94 shRNAs and 17-AAG were performed as described above, but using 300 μL of the indicated lentivirus.

Statistical Analyses

Data are presented as the mean and standard deviation from at least three biological replicates, with the exception of screening data, which were generated in biological duplicate. Statistical significance was assessed on normalized data from immunoblots using an unpaired Student's *t*-test (unequal variances), with significance thresholds of * = $p < 0.05$ and ** = $p < 0.01$.

RESULTS

Luminescent Assay Design and Validation

Because collagen-I lacks enzymatic activity, current methods to assay collagen-I levels in moderate- to high-throughput fashion primarily rely on the binding of hydrophobic dyes to deposited collagen fibers.[34, 35] Other assays are either conducted *in vitro* with purified collagen-I and recombinant collagen-binding proteins[16, 36] or measure collagen transcription,[37] rather than the many posttranscriptional processes key to collagen biosynthesis.

We reasoned that appending a luciferase enzyme to collagen-I would allow for direct monitoring of fusion protein secretion, with increased sensitivity from the enzymatic signal amplification. With this approach, the screen would be suitable for discovering both activators and inhibitors, and would not require prior knowledge of collagen folding, quality control, and secretion pathways. For our reporter molecule, we selected the engineered, or "enhanced," variant of *Gaussia* luciferase (eGLuc2). eGLuc2 is natively secreted, small, bright, ATP-independent, highly stable, and has been used successfully for high-throughput screening.[27, 38–41] As native collagen-I is a 2:1 Colα1(I):Colα2(I) heterotrimer,[42] we attached eGLuc2 to Colα2(I) to control the stoichiometry of fusion protein incorporation. We positioned eGLuc2 at the extreme N-terminus to avoid interfering with triple-helix nucleation by collagen's C-terminal propeptide (Fig. 1A).[43] We also included the eGLuc2 signal sequence to direct eGLuc2.Colα2(I) to the secretory pathway. Finally, we engineered the fusion protein gene to be under control of a tetracycline repressor-regulated, doxycycline (Dox)-inducible promoter to both simplify the long-term propagation of stable cell lines as well as provide temporal control of eGLuc2.Colα2(I) expression.

We next engineered Saos-2 osteosarcoma cells, which are osteoblast-like and produce endogenous collagen-I,[44, 45] to stably and inducibly express eGLuc2.Colα2(I). We had previously shown that Saos-2 cells stably expressing the tetracycline repressor (termed Saos-2-TREx cells) can be used to control the heterologous expression of antibody epitope-tagged collagen-I variants.[25] Building on that work, we transduced Saos-2-TREx cells with an eGLuc2.Colα2(I)-encoding lentivirus, selected for stable gene incorporation, validated protein expression by immnoblot (Fig. S1A), and isolated a genetically homogeneous single colony line for assay development. eGLuc2.Colα2(I) secretion can be assayed in the resulting Saos-2$^{GLuc.Col}$ cells by inducing transcription with Dox and then measuring the extent of secretion via a luminescence assay on the conditioned media (Fig. 1A). We verified that the extracellular luminescent signal is increased in response to sodium ascorbate (Fig. S1B), which induces collagen-I translation and also facilitates collagen-I secretion by

recycling the prolyl-4-hydroxylase metallocofactor.[42, 46, 47] eGLuc2.Colα2(I) secretion is also strongly reduced by treatment with the ER-to-Golgi transport inhibitor Brefeldin A (Fig. S1C).[48] Thus, the secretion of our eGLuc2.Colα2(I) fusion protein is dependent on mechanisms known to be involved in collagen export. In tandem, we also prepared a Saos-2^{GLuc} cell line, which inducibly expresses unfused eGLuc2 under the tetracycline repressor, as a control for our high-throughput screen.

Although we and others[25, 49] have generated N-terminal fusions to collagen-I and shown that such proteins are well behaved, we sought to evaluate whether the presence of eGLuc2 disrupts collagen-I folding or assembly. We first determined whether eGLuc2.Colα2(I) associates with Colα1(I) intracellularly. Immunoprecipitating eGLuc2.Colα2(I) with an α-GLuc antibody from Saos-2$^{GLuc.Col}$ cell lysate revealed that both eGLuc2.Colα2(I) and Colα1(I) are enriched in the eluted fraction (Fig. 1B), indicating formation of a stable heteromeric complex, as expected, and suggesting that eGLuc2.Colα2(I) can successfully assemble with Colα1(I).

We next sought to determine if secreted eGLuc2.Colα2(I) also formed stable heteromers with Colα1(I). As disulfide-bond formation in the C-terminal propeptide is needed to initiate collagen-I triple helix formation,[5] we precipitated collagen-I from conditioned media of Saos-2$^{GLuc.Col}$ cells and assayed for disulfide-dependent assembly by SDS-PAGE (Fig. 1C). We observed that secreted eGLuc2.Colα2(I) associates with Colα1(I), as detected by the co-staining of α-GLuc and Colα1(I) antibodies under non-reducing conditions. While we observe monomeric (~190 kDa) and dimeric (~245 kDa) species for both Colα1(I) and eGLuc2.Colα2(I) under non-reducing conditions, likely due to disulfide shuffling during SDS-PAGE separation, the banding pattern is consistent with previous observations by us and others for native collagen-I.[50, 51] Importantly, in the presence of the reducing agent DTT, both eGLuc2.Colα2(I) and Colα1(I) collapse to the monomeric molecular weight, as expected for dissociation of disulfide-linked trimers.

We also performed pepsin digests of precipitated collagen-I from Saos-2$^{GLuc.Col}$ cells. Properly folded collagen-I triple helices are known to be pepsin-resistant,[52] and indeed we find that upon Dox treatment, a second pepsin-resistant band corresponding to propeptide-cleaved eGLuc2.Colα2(I) appears in addition to propeptide-cleaved Colα1(I) (Fig. S2). Together, these results suggest that the eGLuc2.Colα2(I) fusion protein is incorporated into stable triple helices with Colα1(I), and that eGLuc2 tagging does not significantly disrupt collagen-I assembly or folding.

High-Throughput Screening for Collagen-I Secretion Modulators

We next miniaturized our collagen secretion assay for high-throughput screening in 384-well plates, tested for linearity, and optimized a timeline for induction of collagen expression and compound addition (detailed assay parameters are presented in the Materials and Methods; full, normalized screening data are presented in Table S1). For our initial screen of eGLuc2.Colα2(I) secretion, we employed the Harvard Medical School Institute of Chemistry and Cell Biology (ICCB) Bioactives and the FDA-Approved small molecule libraries, commercially available from Enzo Life Sciences. Together, the libraries contained 1228 unique compounds spanning at least 22 drug target classes. Our results from analyzing

conditioned media after 24 h of eGLuc2.Colα2(I) secretion were quite reproducible, as indicated by a low coefficient of variation (% CV, vehicle-treated wells) and a Pearson's correlation coefficient of 0.9611 between eGLuc2.Colα2(I) replicates for compound treatments across both libraries (Fig. S3A and C).

A number of factors, including compound toxicity or effects on fusion protein transcription/translation, general protein secretion, or luciferase enzyme activity, might produce a response in this high-throughput assay, despite being unrelated to our desired phenotype of selective effects on collagen-I secretion. We performed two counter-screens to filter out such false positives: (1) we used a CellTiter-Glo assay to remove cytotoxic compounds, and (2) we employed an eGLuc2 secretion assay to remove compounds that shift eGLuc2-derived signal in the same direction as eGLuc2.Colα2(I)-derived signal (Fig. S3B). The results are presented in Fig. 1D. Notably, many of the compounds that strongly decreased eGLuc2.Colα2(I) luminescence were also flagged in our CellTiter-Glo secondary screen as cytotoxic (signal >3 standard deviations below the average DMSO signal) at the concentrations used, and were therefore triaged. Meanwhile, many of the compounds that yielded the largest changes in normalized luminescence in our eGLuc2.Colα2(I) secretion assay, some on the order of ~5-fold increases, were triaged because they had similar effects on luminescence signal obtained in media from our control Saos-2^{GLuc} cell line. The striking effects on protein secretion yielded by some of these compounds (see Table S1) suggest that they may prove useful as broad-spectrum modulators of secretory pathway function, although the effects may also be due merely to modification of eGLuc2 luminescence. Regardless, the aim of our screening protocol was to identify compounds that selectively target collagen-I secretion. Those with obviously non-specific effects on protein secretion were not pursued further in this study.

After filtering the primary screening results as described above, we obtained 17 putative hit compounds that selectively modulate eGLuc2.Colα2(I) secretion from Saos-2 cells. A list of these compounds, their effects on eGLuc2.Colα2(I) secretion, and their annotated functions is provided in Table S2.

Structurally Diverse Hsp90 Inhibitors Reduce Endogenous Collagen-I Secretion by Primary Cells

We re-purchased and re-tested 15 of the 17 compounds that passed our screening filters, yielding six validated small molecules that selectively alter eGLuc2.Colα2(I), but not eGLuc2, secretion, and are not cytotoxic in Saos-2 cells at the tested concentrations (Fig. 1E and Fig. S4). 17-Allylamino geldanamycin (17-AAG), a particularly well-characterized inhibitor of the chaperone Hsp90,[53, 54] stood out to us from these validated compound hits because it has opposite effects on eGLuc2.Colα2(I) and eGLuc2 secretion (Fig. 1E). Furthermore, 17-AAG and its analogue 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) have been reported to display beneficial effects in fibrosis models, by a mechanism involving reduction of inflammatory signaling but not by direct inhibition of collagen biosynthesis, folding, or secretion.[20, 22, 23, 55] We, however, hypothesized that Hsp90 inhibition might directly impede collagen-I secretion. To explore

this possibility further, we chose to follow up on the 17-AAG results from our high-throughput screen and investigate the mechanism of action.

To begin, we treated Saos-2$^{GLuc.Col}$ cells with a range of 17-AAG concentrations, and observed a dose-dependent reduction in luminescent signal (Fig. 2A). Next, we focused on testing whether these results would recapitulate in primary cells expressing endogenous collagen-I, instead of an chose to employ primary human fibroblasts, which can be readily cultured *in vitro* and are one of the major producers of collagen-I in humans. We also wanted to confirm that the response to 17-AAG was a result of Hsp90 inhibition, and not an unappreciated off-target effect. We therefore obtained five additional Hsp90 inhibitors spanning diverse molecular scaffolds[53] (Fig. 2B) and tested the entire Hsp90 inhibitor set for effects on collagen-I secretion in primary human fibroblasts.

After 24 h of treatment with pan-isoform Hsp90 inhibitors, immunoblotting revealed significant decreases in endogenous collagen-I secretion relative to vehicle-treated cells for 17-AAG, 17-DMAG, EC 144, and STA-9090 (Fig. 2C and D). We observed no changes in intracellular levels of collagen-I. We also confirmed functional inhibition of cytosolic Hsp90 at the concentrations used, indicated both by the expected increase in Hsp70 levels due to induction of a compensatory heat shock response (HSR) as well as by the loss of signal from the cytosolic Hsp90 client Akt (Fig. 2C).[56, 57] One pan-isoform Hsp90 inhibitor, radicicol, caused a notable but not significant decrease in collagen-I secretion (Fig. 2C and D). However, radicicol was later found to be mildly cytotoxic (see Fig. 3A), and radicicol treatment induced Akt degradation less effectively than the other pan-isoform Hsp90 inhibitors, perhaps explaining its less striking effects on collagen-I secretion. Cumulatively, the data in Fig. 2B–D indicate that reductions in collagen-I secretion mediated by diverse Hsp90 inhibitors do occur in primary cells and that the reduced collagen-I secretion is an on-target consequence of Hsp90 inhibitor treatment.

Finally, to test whether the effects of Hsp90 inhibitor treatment on endogenous collagen-I secretion by primary fibroblasts were cell-line specific, we obtained a second primary fibroblast line. As expected, 17-AAG treatment caused similar reductions in collagen-I secretion from these cells (Fig. 2E), indicating that our results are not dependent on a particular primary fibroblast genotype.

Hsp90 Inhibitors Reduce Collagen-I Secretion Via a Post-Translational Effect

We next investigated the mechanism by which Hsp90 inhibitor treatment reduces collagen-I secretion. Possible trivial explanations we considered were (1) that reduced collagen-I secretion in primary fibroblasts is caused by compound cytotoxicity, (2) that Hsp90 inhibitors reduce the levels of collagen-I transcripts, and/or (3) that Hsp90 inhibitors lower collagen-I translation. Our Saos-2 data (Fig. 1) suggested that these explanations were not applicable, as we had already counter-screened for cytotoxicity and we employed a non-native promoter, but it was nonetheless important to test all three possibilities in primary cells.

We treated primary fibroblasts with pan-isoform Hsp90 inhibitors for 24 h, and then tested for cytotoxicity by staining cells with Annexin-V/Alexa Fluor 488 and propidium iodide

followed by FACS analysis. With the exception of radicicol, which we found to be mildly cytotoxic, none of the Hsp90 inhibitors tested were toxic at the concentrations used, confirming that Hsp90 inhibitors do not decrease collagen-I secretion from primary fibroblasts simply by promoting cell death (Fig. 3A).

Next, we performed qPCR analysis of collagen-I transcripts from Hsp90 inhibitor-treated primary fibroblasts. We found that *COL1A1* and *COL1A2* transcript levels are not significantly altered, indicating that the mechanism of action is post-transcriptional (Fig. 3B). Finally, we used a tritium pulse assay to test whether collagen-I translation is altered by 17-AAG treatment. Briefly, we pulsed treated fibroblasts with [5-^{3}H]proline for 30 min to label the proline-rich collagen-I protein, followed by collection of cell lysate and autoradiography analysis of the collagen-I bands (identified by bacterial collagenase treatment). We observed no significant alterations in levels of newly synthesized, radiolabeled collagen-I upon 17-AAG treatment (Fig. 3C and Fig. S5A). Cumulatively, these data indicate that Hsp90 inhibitors reduce collagen-I secretion via a post-translational mechanism.

Hsp90 Inhibitors Selectively Reduce Collagen-I, But Not General Protein Secretion, in Primary Fibroblasts

Another potential explanation for our results is that Hsp90 inhibitors non-selectively reduce protein secretion. We had established that 17-AAG treatment does not inhibit eGLuc2 secretion in Saos-2 cells (Fig. 1), but it remained unclear whether effects on endogenous collagen-I secretion in primary fibroblasts would be selective. Indeed, prior work suggests that Hsp90 inhibitors can disrupt protein secretion in yeast[58] or inhibit ER-to-Golgi transport by perturbing Rab1 GTPase recycling in mammalian cells,[59] and so we wondered if the effects of Hsp90 inhibitor treatment on collagen-I secretion might extend generally to other proteins.

To examine this question, we transduced primary fibroblasts with adenoviruses expressing FLAG-tagged transthyretin (TTR) or fibulin-3 (two well-characterized and soluble secreted proteins), treated the cells with 17-AAG at a concentration that reduces collagen-I secretion, and assayed for altered secretion of these additional ER client proteins (Fig. 4A). Treatment with 17-AAG did not alter TTR secretion, while fibulin-3 secretion was actually enhanced ~3-fold by 17-AAG treatment (Fig. 4B). We next tested the secretion of fibronectin, an endogenous secreted protein that interacts with collagen-I but is secreted in globular form.[60, 61] Again, 17-AAG robustly decreased collagen-I secretion, but did not alter fibronectin export (Fig. 4C and D).

Although these proteins are not representative of all possible secretory pathway cargo, their response to 17-AAG treatment, in contrast to what we observe for collagen-I from the same cells, strongly suggests that 17-AAG at the concentrations employed reduces collagen-I secretion with considerable selectivity. However, because high concentrations of Hsp90 inhibitors had been reported to induce Golgi fragmentation,[59] we sought to more rigorously test whether global protein secretion was inhibited under our experimental conditions.

We first evaluated Golgi integrity using imaging techniques. We treated primary fibroblasts with ascorbate and DMSO or 17-AAG for 20 h and then stained cells with an antibody raised against the *cis*-Golgi marker GM-130.[59] Confocal microscopy showed that treatment with Brefeldin A resulted in a punctate α-GM-130 distribution, as expected (Fig. S6). By contrast, 17-AAG did not significantly alter Golgi structure relative to DMSO treatment, either at the concentration used in our collagen-I secretion experiments (250 nM) or at a 10-fold higher dose. We also performed electron microscopy on fibroblasts treated with ascorbate and 17-AAG for 6 or 18 h. In addition to the characteristic Golgi stacks, some regions of vesicular tubular networks were visible in all samples; however, neither 6 nor 18 h of 17-AAG treatment resulted in Golgi collapse or loss of morphology relative to DMSO treatment (Fig. S7).

Next, we employed radiolabeling to globally assess protein secretion. We pre-treated fibroblasts with ascorbate and 17-AAG for 2 h, and then incubated the cells with [^{35}S]Met and Cys-labeled media, ascorbate, and 17-AAG for 6 h. The resulting phosphorimages, shown in Fig. 4E and Fig. S5B, reveal that treatment with 17-AAG does not broadly affect the secretion of radiolabeled proteins in our system. Based on these results, we conclude that treatment with Hsp90 inhibitors can reduce collagen-I secretion from primary fibroblasts without globally inhibiting protein trafficking.

Grp94 Knockdown Does Not Inhibit Collagen-I Secretion

There are several Hsp90 isoforms in the cell: Hsp90α and Hsp90β in the cytosol, Grp94 in the ER, and TRAP1 in mitochondria (Fig. 5A).[62] Although 17-AAG and the other Hsp90 inhibitors employed can bind to and inhibit all Hsp90 isoforms, nascent collagen-I molecules are found only in the ER and Golgi. We therefore questioned whether inhibition of Grp94, which is already known to engage collagen-I in the ER,[25, 63] was responsible for the observed effects of pan-isoform Hsp90 inhibitors on collagen-I secretion, possibly by slowing or preventing the folding of collagen-I. To test this hypothesis, we generated lentiviruses encoding a non-mammalian control shRNA or three distinct shRNA constructs targeting Grp94 (Table S3). We examined the levels of cytosolic Hsp90α and Hsp90β and the mitochondrial isoform TRAP1 and showed that they are unaffected by Grp94 knockdown, confirming the specificity of our shRNA constructs (Fig. S8A). We then transduced primary fibroblasts with these lentiviruses, induced collagen-I expression with ascorbate 72 h post-transduction, collected media for 24 h, and then assayed levels of secreted collagen-I by immunoblotting (Fig. 5B and C). Strikingly, we observed no reduction in collagen-I levels in conditioned media, despite strong knockdown of Grp94 at the protein level.

We next evaluated whether Hsp90 inhibitors could reduce collagen-I secretion even in a background of Grp94 knockdown. We repeated the Grp94 shRNA knockdown and then co-treated cells with DMSO or 17-AAG (Fig. 5D). qPCR on these cells confirmed that Grp94 transcript levels decrease upon shRNA knockdown; in addition, we observed that Hsp70 transcript levels increase upon 17-AAG treatment, indicating functional inhibition of Hsp90 (Fig. S8B). We then collected and analyzed conditioned media by immunoblotting, and found that treating fibroblasts with 17-AAG decreased collagen-I secretion for both a non-

targeting control and a Grp94-targeting shRNA (Fig. 5E). These results demonstrate that Hsp90 inhibitors can reduce collagen-I secretion independent of Grp94, and suggest that Grp94 is in fact dispensable for collagen-I secretion under the conditions studied here. Instead, inhibition of another Hsp90 isoform, most likely cytosolic Hsp90, is likely to be responsible for reductions in collagen-I secretion.

DISCUSSION

Given that Grp94 knockdown does not inhibit collagen-I secretion, how might the cytosolic Hsp90 isoforms participate in the process? As the heat shock protein Hsp47 is a well-established collagen-specific chaperone[63] whose expression increases upon HSR activation,[64] it is possible that Hsp90 inhibitors reduce collagen-I secretion by increasing Hsp47 availability through the HSR. However, a recent report revealed that a small molecule inhibitor of the Hsp47–collagen interaction instead reduces collagen secretion,[16] indicating that a different mechanism is likely operating here.

A more likely possibility is that cytosolic Hsp90 influences collagen-I secretion via interactions with cytosolic components of the secretory pathway. Because of its size and rigidity, collagen-I cannot be exported from cells in conventional COP-II secretory vesicles. Recent work has suggested that collagen is instead transported via a unique secretion pathway involving expanded COP-II vesicle coats.[65, 66] A combination of live-cell imaging, correlated light electron microscopy, and super-resolution microscopy techniques showed that enlarged COP-II vesicles are competent to carry collagen-I and are physically separated from the ER.[67] Because the inhibitors we tested selectively reduce secretion of collagen-I, but not secretion of the more compact proteins eGLuc2, TTR, fibulin-3, or fibronectin, we speculate that cytosolic Hsp90 may be required for the assembly of these large, collagen-specific vesicles, a hypothesis we are actively investigating.

Significant efforts by multiple groups have revealed how another large collagen, type-VII, is exported from cells. Specifically, TANGO1 concentrates collagen-VII at ER exit sites, recruits ERGIC membranes to the growing collagen carrier, and cooperates with cytosolic protein partners such as cTAGE5, Sedlin, and Sly1 to regulate vesicle growth.[68–72] A separate study found that the transcription factor BBF2H7 up-regulates the expression of genes involved in COP-II vesicle expansion to facilitate a switch from export of short-chain collagen type-VIII to extended collagen type-II.[73] These results suggest that the mode of collagen secretion may be tailored to specific types, based on developmental signaling[73] or the directionality of collagen secretion.[74] Indeed, the dependence of collagen-I secretion on the Sec16-binding protein TFG implies the existence of analogous, but distinct, mechanisms of endoplasmic reticulum exit site organization for collagen-I and collagen-VII export.[75] Exactly how TFG, TANGO1, and their partners facilitate the secretion of specific collagen types awaits further clarification.[68, 72, 76] The high-throughput screen reported here and Hsp90 inhibitors should prove enabling for such studies.

We note that ~50% reductions in collagen-I levels, on the order of what we observe in primary fibroblasts, are sufficient to reduce fibrotic area in tissue sections from mouse models,[20] suggesting that the effect sizes we detect upon Hsp90 inhibition could be

sufficient to reduce disease burden. Moreover, collagen-I secretion is in some cases reduced by Hsp90 inhibitors to levels similar to that of the ascorbate-untreated control, and thus likely approaches the realistic limit for collagen-I secretion inhibition in our model system.

Looking beyond the specific insights we obtained regarding the capacity of Hsp90 inhibitors to reduce collagen-I secretion, we note that as a large, multimeric protein that simultaneously lacks enzymatic activity and requires highly specialized cellular folding machinery, collagen is a challenging protein to assay. We believe the cell-based, high-throughput screen for collagen-I secretion designed and validated here provides a viable solution to this challenge that will prove useful on numerous accounts. Indeed, in addition to the Hsp90 inhibitors we also identified six other functionally and structurally distinct small molecules that selectively affect collagen-I secretion. These validated hits include two structurally related EGFR tyrosine kinase inhibitors (AG1478 and gefitinib), a Ras farnesyl transferase inhibitor (L-744,832), a dopamine D2 receptor agonist (bromocriptine mesylate), and mitomycin C (Figure S4). While most of these compounds have not been tested in collagen-related disease models, mitomycin C was previously used as a wound healing adjuvant, where it was found to slow the healing process but also to reduce fibrosis.[77–79] Follow-up studies using these compounds are expected to provide additional insights into mechanisms of collagen-I biosynthesis and secretion, as well as new potential opportunities for therapeutic intervention.

In summary, we screened 1228 compounds against our cell-based, high-throughput assay and identified six selective small molecule modulators of collagen-I secretion. Using the parent compound 17-AAG identified in our screen, we further validate Hsp90 inhibition as a viable anti-fibrotic strategy[19–24, 55] that points to a possible role for cytosolic Hsp90 in post-translationally regulating collagen-I secretion. Our screen design employs cells that natively synthesize collagen-I, and uses stably engineered cell lines for improved convenience and consistency. Our high-throughput assay can also be readily adapted to study disease-causing, misfolding variants of collagen-I by mutagenizing the wild-type Colα2(I) gene in our eGLuc2.Colα2(I) fusion protein, and we expect this flexibility to provide a valuable addition to the suite of current tools to study the collagenopathies. Finally, the continued development of more selective Hsp90 inhibitors will aid efforts to determine which Hsp90 isoform(s) are required for collagen-I secretion, and how cytosolic Hsp90 might regulate this process.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

17-AAG	17-allylaminogeldanamycin
Colα1(I)	collagen-α1(I)
Colα2(I)	collagen-α2(I)
CTG	CellTiter-Glo
Dox	doxycycline
DTT	1,4-dithiothreitol
ER	endoplasmic reticulum
eGLuc2	enhanced *Gaussia* luciferase 2
FBS	fetal bovine serum
GFP	green fluorescent protein
Grp94	94 kDa glucose-regulated protein
Hsp90	heat shock protein 90
HSR	heat shock response
IAA	iodoacetamide
nmc	non-mammalian control
PBS	phosphate-buffered saline
PMSF	phenylmethylsulfonyl fluoride
TTR	transthyretin

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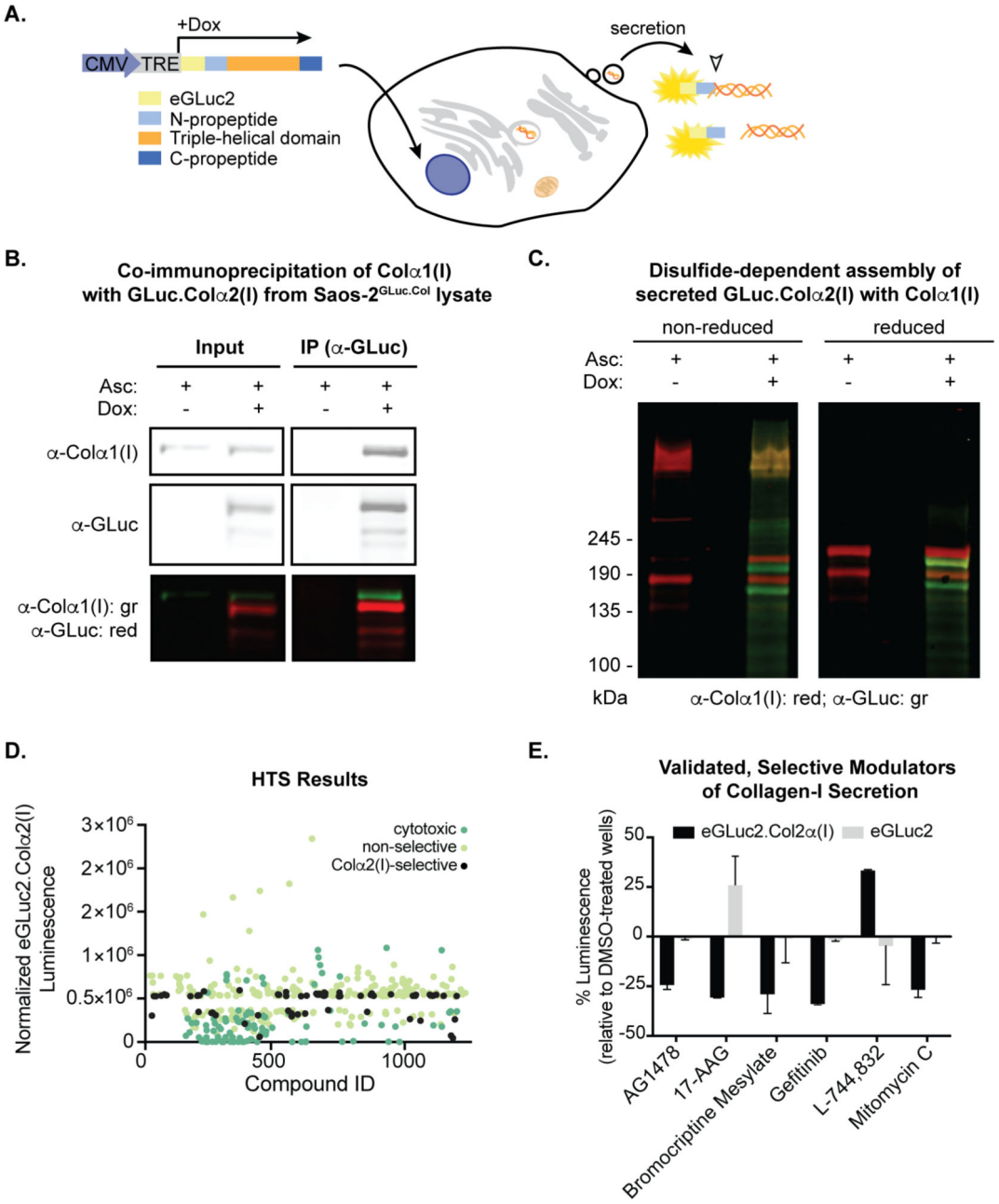

Figure 1.

High-throughput screening strategy and results. (A) Schematic of screen design. Addition of 1 μg/mL doxycycline (Dox) to the stable single-colony Saos-2$^{GLuc.Col}$ cell line induces expression of the eGLuc2.Colα2(I) fusion protein, which can be proteolytically processed post-secretion. Luminescence generated by the fusion protein may be assayed upon addition of the eGLuc2 substrate. (B) eGLuc2.Colα2(I) from Saos-2$^{GLuc.Col}$ lysate was immunoprecipitated with an α-GLuc antibody and analyzed by immunoblotting, revealing that eGLuc2.Colα2(I) associates intracellularly with Colα1(I). (C) Disulfide-dependent assembly of eGLuc2.Colα2(I) with Colα1(I). Collagen-I precipitated from conditioned media of Saos-2$^{GLuc.Col}$ cells was treated with iodoacetamide and heated in Laemmli buffer

with or without DTT. Samples were separated by SDS-PAGE and analyzed by immunoblotting. (D) Plot of filtered screening results. (E) Validated small molecule modulators of collagen-I secretion.

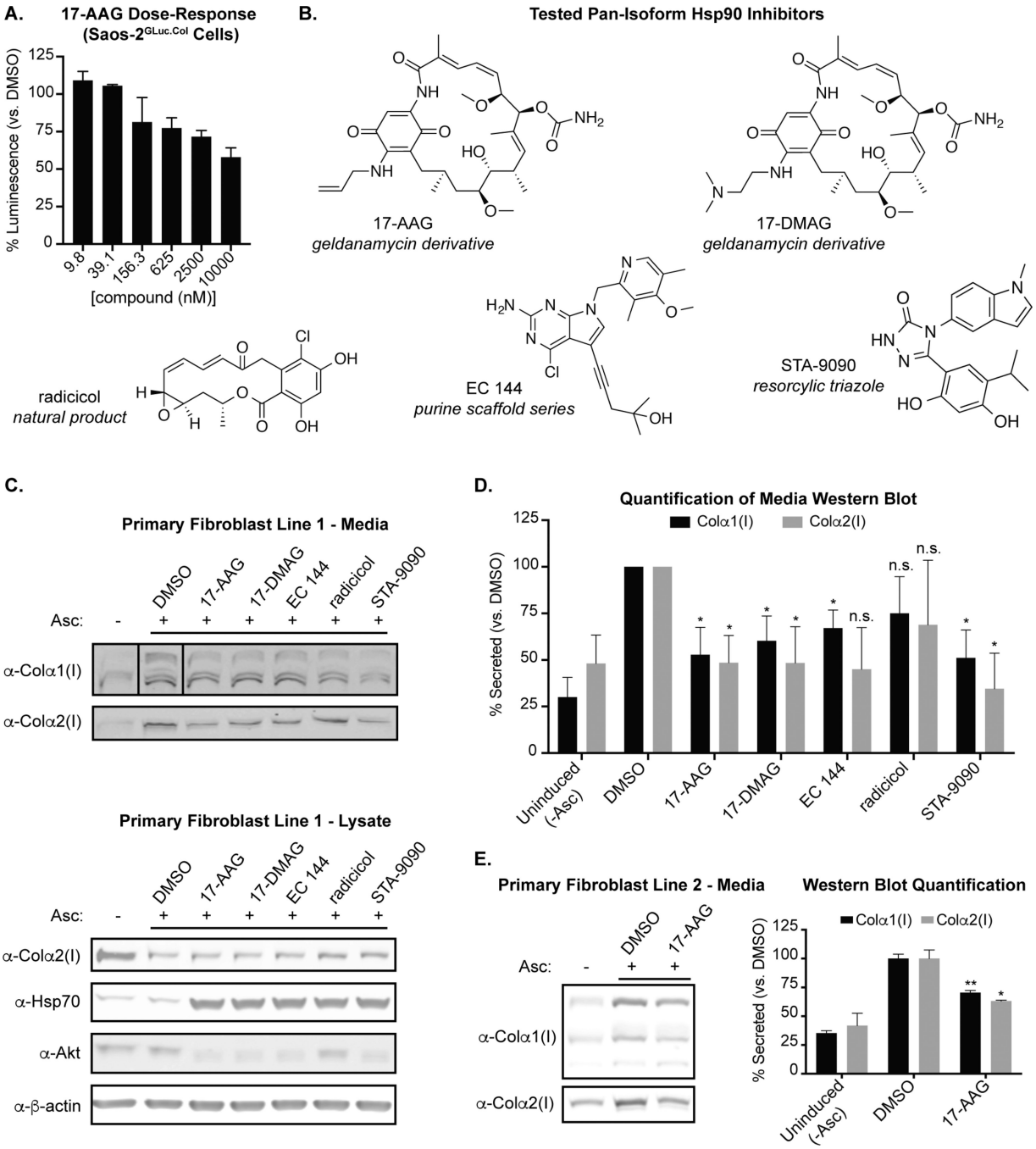

Figure 2.

Pan-Hsp90 inhibitors reduce endogenous collagen-I secretion. (A) Dose-response curve for the screening hit 17-AAG in Saos-2$^{GLuc.Col}$ cells. Cells were plated in 384-well plates and co-treated 5−6 h after plating with 1 μg/mL Dox and 17-AAG. After 23 h, plates were cooled at rt for 1–1.5 h, substrate was added, and luciferase activity was assayed. (B) Structures, names, and compound classes of tested pan-isoform Hsp90 inhibitors. (C) Western blot of conditioned media (*top*) and lysate (*bottom*) from primary dermal fibroblasts. Cells were plated and allowed to adhere overnight before receiving fresh media

with 200 μM ascorbate and the following concentrations of Hsp90 inhibitors, as indicated: 17-AAG (250 nM), 17-DMAG (250 nM), EC 144 (100 nM), radicicol (250 nM), STA-9090 (300 nM). After 24 h of treatment, conditioned media and cells were harvested for immunoblotting. (D) Quantification of Western blot results. * = $p < 0.05$ (n = 3). (E) Western blot of conditioned media from a second healthy fibroblast line. * = $p < 0.05$; ** = $p < 0.01$ (n = 3).

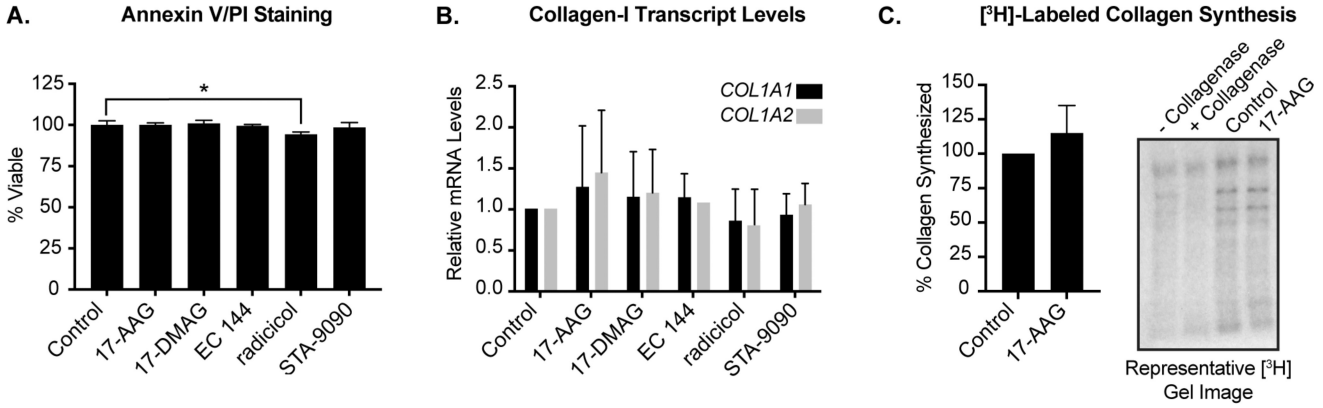

Figure 3.

Effects of Hsp90 inhibition on collagen-I secretion are post-translational. (A) FACS analysis of Annexin-V/Alexa Fluor 488 and propidium iodide staining to detect cell death. Cells were plated in triplicate for each condition and allowed to adhere overnight before being treated as in Fig. 2C. After 24 h, cells were trypsinized and labeled for FACS analysis. (B) qPCR analysis of collagen-I transcript levels from cells treated as in Fig. 2C. Average values are shown with the standard deviation for each condition (n = 3). (C) Measurement of collagen-I synthesis by [5-^{3}H]proline pulse. Cells treated for 6 h with ascorbate and 17-AAG (250 nM) were pulsed with [5-^{3}H]proline for 30 min prior to harvesting and analysis. Quantitation of newly synthesized collagen-I (mean ± standard deviation) is shown along with a representative gel image (n = 3).

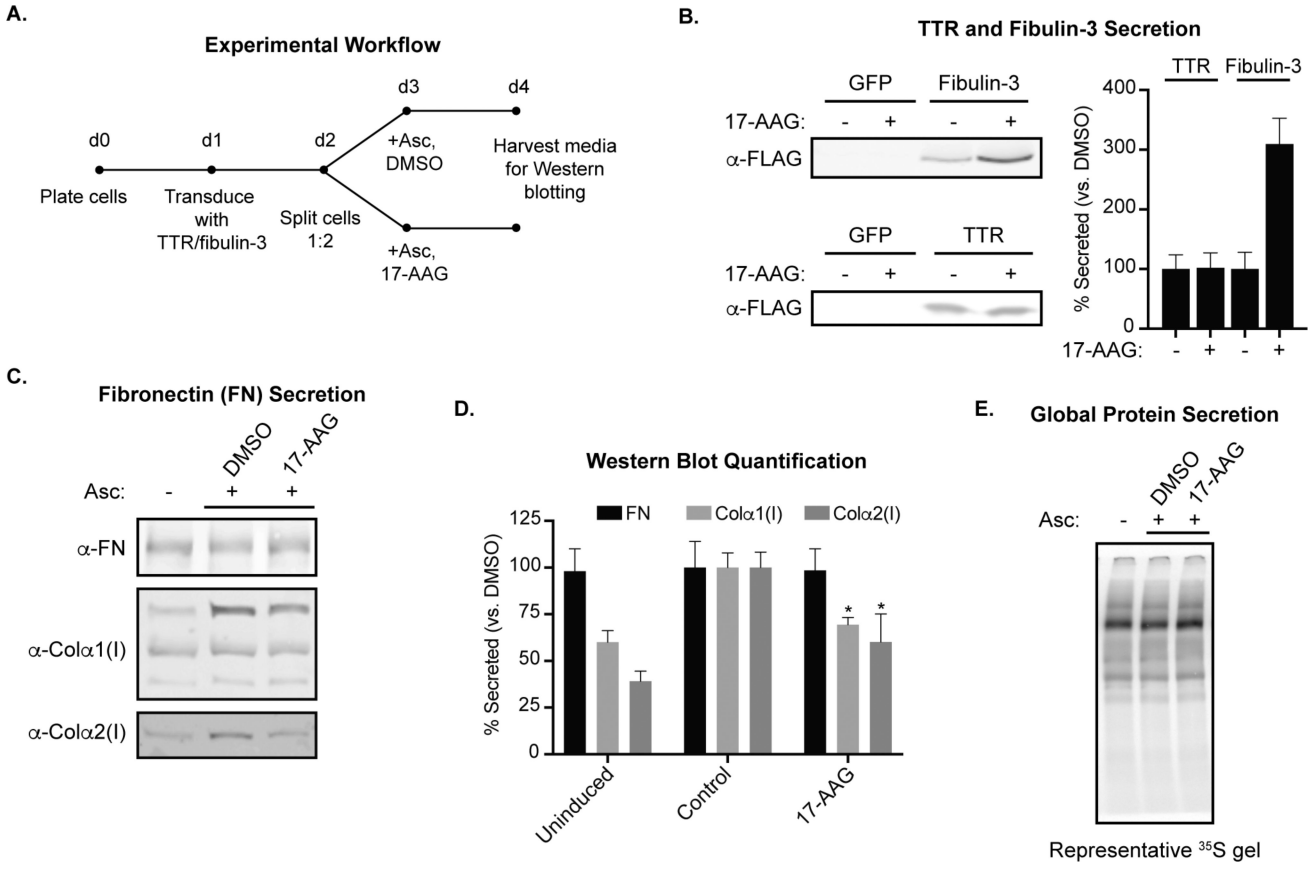

Figure 4.

Pan-Hsp90 inhibitors do not globally affect protein secretion. (A) Experimental workflow. Cells were plated and allowed to adhere overnight before being transduced with either GFP, transthyretin (TTR), or fibulin-3-expressing adenoviruses. The next day, transductions were split 1:2 and allowed to adhere overnight; cells were then treated with ascorbate and DMSO or 17-AAG (250 nM) for 24 h prior to harvesting. (B) Western blot of conditioned media from primary dermal fibroblasts transduced with FLAG-tagged transthyretin (TTR) or fibulin-3. (C) Immunoblot analysis of the secretion of endogenous fibronectin after treatment with 17-AAG. Cells were plated and treated as described in Fig. 2C. (D) Quantification of Western blot results. * = $p < 0.05$ (n = 3). (E) Phosphorimage of ^{35}S-labeled secreted proteins. Cells were plated in triplicate for each condition and allowed to adhere overnight before being pre-treated with ascorbate and DMSO or 17-AAG (250 nM) for 2 h. Cells were then washed and cultured in radiolabeled media containing ascorbate, [^{35}S]-Met/Cys, and DMSO / 17-AAG for 6 h. A representative radiograph is shown; the remaining biological replicates are presented in Fig. S5B.

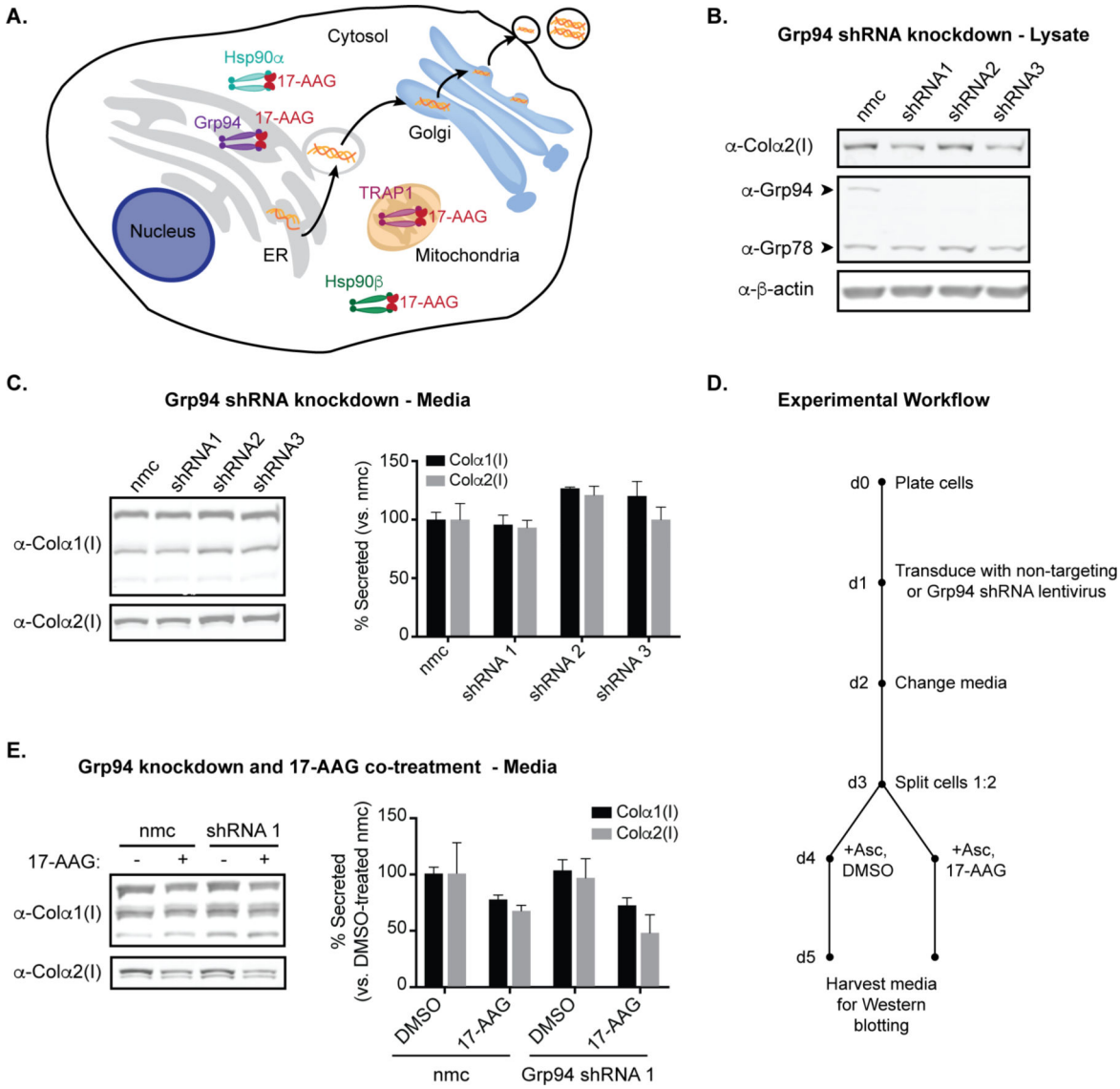

Figure 5.

Grp94 is dispensable for collagen-I secretion from primary fibroblasts. (A) Diagram of cellular Hsp90 isoforms along with the known collagen-I secretion pathway. Grp94, in the ER, is the only Hsp90 isoform capable of directly interacting with nascent collagen-I. (B) Western blot of lysate after lentiviral shRNA knockdown of Grp94. Cells were plated in triplicate for each construct and allowed to adhere overnight before being transduced with non-targeting control (nmc) or Grp94 shRNA lentiviruses. Media was changed the next day, and cells were left until 72 h post-transduction, when they received fresh media with ascorbate for 24 h prior to harvesting. (C) Immunoblots of corresponding media for the experiment described in (B). (D) Experimental workflow. Cells were plated in triplicate for each construct and allowed to adhere overnight before being transduced with non-targeting control or a Grp94 shRNA lentivirus. After 48 h, transductions were split 1:2 and allowed to adhere overnight. Cells subsequently received fresh media and were treated with ascorbate

and DMSO or 17-AAG for 24 h prior to harvesting. (E) Immunoblots of conditioned media from cells co-treated with shRNA-containing lentivirus and DMSO or 17-AAG (n = 3).