Thiolutin inhibits endothelial cell adhesion by perturbing Hsp27 interactions with components of the actin and intermediate filament cytoskeleton

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Abstract Thiolutin is a dithiole synthesized by *Streptomyces* sp. that inhibits endothelial cell adhesion and tumor growth. We show here that thiolutin potently inhibits developmental angiogenesis in zebrafish and vascular outgrowth from tissue explants in 3D cultures. Thiolutin is a potent and selective inhibitor of endothelial cell adhesion accompanied by rapid induction of HSPB1 (Hsp27) phosphorylation. The inhibitory effects of thiolutin on endothelial cell adhesion are transient, potentially due to a compensatory increase in Hsp27 protein levels. Accordingly, heat shock induction of Hsp27 limits the anti-adhesive activity of thiolutin. Thiolutin treatment results in loss of actin stress fibers, increased cortical actin as cells retract, and decreased cellular F-actin. Mass spectrometric analysis of Hsp27 binding partners following immunoaf-

finity purification identified several regulatory components of the actin cytoskeleton that associate with Hsp27 in a thiolutin-sensitive manner including several components of the Arp2/3 complex. Among these, ArpC1a is a direct binding partner of Hsp27. Thiolutin treatment induces peripheral localization of phosphorylated Hsp27 and Arp2/3. Hsp27 also associates with the intermediate filament components vimentin and nestin. Thiolutin treatment specifically ablates Hsp27 interaction with nestin and collapses nestin filaments. These results provide new mechanistic insights into regulation of cell adhesion and cytoskeletal dynamics by Hsp27.

Keywords Hsp27 · Arp2/3 · Intermediate filaments · Angiogenesis · Mass spectrometry

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Abbreviations

ADT	5-[p-methoxyphenyl]-3H-1,2-dithiol-3-thione
D3T	1,2-dithiole 3-thione
Hp	hours post-fertilization
DTT	dithiothreitol
FCS	fetal calf serum
Hsp27	heat shock protein 27 (HSPB1)
HUVEC	human umbilical vein endothelial cells
LC-MS	liquid chromatography-mass spectrometry

Introduction

Dithiols and other dietary organosulfur compounds such as sulforaphane have recently attracted attention due to their potential use for cancer prevention (Kensler et al. 2000; Kwak et al. 2004). 3H-1,2-Dithiole is naturally found in the diet, and a number of derivatives have been synthesized to develop more potent compounds for cancer chemoprevention including 3H-1,2-dithiole-3-thione (D3T), 5[4-methoxyphenyl]-3H-1,2-dithiole-3-thione (ADT), and oltipraz (4-methyl-5-pyrazinyl-3H-1,2-dithiole-3-thione). Oltipraz has been tested for this purpose in clinical trials, but toxicity limited its use (Pendyala et al. 2001; Kelley et al. 2005; Glintborg et al. 2006).

Thiolutin (*N*-(4-methyl-3-oxo-7,8-dithia-4-azabicyclo [3.3.0]octa-1,5-dien-2-yl)acetamide, Fig. 1a) is a naturally occurring bicyclic dithiole that was first isolated from *Streptomyces luteorectuli* based on its antibiotic activities (Celmer and Solomon 1955). Thiolutin inhibits the growth of *Saccharomyces cerevisiae* and several gram-positive and -negative bacteria (Seneca et al. 1952). In yeast, thiolutin at 20–40 μ M reduces RNA synthesis by inhibiting DNA-dependent RNA polymerases I, II, and III (Tipper 1973) but not the transcription of heat shock proteins (Adams and Gross 1991). The antibiotic activity of thiolutin is, therefore, considered to arise from inhibition of transcription.

Subsequently, thiolutin was shown to potently inhibit endothelial cell adhesion with an $IC_{50} < 1 \mu$ M and to inhibit S180 tumor-induced angiogenesis in mice (Minamiguchi

et al. 2001). We recently found that thiolutin potently stimulates phosphorylation of HSPB1/Hsp27 in endothelial cells and that expression of Hsp27 is important for the anti-proliferative activity of thiolutin (Dai et al. 2008). Other dithiols were weaker inducers of Hsp27

Fig. 1 Thiolutin inhibits zebrafish embryonic development and angiogenesis. **a** Structures of thiolutin and ADT. Both have $\alpha\beta$ -unsaturated dithiole moieties. **b–c** Lateral whole mount views of zebrafish embryos treated with DMSO (**b**) or thiolutin (**c**). Each panel contains three pictures. The *top picture* in each panel is a phase contrast image, the *middle picture* is a fluorescent (GFP) image of the same embryo, and the *bottom picture* is the close up view of the trunk region of the treated embryo. Left is head and right is tail region. **d** At lower concentrations of thiolutin (less than 1 μ M), 50% of embryos showed circulatory defects at 26 hpf when compared to 25% of age-matched DMSO treated embryos, but fewer embryos showed such defects at 29 hpf

Thiolutin (μ M)	% Embryos with Circulation		
	26hpf	29hpf	N
1.0	53	76	34
0.5	54	87	68
0.2	75	92	48
0.1	75	95	40
DMSO	70	94	44*

*@29hpf N=31

phosphorylation, proportional to their anti-angiogenic activities. The mechanism by which thiolutin inhibits endothelial cell adhesion is not clear, but two focal adhesion proteins were found to be affected by thiolutin. Thiolutin inhibits the phosphorylation of focal adhesion kinase (FAK) and reduces the expression of paxillin in human umbilical vein endothelial cells (HUVEC) plated on vitronectin (Minamiguchi et al. 2001).

Hsp27 participates in cytoskeletal reorganization, apoptosis inhibition, and acts as a protein chaperone (Huot et al. 1997; Concannon et al. 2003; Nakagomi et al. 2003; Arrigo et al. 2005). The expression and/or phosphorylation of Hsp27 can be up-regulated in response to stress stimuli. In endothelial cells, phosphorylation of Hsp27 is a shared response to a number of angiogenesis inhibitors (Keezer et al. 2003; Bix et al. 2004) including two dithiolethiones that are structurally related to thiolutin (Isenberg et al. 2007). Activation of the p38 MAP kinase signaling pathway leads to human Hsp27 phosphorylation at residues S¹⁵, S⁷⁸, and S⁸² by activated MAPKAP-2 (Landry et al. 1992; Stokoe et al. 1992). Thiolutin-induced phosphorylation requires p38 activity but stimulates this response downstream of p38 (Dai et al. 2008). Non-phosphorylated Hsp27 tends to form large oligomers that mediate its protein chaperone activity, while phosphorylated Hsp27 dissociates into octamers, tetramers, dimers, and monomers (Theriault et al. 2004).

In addition to regulating its chaperone activity, phosphorylation-dependent changes in Hsp27 oligomerization have been implicated in signaling pathways regulating cytoskeletal reorganization and apoptosis. In some cells, Hsp27 colocalizes with cellular F-actin in a phosphorylation-independent manner (Jog et al. 2007), but its ability to induce remodeling of the actin cytoskeleton requires a dynamic cycling of Hsp27 between the phosphorylated and dephosphorylated states (During et al. 2007). Consistent with the reported effect of thiolutin on FAK phosphorylation in endothelial cells, over expression of Hsp27 in 3 T3 fibroblasts increased FAK phosphorylation at several sites (Lee et al. 2008). The molecular interactions through which Hsp27 regulates actin dynamics and FAK activity, however, remain unclear.

In this study, we further examine the anti-angiogenic activity of thiolutin and the mechanism for its anti-adhesive activity in endothelial cells and the role of Hsp27 in this response and its reversibility. We examine effects on zebrafish embryo development and angiogenesis and ex vivo wound- and tumor-driven vascular outgrowth. We use quantitative liquid chromatography/mass spectrometry (LC-MS) analysis to identify proteins in endothelial cells that interact with Hsp27 in a thiolutin-dependent manner and study the roles of these proteins in the regulation of cytoskeletal dynamics and cell adhesion using co-immunoprecipitation and colocalization studies.

Materials and methods

Cells and reagents

HUVEC (Cambrex, Walkersville, MD, USA) were maintained in endothelial cell growth medium containing the manufacturer's additives and 2.5% fetal calf serum (FCS) in 5% CO₂ at 37°C. Cells were utilized between passages 4 and 8. Purity of cultures was monitored by immunochemical staining with monoclonal anti-human CD31 antibody (P8590, Sigma, St Louis, MO, USA). ADT was from Milan University. D3T was obtained from LKT, St. Paul, MN, USA. Thiolutin was purchased from Sigma (T3450). Monoclonal anti-human vinculin (clone V1F9 (7F9)), polyclonal anti-human Hsp27 (06-478), and monoclonal anti-human Ser⁷⁸P-Hsp27 antibodies (clone JBW502) were from Millipore (Billerica, MA, USA).

Zebrafish embryo development

Zebrafish were maintained as described in The Zebrafish Book (Westerfield 1995). Transgenic Tg(Fli: EGFP)^{y10} embryos were dechorinated at 10 h post fertilization (hpf) with 2 mg/mL pronase (Sigma, Cat. No. P-8811) stock in 2–3 mL Blue Water (E3 Solution (Holley et al. 2002)). Embryos were then added to 2 mL of Blue Water containing the desired concentration of drug compound or dimethyl sulfoxide control at either 10 hpf or, in some experiments, 18 hpf. All compounds were dissolved in dimethyl sulfoxide to 1 mM before being diluted in Blue Water. Embryos were incubated at 28°C until 48 hpf and were photographed with a Zeiss AxioCam HRc. All zebrafish studies were performed according to guidelines established by the Animal Care and Use Committees of the National Cancer Institute and the National Institutes of Health.

Mice

C57B16 mice and Cr:(NCr)-athymic nu fBR mice (NCI, Frederick, MD, USA) were housed in a pathogen-free environment and allowed ad libitum access to food and water. Handling and care of the animals was in compliance with the guidelines established by the Animal Care and Use Committees of the National Cancer Institute and the National Institutes of Health.

Explant vascular outgrowth assays

Biopsies (1 mm³) from the pectoralis major muscle of 8-week-old C57BL6 mice were harvested and explanted into type-I collagen gel in 96-well tissue culture plates as described (Isenberg et al. 2005). Following gelation of the collagen, explants were incubated in endothelial basal

medium+2% FCS in the presence or absence of a dose range of the indicated treatment agents at 37°C and 5% CO₂. Maximum cell migration through the matrix was measured following 7 days of incubation. In other experiments, C3H athymic nude mice were injected subcutaneously with 1×10^6 HT29 adenocarcinoma tumor cells in PBS to the lateral thigh. Animals were euthanized when tumors reached 1 cm. Tumors were excised immediately using sterile technique and 1-mm³ tumor biopsies were taken from the outer cortex of each mass and explanted into type I collagen matrix as described above and incubated in basal medium with 1% FCS. Following 7 days of incubation at 37°C and 5% CO₂ in the presence of the indicated treatment agents, maximum vascular cell outgrowth was measured.

Cell adhesion

Cell adhesion was carried out in 96-well culture plates. After pre-coating wells with type I collagen (3 µg/ml, Inamed, Fremont, CA, USA), harvested cells were plated at a density of 1×10^4 cells/well in endothelial basal medium plus 0.1% BSA and treatment agents and incubated at 37°C in 5% CO₂ for 1 h. Wells were washed with PBS, and the cells were fixed with 1% glutaraldehyde for 10 min, washed with PBS, and stained with 1% crystal violet for 20 min. Excess stain was rinsed away, adherent cells were treated with 10% acetic acid, and absorbance was quantified at 570 nm.

The effect of thiolutin on cell adhesion was also measured based on impedance as determined by the RT-CES system (ACEA Biosciences, San Diego, CA, USA). Briefly, HUVEC were collected by trypsin/EDTA and seeded at 2×10^4 cells/well into 16-well cell sensor chambers (ACEA Biosciences). In some experiments, freshly fed HUVEC were heat shocked at 43°C±0.1°C for 30 min in a circulating water bath. Cells were then lifted by trypsin/EDTA and seeded as described above. Cells were incubated at 37°C for 23–24 h to allow recovery and full attachment to wells. Cells were then treated with thiolutin at the indicated dosages. The initial response to thiolutin was collected every minute for 2 h and then every 10 min up to 16–24 h. DMSO was added to the control at a concentration equivalent to that added in the thiolutin solution.

Cell lysis

HUVEC were starved overnight in EBM+1% FCS and then treated with the reagents at the indicated dosages and duration. Immediately following treatment, HUVEC were washed three times with ice-cold TBS and lysed using 1× SDS sample buffer containing protease inhibitors for Western blotting or with RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% NP40, and protease inhibitors) for immunoprecipitation. For thiolutin-treated

samples, floating cells, in culture medium and washes, were collected by centrifugation and combined with the remaining adherent cells. The cell lysates were collected into microcentrifuge tubes and centrifuged at 12,000×g for 5 min. The supernatants were processed for immunoprecipitation and Western blotting.

Yeast-two hybrid screen for Hsp27 binding partners

The yeast-two hybrid screen was performed as previously described (Jia et al. 2001). Using pGal4-BD-Hsp27 as the bait, a rat ortholog of the Arp2/3 complex protein p41-ArpC1a was identified as an Hsp27 binding protein (accession #AF315378) (Li et al. 2003; Thornton et al. 2006).

Immunoprecipitation

Mouse IgG and antibodies against Hsp27 and Hsp27-P78 (Upstate) were cross-linked to Dynabeads coated with protein G (DPG, Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Preclearance was performed by adding 100 µl of DPG-IgG per ml HUVEC lysate and incubating at RT for 30 min with gentle mixing. DPG-IgG was pulled down and washed three times with the RIPA buffer by magnetic separation. The DPG-IgG-bound proteins were eluted with 0.1 M citrate (pH 2–3), and the eluted sample was neutralized with 50 mM Tris pH 9.5 as instructed by the manufacturer. The supernatant, after the preclearance, was collected and equally divided for immunoprecipitation using a ratio of 100 µl of DPG-Hsp27 or DPG-Hsp27-P78 per milliliter of the supernatant. The reactions were incubated at RT for 60 min with gentle mixing. Beads were collected and washed by magnetic pull down. The DPG-Hsp27- or DPG-Hsp27-P78-bound protein(s) were eluted as described above, and the samples were stored at –80°C for mass spectrometry analysis and Western blot analysis.

Western blotting

Cell lysate or immunoprecipitated complex was prepared in 1× SDS sample buffer, boiled, separated by SDS-PAGE, and transferred to an Immobilon-P membrane (Millipore) via semi-dry transfer apparatus (Bio-Rad, Hercules, CA, USA) according to the manufacturers' instructions. Membranes were blocked with 5% non-fat dry milk at RT for 1 h and incubated with primary antibodies overnight at 4°C. After three washes with DPBS, the membranes were incubated with secondary antibodies (Jackson ImmunoResearch) at room temperature for 1 h, and followed by five washes with DPBS. The reacting bands were detected using enhanced chemiluminescence (Pierce Supersignal West Pico). Membranes were then reprobed with anti-actin antibody for sample loading control.

Multiple enzymatic digestions

After immunoprecipitation, proteins eluted from the cross-linked antibody/protein-G beads were buffer exchanged with 6 M guanidine hydrochloride using a Microcon ultracentrifuge device (10 kDa molecular weight cutoff; Millipore) for five cycles (10,000×g for 5 min per cycle). The exchanged protein solution was reduced with 20 mM dithiothreitol (DTT) for 30 min at 37°C and alkylated with 50 mM of iodoacetic acid A in the dark at RT for 1.5 h. After buffer exchange, endoproteinase Lys-C (1:100 w/w) was added to digest the protein for 4 h at 37°C. The Lys-C digests were split into three equal aliquots. One aliquot was used directly for LC-MS analysis, one was digested a second time by trypsin (1:50, w/w), and the final one was digested a second time by endoprotease Glu-C (1:40 w/w). In each case, digestion was halted by the addition of formic acid to a final concentration of 1%, and then the solution was concentrated down to ~10 µL. Approximately 2 µL of the digest was directly loaded onto the LC column for LC-MS analysis. The remainder of the sample was stored at -80°C for further use.

LC-MS

LC-MS experiments were performed on an LTQ-FTMS instrument (ThermoFisher, San Jose, CA, USA) with an Ultimate 3000 nano-LC pump (Dionex, Mountain View, CA, USA) using a C-8 reverse phase column (75 µm i.d.×10 cm, 5 µm particle size, 300 Å pore size, Vydac C8, Grace Davison, Deerfield, IL, USA), which was prepared in-house. Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. The gradient used for all analyses was: (1) 40 min at 0% B for sample loading, (2) linear gradient to 40% B over 40 min, (3) linear gradient to 80% B over 10 min, and finally (4) constant 80% B for 10 min. The flow rate of the column was set at 200 nL/min. The experimental parameters of the LTQ-FT mass spectrometer and the acquisition modes for MS2 and MS3 were similar to those in our previous study (Dai et al. 2008). Briefly, the mass spectrometer was operated in the data-dependent mode to switch automatically between MS, MS2, and MS3 acquisitions. Survey full scan MS spectra with two microscans (m/z 400–2,000) were acquired in the FTICR cell with mass resolution of 100,000 at m/z 400 (after accumulation to a target value of 2×10^6 ions in the linear ion trap), followed by four cycles of sequential MS2 and MS3 scans. Dynamic exclusion was utilized with an exclusion duration of 30 s and two repeat counts.

Peptide assignment

The assignments of peptides (for charge state $\leq 3+$), large peptides (for charge state $\geq 4+$), and phosphopeptides were

similar to our previous report (Dai et al. 2008 4653). Briefly, the Sequest algorithm in the BioWorks software (version 3.3.2, ThermoFisher) was used to search all MS2 and MS3 spectra against spectra of theoretical fragmentations (b and y ions) of a human SwissProt annotated database, downloaded in January 2007 (14,194 protein entries), with a mass tolerance of ± 1.4 Da without any enzymatic specificity. The resultant spectra were filtered using the scores of Xcorr ($1+$ precursor ion ≥ 1.5 , $2+$ ≥ 2.0 , and $3+$ ≥ 2.5) and with semi-enzymatic specificity, containing either Lys-C plus trypsin or Lys-C plus Glu-C specificity. Peptides ($\leq 3+$ ions) were assigned with a probability greater than 95% confidence and a mass accuracy of the precursor ions of less than 5 ppm. Since no rigorous statistics are available for peptides with $\geq 4+$ charges, Sequest was used to select and rank the most probable peptides, and the top assignment was further confirmed by the mass accuracy of the precursor ion (< 5 ppm) and the preferred fragmentation patterns in the observed MS2 and MS3 spectra. For Hsp27 phosphopeptides, the data were searched against a single database corresponding to the sequence of Hsp27 with the parameter of differential modification (Ser, Thr, and Tyr) equal to +80 Da. The locations of the phosphorylated sites in the identified phosphopeptides were further confirmed by manual inspection of related b and y ions.

Peptide quantitation

The peak areas of the peptide ions from the MS spectra (XIC) were extracted for quantitation, in which the charge state with the highest intensity of a given peptide was used for extraction. For phosphorylation quantitation (Dai et al. 2008), the non-phosphorylated counterpart was used for normalization. For interaction partners, the intrinsic protein (i.e., Hsp27) was used for normalization, which was achieved by dividing the XIC peak area of a specific peptide of the interaction partner by the average XIC peak area of three representative Hsp27 peptides. In the experiment of using antibody against pS78 Hsp27 (Hsp27 was not pulled down in the control sample because it lacked phosphorylation), the other abundant intrinsic protein from both the control and thiolutin-treated samples (i.e., Drebrin) was used for normalization. In addition to the use of peak area (XIC), the number of identified peptides per protein (peptide hits) was also used as an alternative means for assessing the protein abundance. The proteins with ratios ≥ 3 by both XIC and peptide hit methods (in agreement between the two methods) are listed in Tables 1 and 2.

Quantitative RT-PCR

HUVEC were cultured in EGM-2% FBS till confluent and then treated with 1 µM Thiolutin for 0.5, 1, 2, 5, 10, 15, 30, and

Table 1 Thiolutin-sensitive interacting partners of S⁷⁸P-Hsp27 in HUVECs

Up-regulated proteins (treated/control $\geq$ 3)
HSB1_HUMAN Heat-shock protein beta-1 (HspB1, Hsp27)
ARC1B_HUMAN ARP2/3 complex 41 kDa subunit (P41-ARC)
CAZA2_HUMAN F-actin capping protein alpha-2 subunit
MYO6_HUMAN Myosin VI
FLNA_HUMAN Filamin A (α -filamin) (Filamin 1)
FLNB_HUMAN Filamin B (FLN-B) (β -filamin)
ANXA2_HUMAN Annexin A2 (Annexin II) (Lipocortin II, calpactin I heavy chain)
RL11_HUMAN 60 S ribosomal protein L11
AR20_HUMAN ARP2/3 complex 20 kDa subunit (p20-ARC)
AR34_HUMAN ARP2/3 complex 34 kDa subunit (P34-ARC)
MYO5A_HUMAN Myosin Va (Myosin 5A) (Dilute myosin heavy chain)
AAC1_HUMAN α -actinin 1
PTRF_HUMAN Polymerase I and transcript release factor
Down-regulated proteins (treated/control $\leq$ 0.3)
HNRPC_HUMAN Heterogeneous nuclear ribonucleoproteins C1
HNRPU_HUMAN Heterogeneous nuclear ribonucleoprotein U (scaffold attachment factor A)
NEST_HUMAN Nestin
RLA2_HUMAN 60 S acidic ribosomal protein P2
DCD_HUMAN Dermcidin precursor (Preproteolysin)
ELMO1_HUMAN Engulfment and cell motility protein 1

Only proteins with more than threefold changes (up- or down-regulation) in response to thiolutin treatment are included

60 min. RNA was isolated by Trizol according to the manufacturer's protocol (Gibco BRL, Carlsbad, CA, USA). Reverse transcription was conducted in a 13- μ l reaction mixture containing 1 μ l oligo dT, 1 μ l 10 mM dNTP, and 5 μ g total RNA in 11 μ l H₂O. The mixture was heated at 65°C for 5 min and transferred to an ice bath. To each mixture, 2 μ l 10 $\times$ RT buffer, 2 μ l 0.1 M DTT, 1 μ l 0.1 M MgCl₂, 13 μ l

RNase OUT, and 1 μ l Superscript III (Invitrogen) were added, for a total volume of 20 μ l. The mixture was incubated at 50°C for 1 h and denatured at 85°C for 5 min. Then, 1 μ l of RNase H was added to each reaction, incubated at 37°C for 20 min, and finished by adding 80 μ l H₂O to each tube.

Real-time PCR reactions were carried out in a volume of 25 μ l containing 4.5 μ l of cDNA, 4 μ l mixture of 4 μ l H₂O, 12.5 μ l SYBR green reagent (Invitrogen), and 1 μ M of the forward (CTGAGGGCACACTGACCG) and reverse (TTACTTGGCGGCAGTCTC) Hsp27 primers. As the endogenous control for real-time PCR, primers of *HPRT1* gene were used to standardize the amount of RNA in each reaction. DNA was quantified by measuring fluorescent intensity using an MJ Research Opticon I instrument (Bio-Rad). Data were processed using the Opticon I software supplied with the instrument.

Immunofluorescent confocal microscopy

HUVEC (1.3×10^5) were seeded onto Lab-Tek glass chamber slides precoated with 0.1% gelatin (ICN) in Clonetics EGM medium and allowed to adhere overnight at 37°C in 5% CO₂. The chambers were aspirated, and pre-warmed medium containing thiolutin at the indicated concentrations was added for 5 min at 37°C in 5% CO₂. The medium was gently aspirated, and cells were fixed in 4% formaldehyde in 10 mM MES pH 6.1, 138 mM KCl, 3 mM MgCl₂, 2 mM EGTA, 329 mM sucrose for 20 min. After rinsing in TBS, the cell membranes were permeabilized in TBS-0.1% Triton X-100 for 10 min and then blocked for 20 min in TBS-2%

Table 2 Thiolutin-sensitive interacting partners of Hsp27 in HUVECs

Up-regulated proteins (treated/control $\geq$ 3)
CYR61_HUMAN CYR61 protein
CRTC_HUMAN Calreticulin
FETUA_HUMAN Alpha-2-HS-glycoprotein
ANXA2, Annexin A2
TBBQ_HUMAN Tubulin beta-4q chain
PDIA1_HUMAN Protein disulfide-isomerase
CAZA2, F-actin capping protein Z α 2 subunit
CYTA_HUMAN Cystatin A (Stefin A, Cystatin AS)
TGM2, Protein-glutamine γ -glutamyl transferase
PTRF_HUMAN Polymerase I and transcript release factor
Down-regulated proteins (treated/control $\leq$ 0.3)
EPLIN, Epithelial protein lost in neoplasm
KAPCA, cAMP-dependent protein kinase, α -catalytic
NEST, Nestin
PLE1, Plectin 1 (PLTN/PCN)
SRC8, Src substrate cortactin (amplaxin, oncogene EMS1)
MPT1, Protein phosphatase 1 regulatory subunit 12A

Only proteins with more than threefold changes (up- or down-regulation) in response to thiolutin treatment are included

BSA (Sigma). Cells were stained with Anti-phospho-Hsp27 (Ser⁷⁸) and Anti-Hsp27 from Upstate Biotechnology, Anti-vinculin and Anti-Nestin from Chemicon, and Anti p34-Arc from Synaptic Systems. After washing three times with TBS-0.01% Triton X-100, 0.1% BSA, the cells were stained with either Alexa Fluor 594 or Alexa Fluor 488 secondary antibodies. Actin labeling with either Alexa Fluor® 594 phalloidin or Oregon Green 488 phalloidin from Molecular Probes was performed for 20 min followed by washing four times with TBS. Slides were mounted with Vectashield (Vector). Confocal images were collected using either a Zeiss LSM 510 UV system, or a Zeiss/Bio-Rad MRC 1024 confocal scan head (Bio-Rad) mounted on a Nikon Optiphot microscope with a 63× plan-Apochromat lens.

Colocalization was quantified using the Zeiss LSM Image Browser colocalization coefficient software. Colocalization coefficients were calculated after subtracting all non-cell and background pixels. The relative number of colocalizing pixels in the red (Ch4) and green (Ch3) channels was calculated and compared to total number of pixels above the lower limit threshold.

Phalloidin binding assay

Total cellular F-actin was determined by a modified phalloidin binding assay (Singhal et al. 1992). Briefly, cells (1×10^4 /well) were plated in 96-well plates and cultured overnight. Cells were treated with 0.25 μ M thiolutin for the time indicated. Plates were centrifuged between each following step to prevent cell loss. Cells were washed with PBS and fixed with 4% paraformaldehyde for 30 min, followed by three PBS washes. Cells were then incubated in 0.1% (w/v) saponin in PBS containing a saturating amount Oregon green phalloidin (Molecular Probes, Eugene, OR, USA) for 1 h at room temperature in darkness with gentle agitation. After three washes with PBS-saponin, the labeled phalloidin was extracted from cells in 200 μ l methanol by agitation for 30 min in darkness and the supernatant was collected and fluorescence was measured in LS-50B luminescence spectrometer. The amount of bound phalloidin was normalized based on total protein analyses of duplicate cell cultures. The experiments were performed four times with triplicates and the data were analyzed by the two-tailed Student *t* test.

Results

Thiolutin disrupts zebrafish embryonic development and disturbs vessel formation

Based on the structural similarity between thiolutin and dithioles with known anti-angiogenic activities (Fig. 1a) and its effects on endothelial cells in vitro (Minamiguchi et al.

2001; Dai et al. 2008), we investigated the effect of thiolutin on vertebrate development using the transparent zebrafish model system. In addition to obtaining toxicology data, the effect of thiolutin on developmental angiogenesis was assessed using the transgenic zebrafish line *Tg(fli1:EGFP)^{v1}*, which carries a 15-kb promoter of friend leukemia integration-1 transcription factor (fli1) that drives green fluorescent protein (GFP) expression in the developing vasculature (Lawson and Weinstein 2002). Thiolutin was added to *Tg(fli1:EGFP)^{v1}* zebrafish embryos 10 h post fertilization when gastrulation is complete. Doses as low as 1 μ M were toxic, causing death in more than 40% of the treated embryos. Lethality was 100% at 5 μ M thiolutin. Similar concentrations of two other dithioles, D3T and ADT, had little toxicity (Isenberg et al. 2007). At 1 μ M concentration, thiolutin-treated embryos (Fig. 1c) display spinal curvature when compared to DMSO (Fig. 1b)-treated embryos, with minimal disruption of the trunk vasculature. ADT-treated embryos showed vascular and morphological defects only at a fivefold-higher concentration (Isenberg et al. 2007, and data not shown). Vascular defects in thiolutin embryos could not be independently assessed above 1 μ M due to lethality, but thiolutin concentrations of 1 μ M or less showed dose-dependent circulatory defects at 26 hpf when compared to age-matched DMSO-treated embryos (Fig. 1d). As the embryo matured further (29 hpf), a dose-dependent increase in embryos with circulatory defects remained, although with fewer embryos showing such defects (Fig. 1d). Therefore, it is possible that the circulatory defects are not primary in thiolutin-treated embryos. These results, when taken together, suggest that thiolutin is a potent agent that targets a major pathway necessary for zebrafish development in addition to inhibiting angiogenesis.

Thiolutin inhibits ex vivo wound- and tumor-driven vascular outgrowth

Muscle biopsies from C57Bl6 mice were explanted into 3D type I collagen in a 96-well plate format and incubated in the presence of endothelial growth medium and the indicated concentrations of treatment agents (Fig. 2a, b). Thiolutin was somewhat more potent than the dithiole-thione ADT for inhibiting wound-driven vascular cell outgrowth. Utilizing an ex vivo model of tumor-driven angiogenesis, vascular outgrowth from HT-29 tumor explants was also sensitive to inhibition by thiolutin and ADT (Fig. 2c).

Endothelial cell adhesion to type I collagen is differentially regulated by thiolutin, D3T, and ADT

Thiolutin at 0.1 μ M was sufficient to dramatically inhibit endothelial cell adhesion to type I collagen (Fig. 2d). In

Fig. 2 Thiolutin blocks both wound-driven and tumor-driven vascular outgrowths.

a, b C57BL6 murine muscle biopsies were explanted in three-dimensional collagen matrix and incubated in the presence of the indicated treatments and cell migration measured at day 7. **c** Biopsies from HT29 adenocarcinoma xenografts harvested from Cr: (NCR)-athymic nu fBR mice were similarly treated. Explants were imaged using a 20 $\times$ objective. Results represent the mean $\pm$ SD of three separate experiments. **d** HUVEC (1×10^4 cell/well) were plated in 96-well culture dishes pre-coated with type I collagen (5 μ g/ml) in the presence of the indicated treatment agents. Following incubation for 1 h at 37 $^{\circ}$ C plates were washed and the attached cells were fixed, developed, and read at 570 nm. Results are expressed as percentage of control and represent the mean $\pm$ SD of at least three separate experiments

contrast, D3T and ADT did not inhibit adhesion of endothelial cells to collagen even up to 100 μ M.

The effect of thiolutin on HUVEC adhesion is reversible

Consistent with the previously reported effect of thiolutin on endothelial cell adhesion on vitronectin (Minamiguchi et al. 2001), we found that ≥ 0.4 μ M thiolutin was sufficient to rapidly and completely disrupt adhesion of attached HUVEC monolayers (Fig. 3a). Disruption of cell adhesion by thiolutin was highly reversible. Figure 3b shows that 0.5 μ M thiolutin completely disrupts HUVEC adhesion on tissue culture plastic within 1 h, but the cells were able to reattach to the plate even in the continued presence of thiolutin. Removing thiolutin from the medium after the initial treatment resulted in rapid cell re-attachment (bottom panel of Fig. 3b). Thiolutin also induced rapid dose-dependent detachment of endothelial cells from the gold-coated ACEA substrate, and cells treated with 0.2 μ M showed partial recovery over the following 25 h (Fig. 3c).

Because similar doses of thiolutin are known to induce phosphorylation of Hsp27 in endothelial cells (Dai et al. 2008) and Hsp27 is a known regulator of the actin cytoskeleton (During et al. 2007), we examined whether increasing Hsp27 phosphorylation and expression using heat shock would alter the response of HUVEC to

thiolutin (Fig. 3d). HUVEC subjected to heat shock and allowed to recover for 24 h were less sensitive at all concentrations of thiolutin examined, and the anti-adhesive activity of thiolutin was more rapidly reversed in these cells. Increased resistance to thiolutin indicated that the cells were in a cytoprotected state.

Thiolutin limits Hsp27 mRNA but increases Hsp27 protein expression

This observation suggested that increased Hsp27 expression could overcome the anti-adhesive activity of thiolutin. To determine whether altered Hsp27 expression played a role in recovery of cell adhesion following thiolutin treatment, we examined Hsp27 mRNA and protein levels in thiolutin-treated HUVEC (Fig. 4). At concentrations comparable to those that resulted in transient de-adhesion of HUVEC in Fig. 3, the expression of Hsp27 protein increased in a dose- and time-dependent fashion (Fig. 4a). However, the increased Hsp27 protein expression was not due to increased transcription because Hsp27 mRNA levels fell approximately fourfold within the first 10 min following treatment of HUVEC with thiolutin (Fig. 4b). The response was specific to Hsp27 in that Hsp90 mRNA levels were unchanged. Therefore, thiolutin increases Hsp27 protein levels in HUVEC at a post-transcriptional level.

Fig. 3 Thiolutin disrupts cell attachment and focal adhesions. HUVEC were cultured in 24-well plates in EGM-2%FBS media to 90% confluence and then incubated overnight in EBM-1%FBS. **a** Cells were treated with thiolutin at 0.5 μ M and digital images were acquired every 15 min. **b** Cells were treated with thiolutin at 0.5 μ M for 1 or 12 h (*top panel*), and then cells were collected and placed into fresh media to recover for 24 h (*bottom panel*). **c** HUVEC were treated with thiolutin at the indicated concentrations 23 h following plating, and adhesion was assessed continuously by impedance measurement using an ACEA RT-CES and presented in cell index units. **d** Heat-shocked HUVEC were allowed to recover for 24 h and then treated with thiolutin. Adhesion was assessed over time by changes in impedance as in **c**. The experiments were repeated at least three times with duplicates. Identical results were observed for each experiment

Thiolutin modulates Hsp27 interactions with the actin cytoskeleton

The known effects of thiolutin on FAK phosphorylation and paxillin levels in HUVEC suggested that thiolutin might disrupt focal adhesions in endothelial cells. HUVEC were stained, therefore, to detect vinculin, a focal adhesion protein that binds to integrins via α -actinin and talin (Kelly

et al. 2006). Figure 5a shows a dramatic decrease in punctate vinculin staining localized near the periphery (arrows) of thiolutin-treated HUVEC. In the treated cells, vinculin became diffusely localized in the cytoplasm or in the uropod of retracting cells (asterisk in Fig. 5a).

Consistent with previous studies using several cell types (Landry and Huot 1999; Chaudhuri and Smith 2008), Hsp27 associated with actin stress fibers and cortical actin in

Fig. 4 Thiolutin treatment increases Hsp27 protein levels but decreases Hsp27 mRNA. HUVEC were cultured as described in the “Materials and methods” section and treated with thiolutin at 0.25, 0.5, and 1 μ M for the time indicated. **a** The expression of Hsp27 was examined by blotting using an anti-Hsp27 antibody. The membranes were reprobed with an anti-actin antibody for loading control. **b** Real-time RT-PCR was performed as described in the “Materials and methods” section. Changes in the levels of Hsp27 and Hsp90 mRNA were determined at the indicated times following thiolutin treatment by the normalizing to HPRT mRNA levels in each sample and presented as mean $\pm$ SD of triplicates

untreated HUVEC (arrows in Fig. 5b upper panels). Staining for S⁷⁸ phosphorylated Hsp27 was highly heterogeneous in untreated HUVEC, but in those cells showing staining, it was mostly associated with cortical actin (Fig. 5c, upper panels). Coincident with the loss of focal adhesion, Hsp27 staining was lost from the stress fibers in thiolutin-treated HUVEC (Fig. 5b, lower panels), and S⁷⁸ phosphorylated Hsp27 was primarily localized to the periphery of treated cells, where it strongly colocalized with cortical actin (Fig. 5c, lower panels).

Consistent with this colocalization, thiolutin treatment induced co-immunoprecipitation of actin with both Hsp27 and S⁷⁸P-Hsp27 antibodies (Fig. 6a). Immunoprecipitation and blotting with actin antibody confirmed similar actin levels in treated and untreated HUVEC (Fig. 6a, lower panel).

The stability of focal adhesions is directly associated with the dynamics of stress fiber and cytoskeleton reorganization. Thus, changes of focal adhesion affect the content of filamentous actin (F-actin). A quantitative phalloidin binding assay showed that thiolutin significantly reduces the amount of total cellular filament actin (F-actin, Fig. 6b), but this loss was transient and reversed as the cells recovered.

Identification of thiolutin-dependent interaction partners of Hsp27 by MS

Although Hsp27 has been localized to the actin cytoskeleton during remodeling processes, and phosphorylation of Hsp27 is known to play an important role in this activity (During et al. 2007; Jog et al. 2007), the molecular interactions

responsible for this activity of phosphorylated and non-phosphorylated Hsp27 are unclear. To identify thiolutin-dependent direct or indirect binding partners of Hsp27 and phospho-Hsp27, we performed a quantitative analysis of proteins that co-immunoprecipitated with Hsp27 in control and thiolutin-treated HUVEC. LC-MS analysis of proteolytic digests was employed to identify and quantify the associated proteins. Treatment for 60 min using 1 μ M thiolutin was chosen as a standard condition for these analyses based on its maximal effect on Hsp27 phosphorylation (Dai et al. 2008). In the IP from the antibody against pS⁷⁸ Hsp27 89 proteins were identified, of which 30 proteins were significantly up-regulated and 10 proteins were down regulated following thiolutin treatment (Supplemental Table 1). In the IP from the antibody against the C-terminal end of Hsp27 89 proteins were also identified; of which 10 proteins were up-regulated and 13 proteins were down regulated (Supplemental Table 2). Those up- or down-regulated proteins differing in abundance by at least three fold between the thiolutin-treated and the control samples are summarized in Tables 1 and 2.

Hsp27 peptides were detected with similar abundances in the anti-Hsp27 IP of thiolutin treated and control endothelial cells (supplemental Table 2), confirming similar efficiencies of IP from HUVEC in both conditions. Similarly, the strongly increased Hsp27 signal in the anti-S⁷⁸P-Hsp27 IP from thiolutin treated cells confirmed the specificity of this IP (supplemental Table 1).

The method was further validated by detection of several known binding partners of Hsp27. In addition to actin, studies

Fig. 5 Thiolutin disrupts focal adhesions and induces association of phosphorylated Hsp27 with cortical actin. Confocal images were obtained for untreated HUVEC and HUVEC treated with 300 nM thiolutin for 5 min. **a** Vinculin (red) and actin (green) and nuclei (blue) staining in untreated HUVEC (left) and in HUVEC treated with thiolutin (right). **b** Hsp27 (red) and actin (green) were imaged in untreated (upper) and treated HUVEC (lower panels). **c** Hsp27-S⁷⁸P (red) and actin (green) were imaged in untreated HUVEC (upper panels) and in HUVEC treated with thiolutin for 5 min (lower panels). Scale bars=10 μ m

Fig. 6 Thiolutin disrupts actin stress fibers and affects actin polymerization. **a** Lysates of HUVEC treated with 1 μ M thiolutin for 60 min were prepared and equally divided for immunoprecipitation. Anti- Hsp27, Hsp27-S⁷⁸P, and actin antibodies were used at a 1:5,000 dilution and incubated for 1 h at 4°C. Protein-G was then added at a 1:500 dilution and incubated overnight at 4°C. After three washes by a quick spin, the resulting immune complexes were dissolved into 1 $\times$ SDS sample buffer, and Western blotting (WB) was performed using the indicated antibodies. HC Ig heavy chain. **b** HUVEC cells were treated with 0.25 μ M thiolutin for the time indicated. After centrifugation, the cells were fixed with 4% paraformaldehyde and incubated with phalloidin at saturated concentration for binding to F-actin. The bound phalloidin was extracted using methanol and quantified using a fluorescence plate reader. The experiments were performed four times with triplicates. ** $p < 0.05$

using purified proteins have established direct binding of Hsp27 with tropomyosin (Somara and Bitar 2004). Consistent with this, myosin heavy chain represented the most abundant peptides in the Hsp27 and S⁷⁸P-Hsp27 IPs, and several other

tropomyosin subunits were also well represented. Consistent with its known binding to nonphosphorylated Hsp27 (Somara and Bitar 2004), the number of myosin heavy chain peptides in the anti-Hsp27 IP was not significantly affected by thiolutin treatment. Tropomyosin binding was reported to increase following Hsp27 phosphorylation (Somara and Bitar 2004), and myosin VI and nonmuscle type B myosin heavy chain showed a corresponding increased abundance in the anti-S⁷⁸P-Hsp27 IP following thiolutin treatment, but other tropomyosin subunits did not show similar increases (supplemental Table 1). Vimentin is another cytoskeletal protein that is known to interact with Hsp27 (Perng et al. 1999), and vimentin peptides were highly represented in the anti-S⁷⁸P-Hsp27 and anti-Hsp27 IPs for both control and thiolutin-treated cells, with the peptide numbers decreasing moderately following thiolutin treatment (Supplemental Tables 1 and 2). Finally, the presence of ubiquitin in all four Hsp27 IPs and transglutaminases 2 in IPs of thiolutin-treated endothelial cells is consistent with the previous isolation of Hsp27 as a covalent adduct with ubiquitin via transglutaminase-catalyzed cross linking to Lys²⁹ and Lys⁴⁸ of ubiquitin (Nemes et al. 2004).

Western blotting confirmation of co-immunoprecipitation

To confirm the MS results, differential co-immunoprecipitation with Hsp27 in thiolutin-treated HUVEC was analyzed by Western blotting for several proteins that could account for the effects of thiolutin on cell adhesion and cytoskeleton disruption (Fig. 7a). The most dramatically down-regulated association identified by MS was with nestin, a developmentally regulated component of intermediate filaments (Michalczyk and Ziman 2005). Nestin was associated with Hsp27 in both thiolutin-treated and control cells, but its abundance was dramatically decreased following thiolutin treatment in the S⁷⁸-Hsp27 immunoprecipitates (lane 3 vs. lane 5 in Fig. 7a). Nestin colocalizes with vimentin in intermediate filaments and promotes their disassembly during mitosis (Chou et al. 2003). Vimentin was highly abundant in the MS analyses of Hsp27-associated proteins, and was down-regulated by thiolutin treatment. Consistent with these data, vimentin efficiently co-immunoprecipitated with both Hsp27 and S⁷⁸P-Hsp27 antibodies and showed a moderate decrease following thiolutin treatment in the case of Hsp27 immunoprecipitation (Fig. 7a).

Up-regulation of actin interaction with Hsp27 and S⁷⁸P-Hsp27 following thiolutin treatment was confirmed in the same immunoprecipitates. In addition to actin, most members of the actin regulatory protein complex Arp2/3 were identified in Hsp27 and S⁷⁸P-Hsp27 complexes analyzed by MS. Among these, p20-ArpC4, p34-ArpC2, and p41-ArpC1 were significantly increased in Hsp27 complexes analyzed by MS following thiolutin treatment (Table 1).

Fig. 7 Thiolutin alters Hsp27 binding to actin cytoskeleton and intermediate filament regulatory components. **a** Control HUVEC (lanes 1, 2, 3, and 6) or HUVEC treated with 0.5 μ M thiolutin for 60 min (lanes 4, 5, and 7) were lysed, and supernatants following centrifugation were immunoprecipitated using control IgG (lane 1), anti-Hsp27 (lanes 2 and 4), and Hsp27-S⁷⁸P antibodies (lanes 3 and 5). Associated levels of the indicated Hsp27-binding proteins in the immunoprecipitates (lanes 2–5) or whole cell lysates (lanes 6 and 7) were determined by Western blotting using the indicated antibodies. **b** Actin (red) and Arp2/3 component p34 (green) were imaged by confocal microscopy in untreated HUVEC (top) and in HUVEC treated with 200 nM thiolutin for 5 min (bottom). Inset shows a higher magnification of the retracting cell edge. **c** Nestin (red) and Hsp27 (green) were imaged in untreated HUVEC (upper panels) and HUVEC treated using 100 nM thiolutin for 5 min (lower panels). Representative images are presented. Scale bar=10 μ m. Right panels show plots of pixel intensities for the red and green channels that were used to calculate colocalization coefficients

A yeast-two hybrid screen was performed previously to identify direct binding partners of Hsp27 (Jia et al. 2001). Among the positive clones identified in that screen, one encoded a rat ortholog of the Arp2/3 actin initiation complex protein p41-ArpC1a (Balasubramanian et al. 1996). Therefore, we propose that Hsp27 associates with the Arp2/3 complex by directly interacting with ArpC1, and based on the immunoprecipitation/mass spectrometry data,

this interaction can occur while ArpC1 is part of the Arp2/3 complex.

Of the available Arp antibodies, one recognizing the subunit p16-ArpC5 was most effective for Western blot analysis, which confirmed its association with Hsp27 and S⁷⁸P-Hsp27 (Fig. 7a). Although p16-ArpC5 did not differ in Hsp27 immunoprecipitates following thiolutin treatment, it was increased in the S⁷⁸P-Hsp27 immunoprecipitate.

Therefore, the increased association of Hsp27 with actin following thiolutin treatment may in part be mediated through phosphorylated Hsp27 association with Arp2/3.

Filamins are high-molecular-weight actin cross-linking proteins that serve as a scaffold to mediate actin cytoskeleton interactions with membrane components such as integrins (Popowicz et al. 2006). Consistent with the MS data, co-immunoprecipitation of filamin A with Hsp27 and $S^{78}P$ -Hsp27 was increased following treatment of HUVEC with thiolutin (Fig. 7a). This may be an indirect reflection of increased Hsp27 binding to p41-ArpC1, but the greater increase in filamin A relative to p16-ArpC5 in the Hsp27 complex from thiolutin-treated cells (Fig. 7a lane 4) suggests differential binding to filamin itself or to unidentified filamin-associated proteins.

Localization of Hsp27-associated proteins in thiolutin-treated cells

We examined the effect of thiolutin treatment on Arp2/3 localization using an antibody against the subunit p34-ArpC2 (Fig. 7b). In quiescent untreated HUVEC, p34-ArpC2 exhibited punctate localization throughout the cells with no staining at the cell periphery (Fig. 7b upper panel). Following thiolutin treatment, p34-ArpC2 relocated rapidly to the periphery, where it associated with cortical actin (Fig. 7b, lower panel). Notably, p34-ArpC2 staining frequently localized to the inner margin of the cortical actin in these retracting edges. As cells further retracted in response to thiolutin treatment, p34-ArpC2 reactivity tended to move away from the periphery and finally localized near the nucleus as cells rounded (results not shown). Localization in the cortical actin of treated cells is consistent with preferential localization of $S^{78}P$ -Hsp27 to the same region (compare Fig. 5c) and with the increased association of several Arp2/3 subunits with $S^{78}P$ -Hsp27 immunoprecipitates observed by MS and Western analysis.

Additional studies using a nestin antibody showed rapid collapse of intermediate filaments containing nestin following thiolutin treatment in the subset of endothelial cells expressing this component (Fig. 7c, left panels). However, since not all HUVEC express nestin, the general effects of thiolutin on cell spreading appear to depend more on disruption of the actin rather than the intermediate filament cytoskeleton in HUVEC. In untreated cells, significant colocalization of Hsp27 with nestin-containing intermediate filaments was detected (Fig. 7c, upper right panels). Due to the intense cytoplasmic staining for Hsp27, this colocalization is most evident for the stacked image in intermediate filaments crossing over or under the nucleus (see arrows). Quantitative analysis of the primary confocal data gave a colocalization coefficient for Hsp27 with nestin of 0.783 in untreated cells (Fig. 7c upper right). Following treatment with 0.1 μ M

thiolutin, no colocalization was detected in the stacked image (Fig. 7c lower panels), and the colocalization coefficient decreased to 0.183 (Fig. 7c lower right), consistent with the co-IP data. No colocalization of phosphorylated Hsp27 with nestin was detected (results not shown).

Discussion

Although previous studies have implicated Hsp27 in regulation of actin polymerization, the primary molecular interaction considered was direct binding of Hsp27 to actin subunits (Landry and Huot 1999; Butt et al. 2001; Bitar 2002; Van Why et al. 2003; Pichon et al. 2004; Chaudhuri and Smith 2008) or binding to actin-associated tropomyosin and caldesmon (Somara and Bitar 2004, 2006). Using purified Hsp27 and actin, *in vitro* studies have shown that non-phosphorylated Hsp27 inhibits actin polymerization, while phosphorylated Hsp27 permits actin polymerization. However, subsequent studies have concluded that Hsp27 plays a more dynamic role in both the assembly and disassembly of actin filaments (During et al. 2007; Doshi et al. 2009). Employing a quantitative LC-MS analysis of Hsp27-associated proteins combined with thiolutin treatment to rapidly stimulate Hsp27 phosphorylation, we have now identified several components of the actin and intermediate filament cytoskeletons that differentially interact with phosphorylated and nonphosphorylated forms of Hsp27. These phosphorylation-dependent interactions of Hsp27 may account for the potent activity of thiolutin to inhibit endothelial cell adhesion and angiogenic responses *in vitro* and *in vivo*.

A number of regulatory components of the actin cytoskeleton were identified by this approach, including several subunits of the Arp2/3 complex. Combined with yeast-two hybrid evidence for direct binding of Hsp27 to p41-ArpC1 (Jia et al. 2001) and the recent colocalization of p34-Arp with Hsp27 in membrane ruffles of platelet-derived growth factor-stimulated smooth muscle cells (Berrou and Bryckaert 2008), these results suggest that Arp2/3 is a major mediator of the effects of Hsp27 on actin cytoskeletal dynamics. The rapid relocation of Hsp27 to retracting borders of thiolutin-treated HUVEC suggests a specific function in actin reorganization accompanying membrane retraction.

Significant changes in Hsp27 interaction with the $\alpha 2$ subunit of actin capping protein Cap Z (Casella and Torres 1994), the myosin-binding regulatory subunit 12A (MYPT1) of the phosphatase PP1 that regulates the interaction of actin with myosin, and the ELMO1 component of the Rac1 GEF ELMO/DOCK180 (Jarzynka et al. 2007) were also identified. MYPT1 localizes with actin stress fibers in growing endothelial cells and at cell–cell contacts in confluent endothelium (Hirano et al. 1999), and it is a target for inhibition of angiogenesis by Rho kinase inhibitors (Somlyo et al. 2003).

Further studies will be required to determine the respective roles of these three actin regulatory proteins in altering the actin cytoskeleton to cause loss of adhesion in endothelial cells treated with thiolutin.

We also identified changes in the interactions of Hsp27 with three isoforms of filamin. Because filamin plays an important role in linking the actin cytoskeleton with integrins (Tseng et al. 2004; Kiema et al. 2006), these results suggest that Hsp27 interactions with filamin could regulate cell-matrix adhesion by regulating signaling between the actin cytoskeleton and extracellular matrix via integrins. Consistent with these results, the actin binding protein nebulin, which was recently shown to interact with filamin C (Holmes and Moncman 2008), also showed increased interaction with Hsp27 in thiolutin-treated cells.

Because we detected no interactions of Hsp27 with focal adhesion components, the previously reported effects of thiolutin on focal adhesions may be secondary responses to the phosphorylation-dependent interactions of Hsp27 identified here or a more general response to the reorganization of the actin cytoskeleton induced by thiolutin. The focal adhesion complex is organized by integrin signal-mediated protein-protein interactions and linked directly to the intracellular cytoskeletal system (Sastry and Burridge 2000; Wehrle-Haller and Imhof 2002).

Intermediate filament proteins were an unexpected target of thiolutin, but several previous studies have suggested a link to Hsp27. Another member of the small heat shock protein family, α B-crystallin, was first found to interact with the intermediate filament proteins peripherin and vimentin in a co-sedimentation assay (Djabali et al. 1997). This interaction was subsequently extended to Hsp27, and both proteins were shown to prevent gel formation by intermediate filament proteins in vitro (Perng et al. 1999). These small heat shock proteins did not prevent filament formation, but were proposed instead to regulate lateral interactions between filaments. We found no general loss of intermediate filaments in thiolutin-treated endothelial cells, although our assays cannot assess lateral interactions in vivo. However, the profound effect of thiolutin on interaction of Hsp27 with nestin and the organization of nestin intermediate filaments suggests that nestin might mediate such effects of Hsp27 on intermediate filament dynamics. Because nestin expression is a marker of stem cells and other cells in early differentiation states (Wiese et al. 2004), thiolutin might be expected to selectively act on these cells. Nestin is also a marker of stem cells in zebrafish (Mahler and Driever 2007), so this may explain some of the embryonic lethality that we observed in thiolutin-treated zebrafish embryos. Regulated Hsp27 interaction with nestin might also be relevant to the pathogenesis of the subset of Charcot-Marie-Tooth disease that is caused by missense mutations in Hsp27 (Evgrafov et al.

2004). Notably, transfection of neuronal cells with the mutant Hsp27 along with neurofilament light chain resulted in altered neurofilament assembly.

In addition to its interaction with nestin, Hsp27 may regulate intermediate filaments through its interaction with plectin-1. Plectin in endothelial cells plays an important role in linking vimentin intermediate filaments to α 6 β 4 integrin by binding to the cytoplasmic tail of the β 4 subunit (Homan et al. 2002). We found that thiolutin significantly decreases plectin-1 association with Hsp27. Because Hsp27 associates with vimentin independent of its phosphorylation, this suggests that the interaction with plectin-1 is direct rather than due to both binding to vimentin. Whether Hsp27 interaction with plectin regulates plectin binding to α 6 β 4 or intermediate filaments remains to be determined.

Although a majority of the Hsp27 interaction partners identified in this study that are regulated by thiolutin are involved in cytoskeletal dynamics, we also identified several targets, including a subunit of cAMP-dependent protein kinase, nucleophosmin, annexin II, Hsp90, hnRNP-U, and the ribosomal proteins L11 and P2 that play broader roles in cell regulation. Among these, nucleophosmin and L11 play important roles in mediating cellular responses to stress by regulating Hdm2/p53 localization to the nucleolus (Colombo et al. 2002; Lohrum et al. 2003). To our knowledge, interactions of these proteins with Hsp27 have not been previously reported. Further work will be required to determine which are direct binding partners and whether Hsp27 interaction regulates their function in cellular responses to stress. We found that endothelial cells efficiently recover from thiolutin-induced detachment. This was surprising, given that endothelial cells typically undergo rapid death by a process known as anoikis following loss of substrate adhesion (Michel 2003). These Hsp27 interactions are candidates to account for the recovery of endothelial cells following exposure to thiolutin.

In summary, these studies provide new insights into the potent anti-angiogenic activity of thiolutin and identify several components of the actin and intermediate filament cytoskeleton as Hsp27-dependent targets. Increased Hsp27 phosphorylation is a convergent target of a number of well known angiogenesis inhibitors (Keezer et al. 2003; Bix et al. 2004; Isenberg et al. 2007), and one of their common effects on endothelial cells is to inhibit cell migration. The cytoskeletal Hsp27 targets identified in the present study, therefore, should be considered as potential mechanisms through which endothelial cell motility is limited by these agents.

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