Development of a Grp94 inhibitor

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INTRODUCTION

Molecular chaperones play a critical role in cellular homeostasis by modulating the folding, stabilization, activation, and degradation of protein substrates. Heat shock proteins (Hsps) represent a class of molecular chaperones whose expression is upregulated in response to cellular stress, including elevated temperatures that disrupt protein folding. Among the various Hsps, the 90 kDa heat shock proteins (Hsp90) are considered promising anticancer targets due to the unique secondary binding pocket that may provide an opportunity to develop isoform-selective inhibitors. Several of these Hsp90 N-terminal inhibitors, e.g. 17-AAG (Phase I–III), SNX-5422 (Phase I), CNF2024 (Phase II), and NVP-AUY922 (Phase I/II) have been evaluated in clinical trials for various indications, including melanoma, multiple myeloma, refractory (Phase I/II) have been evaluated in clinical trials for various indications, including melanoma, multiple myeloma, refractory (Phase I/II) have been evaluated in clinical trials for various indications, including melanoma, multiple myeloma, refractory (Phase I/II) have been evaluated in clinical trials for various indications, including melanoma, multiple myeloma, refractory (Phase I/II) have been evaluated in clinical trials for various indications, including melanoma, multiple myeloma, refractory (Phase I/II) have been evaluated in clinical trials for various indications, including melanoma, multiple myeloma, refractory

pan-Hsp90 inhibition may be the cause for these effects, as clinical inhibitors are known to target all four human isoforms: Hsp90α, Hsp90β, Trap-1, and Grp94. Hsp90α (inducible) and Hsp90β (constitutively active) are the cytosolic isoforms, whereas tumor necrosis factor receptor associated protein (Trap-1) is localized to the mitochondria, and glucose-regulated protein, Grp94, resides in the endoplasmic reticulum. Little is known about the client protein selectivity manifested by each of the four isoforms, and this gap in understanding may underlie the toxicity concerns that have arisen in clinical trials. Despite the clinical significance of Hsp90 inhibition, little investigation toward the development of isoform-selective inhibitors has been reported to delineate isoform-dependent substrates or as an opportunity to reduce the potential side effects that result from pan-inhibition.

Unlike the cytosolic chaperones, Hsp90α and Hsp90β, which have been well-studied, little is known about Trap-1 and Grp94. At present, no isoform-specific clients have been described for Trap-1; in fact, neither the crystal nor the solution structure has been solved. In contrast, Grp94 co-crystal structures have recently been determined and demonstrate that it contains a unique secondary binding pocket that may provide an opportunity to develop isoform-selective inhibitors. Unlike
Trap-1, several substrates dependent upon Grp94 have been identified and include Toll-like receptors (TLR1, TLR2, TLR4, and TLR9), integrins (CD11a, CD18, CD49d, α4, β7, αL, and β2), IGF-I and -II, and immunoglobulins. Since these clients play key roles in cell-to-cell communication and adhesion, Grp94-selective inhibitors may disrupt malignant progression by preventing metastasis, migration, immune evasion, and/or cell adhesion. Interestingly, many of these Grp94-dependent clients have also been identified as key contributors to inflammatory disorders such as rheumatoid arthritis, diabetes, and asthma. Therefore, the ability to develop a Grp94-selective inhibitor may provide not only a new paradigm for Hsp90 inhibition but also new opportunities for the treatment of diseases other than cancer.

The biological roles manifested by Grp94 have been primarily elucidated through the use of RNAi-induced Grp94 knockdown, immunoprecipitation experiments, or through pan-inhibition of all four Hsp90 isoforms. A selective small molecule inhibitor of Grp94 would provide an alternative and potentially powerful method for further elucidation of the roles manifested by Grp94, as well as the identity of other Grp94-dependent processes/substrates. Recently, the co-crystal structures of the chimeric inhibitor, radamide (RDA), bound to the N-terminal domain of both the yeast ortholog of cytosolic Hsp90 (yHsp82) and Grp94 from canine (cGrp94NΔ41) by the Gewirth laboratory. Analyses of the two co-crystal structures (Figure 3A–C) revealed the

Figure 1. Some Hsp90 inhibitors previously or currently under clinical evaluation.

Figure 2. Chimeric approach to Hsp90 inhibition.

Figure 3. RDA quinone (green) hydrogen-bonding network comparison between yHsp82N (A) and cGrp94NΔ41 with RDA cis-amide (teal, B) and RDA trans-amide (teal, C). Red spheres represent water molecules, and hashed lines represent a hydrogen-bonding interaction.

RESULTS AND DISCUSSION

Design and Synthesis of Grp94 Inhibitors. Co-crystal structures of the natural products geldanamycin (GDA) and radicicol (RDC) bound to the highly conserved N-terminal region have been solved. Subsequent studies showed that chimeric inhibitors containing the quinone moiety of GDA and the resorcinol of RDC (Figure 2) also target this domain. Three chimeric scaffolds were identified as Hsp90 inhibitors that manifested anti-proliferative activity against various cancer cell lines. Radamide (RDA) was the first chimera produced, and the first co-crystallized with cytosolic Hsp90 from yeast (yHsp82) and Grp94 from canine (cGrp94NΔ41) by the Gewirth laboratory. Analyses of the two co-crystal structures (Figure 3A–C) revealed the
resorcinol ring to bind similarly to both isoforms, making a direct hydrogen bond with the conserved aspartic acid residue (Asp79 in yHsp82 and Asp149 in cGrp94NΔ41) involved in ATP binding. However, the quinone moiety was found to bind yHsp82N in a linear, trans-amide conformation, which was distinct from one conformation observed in the cGrp94NΔ41 co-crystal structure. Upon binding cGrp94NΔ41, two opposing conformations of RDA were observed (50% occupancy each). One conformation exhibited a cis-amide orientation and predicted the quinone moiety into a hydrophobic pocket that exists solely in Grp94 due to a five amino acid insertion into the primary sequence. The second conformation of RDA observed in the RDA-cGrp94NΔ41 co-crystal structure presented the amide in a trans-configuration and projected the quinone toward the outside of the binding pocket, similar to that observed for RDA in the yHsp82N co-crystal structure.21 Interestingly, RDA was found to exhibit an approximately 2-fold higher binding affinity for full-length Grp94 than yHsp82.21

Further analyses of the RDA-yHsp82N co-crystal structure revealed the quinone to mediate an intricate hydrogen-bonding network, whereas its interaction with cGrp94NΔ41 was limited (Figure 3). For example, in the RDA-yHsp82N structure, direct hydrogen bonds between the RDA quinone and Lys98 and Lys44 were observed. In contrast, no direct hydrogen bonds were observed between cGrp94NΔ41 and the cis-amide quinone (Figure 3B), suggesting that functionalities on the quinone ring may be dispensable for Grp94 binding but obligatory for cytosolic Hsp90 binding. In addition, this Grp94 hydrophobic pocket contains aromatic amino acids (Phe199, Tyr200, and Trp223) that are likely to facilitate hydrophobic, π-rich surrogate for the quinone, the latter of which would be incapable of providing the requisite hydrogen-bonding interactions with cytosolic Hsp90 and should therefore facilitate binding to the π-rich region of Grp94.

Utilizing Surfex molecular docking software, analogues 1–5 were docked to the RDA-cGrp94NΔ41 complex (PDB: 2FXS). As shown in Scheme 1, the Surfex binding scores for compounds 1 and 2 were 1–2 units higher than that of RDA, suggesting binding affinities 10- to 100-fold higher for cGrp94NΔ41, respectively. Furthermore, 1–5 failed to dock to the RDA-yHsp82N complex (PDB: 2FXS), supporting our hypothesis that these phenyl imidazole analogues may exhibit selective inhibition. Although 1 and 2 were the only compounds predicted to bind cGrp94NΔ41, prior studies demonstrated the Grp94 lid region to undergo significant variations that are capable of accommodating various ligand sizes and chemotypes. Unfortunately, available modeling programs could not account for this phenomenon, and therefore all five analogues were constructed. Aldehyde 6 (Scheme 1), which was utilized during the synthesis of RDA,41,42 was readily available and allowed for the rapid preparation of analogues. As shown in Scheme 1, a Radziszewski-like condensation of aldehyde 6 with the requisite aniline/primary amine in the presence of glyoxal and ammonium bicarbonate provided the desired compounds as protected silyl ethers.35,46 Addition of tetrabutylammonium fluoride to the reaction mixture yielded the desilylated compounds 1–5 in moderate yields.

**Scheme 1. Synthesis and Surfex Molecular Docking Scores for Compounds 1−5**

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<th>Analog</th>
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<th>Grp94 Score (logKd)</th>
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Binding of Compounds 1–5 to Grp94. Upon preparation of compounds 1–5, their ability to bind Grp94 was investigated. Using fluorescence polarization competition assays with recombinant cGrp94 and FITC-GDA, the ability of each compound to bind Grp94 and displace FITC-GDA was determined (Figure 4).27 As evidenced in Figure 4, compounds...
and 2 were the only analogues that bound Grp94 and displaced FITC-GDA. These results are consistent with the Surflex-generated docking scores shown in Scheme 1. Although fluorescence polarization can be used to confirm binding affinity for Grp94, prior studies have shown that Hsp90 inhibitors bind preferentially to the entact heteroprotein complex found in cells. Therefore, compounds 1–5 were further investigated in cell-based assays.

**Effect on Trafficking of the Toll Receptor.** Once compounds 1–5 were evaluated for Grp94 binding, studies commenced to validate our hypothesis that imidazoles containing a phenyl moiety inhibit Grp94 in cells. Unlike cytosolic Hsp90 inhibitors that exhibit anti-proliferative effects, RNAi experiments have shown that in culture, cell viability is unhampered by knockdown of Grp94. Thus, a functional assay was necessary to determine Grp94 inhibition.

Grp94 is required for the functional maturation and trafficking of select TLRs. Therefore, TLR dependence upon Grp94 was utilized to develop an assay to quantify Grp94 inhibition. As proof of concept, HEK293 cells were stably transfected to express Grp94 directed or scrambled shRNA. Both cell lines were then transfected with a plasmid encoding expression of the Toll protein, the Drosophila homologue of the interleukin 1 receptor and the founding member of the TLR family. Grp94 knockdown prevented presentation of the Toll receptor at the cell surface (Figure 5A) as indicated by immunostaining and fluorescence microscopy. In order to investigate this inhibition of trafficking, cells were permeabilized with Triton X to effect intracellular staining for Toll. Results clearly indicated that the Toll receptor was expressed in the absence of Grp94 but was unable to be trafficked to the cell surface.

Figure 4. Binding of compounds 1–5 to Grp94. Compounds 1–5 (25 μM) were incubated with cGrp94 and FITC-GDA (tracer) for 5 h before fluorescence polarization values were determined. DMSO (1%) served as a negative control (vehicle), and GDA (500 nM) served as the positive control.

Figure 5. (A) Representative fluorescence confocal microscopy images of HEK293 cells stably transfected to produce either scrambled shRNA or Grp94-targeted shRNA and transfected to express the Toll receptor (green) (blue = DAPI, 100x, TIRF oil immersion). (B) Western blot analysis of cells treated as in panel A. (C) Table of activities for compounds 1–5 to inhibit the trafficking of toll (error bars = ±SEM for at least 100 different cell populations. (D) Representative epifluorescence microscopy images of HEK293 cells transfected to express the Toll receptor (green) and then treated with increasing concentration of compound 2 for 24 h prior to staining (blue = DAPI, 60x, air objective). (E) Dose–response curve for Toll-trafficking inhibition of compound 2.
membrane. Western blot analyses of lysates from Grp94 knockout cells indicated a difference in the glycosylation pattern of the Toll protein, consistent with ER-retention and providing evidence for impaired trafficking to the cell membrane (Figure 5B). This may indicate that Grp94 interacts with a chaperone or partner protein that is involved in the glycosylation of its clients.

Once functional knockdown of Grp94 was established and a reduced cell surface expression of Toll was observed, this assay served as readout for Grp94 inhibition. HEK293 cells were transfected with the same Toll-expressing plasmid and subsequently exposed to compounds 1–5 for 24 h prior to surface staining. The extent of surface expression was then quantified by measuring fluorescence intensity at the cell surface with CellProfiler. Representative fluorescent microscopic images and a dose–response curve are shown for compound 2 in Figure 5. Interestingly, the observed IC\textsubscript{50} values for this series of compounds correlated well with the increased binding affinity of the compounds that inhibited at least 50% of Toll trafficking at 5 μM, as shown in Figure 6. IC\textsubscript{50} values for the compounds evaluated are shown in Figure 5C. Representative dose–response curves for each of the compounds that inhibited at least 50% of Toll trafficking at 5 μM were generated to obtain IC\textsubscript{50} values (Figure 5B). A dose–response curve for each of the compounds that inhibited at least 50% of Toll trafficking at 5 μM was generated to obtain IC\textsubscript{50} values (Figure 5C).

**Inhibition of IGF-II Secretion by 2.** IGF-II is a second well-defined Grp94-dependent client protein, and active Grp94 is required for the secretion of IGF-II.\textsuperscript{55} It has been previously demonstrated that pan-Hsp90 inhibitors, such as 17-AAG, prevent the secretion of IGF-II in serum-starved C2C12 myoblast cells.\textsuperscript{56} Accordingly, serum-starved C2C12 cells were treated with increasing concentrations of compound 2, and the secretion of IGF-II was measured by ELISA (Figure 6A). Approximately 60% reduction of IGF-II was observed already at 10 μM 2, while little effect on cell viability was observed (Figure 6B). The effect on IGF-II secretion is consistent with previous observations using pan-Hsp90 inhibitors, while the lack of effect on cell viability by 2 indicates that this compound is working through a Grp94-dependent mechanism and does not exhibit pan-inhibition.

**Effect on Grp94 Conformation.** Prior studies have shown that occupation of the Grp94 N-terminal ATP binding pocket by inhibitors results in an altered conformation of this domain. Anti-Grp94 (9G10) is an antibody that recognizes the acidic region (residues 290–350) in the second domain of Grp94.\textsuperscript{59} Occupation of the ATP binding site causes a conformational switch in this region and prevents the 9G10 antibody from recognizing Grp94.\textsuperscript{58} Therefore, lysates of C2C12 cells treated with increasing concentrations of compound 2 were immunoprecipitated to assess whether it induces a conformational switch in Grp94. As observed in Figure 7, compound 2 induces a conformational switch in Grp94, as the 9G10 antibody is unable to recognize and immunoprecipitate the Grp94 in cells treated with 2. This result parallels the IGF-II secretion data shown in Figure 6, suggesting that an alteration in Grp94 conformation is incompatible with IGF-II secretion. Interestingly, this activity of Grp94 inhibitors appears to be cell-specific, as analogous experiments performed in CHO cells failed to show an effect on the conformation of Grp94 (data not shown).

**Hsp90 \(\alpha/\beta\) Inhibitory Activity of Compound 2.** As previously mentioned, it has been shown that Grp94 is not essential for tissue culture cell viability. In contrast, loss of functional Hsp90\(\alpha\) or Hsp90\(\beta\) results in cell death. Therefore, we investigated the anti-proliferative effects of compounds 1–5 against two breast cancer cells, MCF7 (ER+) and SKBR3 (Her2 overexpressing, ER−), and against the non-transformed HEK293 cells. None of the compounds evaluated manifested anti-proliferative activity at 10 μM, indicating these compounds do not target Hsp90\(\alpha\) or Hsp90\(\beta\). To support these findings, Western blot analyses of Hsp90\(\alpha/\beta\) client proteins were performed from HEK293 cell lysates. Prototypical pan-Hsp90 inhibitors induce proteasome-mediated degradation of Hsp90\(\alpha/\beta\) client substrates.\textsuperscript{53} As shown in Figure 8, compound 2 does not induce the degradation of Raf or Akt, two well-documented Hsp90\(\alpha/\beta\)-dependent client proteins until 100 μM concentration (see also Figure 9). At this concentration, induction of Hsp70, similar to the one induced by GDA, is presumably mediated by targeting of cytosolic Hsp90. As shown in Figure 8B, the effect on Akt cannot be attributed to ablation of Grp94.
approximately 30 nM and does not affect cytoplasmic proteins until 100 μM in HEK293 cells, providing evidence for Grp94-selective inhibition. To further understand the implications of Grp94-selective inhibition, compound 2 was analyzed in other Grp94-dependent processes.

**Induction of BiP Expression.** Inhibition of Hsp90 is also known to induce expression of Hsp70, and this response is useful as a diagnostic tool (Figure 8A, GDA). A parallel response exists when Grp94 expression is ablated by RNAi, or when its activity is inhibited by RDC or 17-AAG: a transcriptional response is initiated that leads to up-regulation of expression of BiP, the ER member of the Hsp70 family (Eletto et al., submitted). We therefore assessed the ability of 2 to cause BiP up-regulation, in comparison to pan-Hsp90 inhibitors. As shown in Figure 9, treatment of C2C12 cells with 0–75 μM of compound 2 did not lead to up-regulation of BiP, while treatment with 10 μM RDC (or 25 μM of 17-AAG, data not shown) did cause BiP up-regulation. Only at concentrations above 200 μM did compound 2 resemble RDC and induce BiP expression. However, at these concentrations, the compound also destabilized Akt, a hallmark of inhibition of cytosolic Hsp90 (Figure 9). The inability of 2 to up-regulate BiP at the 0–75 μM concentration range was surprising, because this transcriptional response was shown to be a property of Grp94 ablation and not Hsp90 (Eletto et al., submitted).

**Effect on Drosophila Development.** Previous studies have demonstrated that Gp93, the Drosophila ortholog of Grp94, is an essential gene. In the Drosophila model, maternal Gp93 is sufficient to support embryogenesis in Gp93 homozygous null embryos. In the absence of zygotic expression of Gp93, however, larvae display a pronounced growth defect, commensurate with disrupted gut epithelial morphology, decreased gut nutrient uptake, and marked aberrations in copper cell structure and function. As a consequence, loss of Gp93 expression is larval lethal in Drosophila. To determine the effects of compound 2 on Drosophila larval growth, first instar wild type (w1118) larvae were placed onto fly food supplemented with either no supplement (A), 0.1% (B), 0.3% (C), or 0.5% (D) DMSO (vehicle controls) or fly food supplemented with 250 μg/mL (E), 500 μg/mL (F), 750 μg/mL (G), or 1 mg/mL (H) compound 2 (Figure 10A–H). As is
evident from the micrographs of representative larvae, dietary uptake of 2 was associated with a dramatic growth phenotype (Figure 10). In parallel experiments, larval gut tissue was obtained from each of the feeding conditions, and gut epithelial morphology was evaluated by fluorescence microscopy. No grossly discernible effects on copper cell structure were observed, indicating that under these feeding conditions, the inhibition of Gp93 function was incomplete (data not shown). Pharmacokinetic studies of compound absorption and metabolism may provide additional insights into this partial phenotypic behavior.

**CONCLUSIONS**

Hsp90 inhibitors have been the subject of intense pharmaceutical research, not only for cancer but also neurodegeneration. All Hsp90 inhibitors that have reached clinical trials bind to the Hsp90 N-terminal ATP-binding pocket and demonstrate pan-Hsp90 inhibition, i.e., they inhibit all human Hsp90 isoforms simultaneously. Toxicities and off-target effects resulting from Hsp90 inhibition may be a consequence of pan-inhibition. Therefore, the design of Hsp90 isoform-selective inhibitors may provide a valuable pharmacological tool to dissect the roles of each isoform and may lead to more clinically useful inhibitors.

Comparing the crystal structures of several known Hsp90 inhibitors bound to either cytosolic Hsp90 or to the ER-resident Grp94 provided a rational design platform for the development of Grp94 inhibitors. Using structure-based drug design, five compounds were identified as potential leads that contain a phenyl ring appended to an imidazole ring, which serves as a cis-amide bioisostere. The predisposed orientation of the phenyl ring was postulated to allow interactions with the unique Grp94 π-rich pocket. Since Grp94 has previously been shown to be responsible for the trafficking of TLRs to the cell membrane, this activity was used as a functional assay for Grp94 inhibition. Of the five compounds evaluated, compound 2 manifested the best activity in this assay (32 nM). In subsequent, direct readout assays, including an in-cell conformational assay, compound 2 affected Grp94 itself at the same concentration as that needed to inhibit chaperone activity.

Once the Grp94 inhibitory activity of compound 2 was established by these parameters, we evaluated the isoform selectivity of the compound. Inhibitors of cytosolic Hsp90 (Hsp90α/β) manifest anti-proliferative activity in cell culture. At concentrations wherein the assays observed activity for compound 2, there were no cytotoxic effects against any cell line tested. In addition, compound 2 exhibited no effect on the prototypical Hsp90α/β client kinases, Akt or Raf, until concentrations 100x greater than the IC50 for Grp94 inhibition. Therefore, compound 2 appears to manifest considerable selectivity for Grp94 versus Hsp90α/β, perhaps explaining its low toxicity. Lastly, compound 2 stunted the growth of Drosophila larvae in a dose-dependent manner, suggesting that it may be a useful Grp94 inhibitor in vivo. Future studies with 2 will help dissect the roles played by Grp94 and will shed light into the validity of Grp94 as a therapeutic target.

**EXPERIMENTAL SECTION**

**General Method for the Synthesis of Compounds 1−5.**

Aldehyde 6 (1 equiv) was dissolved in wet MeOH at 25 °C. The required aniline/amine (1 equiv) was added dropwise by a syringe to the reaction flask followed by addition of ammonium bicarbonate (1 equiv). Glyoxal (1 equiv) was then added dropwise by a syringe, and the reaction was allowed to sit at 25 °C for 8 h. Upon complete conversion of the aldehyde, as observed by thin-layer chromatography, tetrabutylammonium fluoride was added dropwise by syringe, and the reaction was allowed to sit at 25 °C for 30 min, at which time the reaction was quenched with satd aq NH4Cl and extracted with EtOAc. The organic layers were combined, dried over Na2SO4, and concentrated in vacuo. All compounds were purified via flash chromatography utilizing 95:5 CH2Cl2/MeOH as the eluent. Yields and characterization for all compounds are provided in the Supporting Information.

**Cell Culture.** HEK293 and C2C12 cells were maintained in DMEM supplemented with non-essential amino acids, d-glutamine (2 mM), streptomycin (500 µg/mL), penicillin (100 units/mL), and 10% FBS. Cells were grown to confluence in a humidified atmosphere (37 °C, 5% CO2). Stable Grp94-shRNA knockdown cell lines were generated as follows: the shRNA sequence 5'-GGCUCAAGACAGAUGAUC-3' was cloned into the AAVSilencer 2.0-U6 vector (Ambion), and positive clones were confirmed by sequencing. The pSilencer 2.0-U6-Grp94 shRNA vector and a control, non-targeting pSilencer 2.0-U6 shRNA vector (scrambled, control), were transfected into HEK293 cells using Lipofectamine 2000 using the manufacturers protocol. Cell cultures were selected 36 h post-transfection by the addition of 1 µg/mL puromycin to the media. Puromycin-resistant clones (both Grp94 shRNA and non-targeting shRNA) were subsequently expanded and screened for knockdown efficiency by immunoblotting, using the Grp94 antibody, DU120. Clones displaying greater than 90% knockdown were selected. Puromycin-resistant clones from the nontargeting shRNA were obtained in parallel and screened for normal Grp94 expression, also by immunoblotting with DU120. C2C12 Cells were maintained and induced to differentiate into myoblasts as previously described.73

**Fluorescence Polarization.** Assay buffer (25 µL, 20 mM HEPES pH 7.3, 50 mM KCl, 5 mM MgCl2, 1 mM DTT, 20 mM Na2MoO4, 0.01% NP-40, and 0.5 mg/mL BSA) containing compounds 1−5 or GDA were plated in 96-well plates (black well, black bottom) to give final concentrations of 25 µM or 500 nM, respectively (1% DMSO final concentration). Recombinant cGrp94 and FITC-GDA were then added (50 and 25 µL) to give final concentrations of 60 and 5 nM, respectively. Plates were incubated with rocking for 5 h at 4 °C. Fluorescence polarization values were then read using excitation and emission filters of 485 and 528 nm, respectively. Percent FITC-GDA bound was determined by using the DMSO millipolarization unit (mP) as the 100% bound value, and the mP value of free FITC-GDA as the 0% bound value.

**Toll-Trafficking Assay.** HEK293 cells were plated in 6-well cell culture treated plates in Dulbecco’s Modified Eagle Medium (1x DMEM) supplemented with 10% FBS containing no antibiotics and were maintained at 37 °C, 5% CO2, and 95% relative humidity. After 24 h, the cells (95% confluence) were transfected with pcDNA6B-Toll-Flag using Lipofectamine 2000 according to the manufacturer’s instructions. Cells were transfected for 16 h and then were trypsinized and plated in 96-well microscopy-quality, black-walled plates that had been pretreated with attachment factor. After 3 h of incubation at 37 °C to allow the cells to attach, compound at varying concentrations in DMSO (1% DMSO final concentration) was added, and cells were returned to incubate for 24 h. After 24 h, the media was removed, and cells were fixed in freshly made 4% paraformaldehyde in Dulbecco’s Phosphate Buffered Saline (DPBS) for 10 min at 25 °C. Cells were washed twice with DPBS and then stained with Wheat Germ Agglutinin-Texas Red (5 µg/mL in DPBS, 60 min, 25 °C). Cells were washed twice with DPBS and blocked in 5% bovine serum albumin (BSA, 10 min, 25 °C), followed by staining for 16 h with an anti-Toll antibody (1:200 in 5% BSA/DPBS, 4 °C, Santa Cruz, sc-33741). Cells were washed twice with DPBS and stained with an anti-rabbit-AlexaFluor488 antibody (1:300 in DPBS, 25 °C, Invitrogen, A-11008) for 3 h at 25 °C. Cells were then washed twice with DPBS after which DAPI was added (1 µM in DPBS). Cells were imaged using an inverted Olympus IX-81 microscope with a 60X long working distance air objective using appropriate filter sets for the various tags.
Lysates were clarified with the reagent (MPER, Pierce) and protease inhibitors (Roche) on ice for 1 h according to the manufacturer. 

IGF-II was detected with a biotinylated anti-IGF-II antibody (BAF792, R&D Systems). Data were acquired in duplicate on a microtiter-plate reader and developed with streptavidin-HRP (R&D Systems) with a standard curve generated with recombinant IGF-II (792-MG). Density units were converted to concentrations of the growth factor.

**GFP94 Immunoprecipitation.** Detergent lysates of the indicated cells were immunoprecipitated with 9G10 monoclonal anti-Grp94 (StressGen, Vancouver, BC) followed by protein G-Sepharose (Sigma Chemicals or Pierce) as previously described. 

**IGF-II Secretion.** C2C12 cells (ATCC, Rockville, MD) were induced to differentiate either by complete withdrawal of serum or by shifting to medium supplemented with 5 mM EGTA and imaged on a Leica MZ FLIII stereomicroscope.

**Drosophila.** Compound effects on Drosophila larval growth were examined as previously described. 

**Notes**

The authors declare no competing financial interest.

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