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AEG3482 Is an Antiapoptotic Compound that Inhibits Jun Kinase Activity and Cell Death through Induced Expression of Heat Shock Protein 70

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Summary

We describe a group of small-molecule inhibitors of Jun kinase (JNK)-dependent apoptosis. AEG3482, the parental compound, was identified in a screening effort designed to detect compounds that reduce apoptosis of neonatal sympathetic neurons after NGF withdrawal. We show that AEG3482 blocks apoptosis induced by the p75 neurotrophin receptor (p75NTR) or its cytosolic interactor, NRAGE, and demonstrate that AEG3482 blocks proapoptotic JNK activity. We show that AEG3482 induces production of heat shock protein 70 (HSP70), an endogenous inhibitor of JNK, and establish that HSP70 accumulation is required for the AEG3482-induced JNK blockade. We show that AEG3482 binds HSP90 and induces HSF1-dependent HSP70 mRNA expression and find that AEG3482 facilitates HSP70 production while retaining HSP90 chaperone activity. These studies establish that AEG3482 inhibits JNK activation and apoptosis by a mechanism involving induced expression of HSP proteins.

Introduction

Multicellular organisms eliminate superfluous cells during development through the process of apoptosis. Most forms of apoptosis converge on caspases as downstream effectors, and a significant body of work indicates that caspases are activated by one of two main pathways. The intrinsic pathway involves induced permeabilization of the outer mitochondrial membrane, leading to the release of several proapoptotic factors, including cytochrome C and SMAC, that cooperate to facilitate activation of initiator caspase-9, which, in turn, leads to activation of effector caspases such as caspase-3. The extrinsic pathway is initiated by ligand binding to cell surface death receptors, and this binding induces the assembly of a death-inducing signaling complex that facilitates activation of initiator caspase-8. There is extensive cross-talk between these pathways, and activation of the extrinsic apoptotic pathway often leads to induction of the intrinsic pathway (reviewed in [1]).

Activation of Jun kinase (JNK) has emerged as a central event in neuronal apoptosis (reviewed in [2, 3]). The role of JNK, mechanisms of its activation, and the subsequent events leading to apoptosis have been particularly well elucidated in sympathetic neurons withdrawn from nerve growth factor (NGF). NGF withdrawal results in the activation of small GTPases, such as Rac1 [4], and the subsequent activation of a JNK signaling module that ultimately results in phosphorylation of transcription factors that include c-Jun [5–9]. The phosphorylated transcription factors function, in part, to facilitate production of BH3 domain-only proteins, which are proapoptotic members of the Bcl-2 family [10–12]. Induction of BH3 domain-only proteins leads to the release of mitochondrial contents and thereby initiates the intrinsic apoptotic cascade.

The p75 neurotrophin receptor (p75NTR) and its downstream interacting partner, NRAGE, can initiate signaling events leading to neuronal apoptosis (reviewed in [13]). Work by ourselves and others have established that, in both primary neurons and PC12 cells, p75NTR- and NRAGE-induced cell death occurs through induction of the JNK pathway, BH3 domain-only protein activation, and release of mitochondrial contents [14–16].

Because of the central role of JNK in neuronal cell death, considerable attention has been focused on developing strategies to attenuate JNK signaling. One widely pursued strategy is to develop chemical inhibitors of JNK activity. Several kinase inhibitors that target elements of the JNK activation pathway have emerged [17, 18], and some of these compounds function as antiapoptotic compounds within in vitro and in vivo models [19, 20]. Another approach is to identify endogenous inhibitors of JNKs that attenuate JNK pathway signaling. Several proteins that directly modulate JNK signaling have been identified [21–25], and the best characterized of these is heat shock protein 70 (HSP70). HSP70 expression is often induced in cells exposed to stressful stimuli, and its role in reducing stress-induced damage through its chaperone function is well established [26]. Independent of its chaperone function, HSP70 can directly bind and inhibit JNK and thereby reduce apoptosis induced by a variety of insults [27–31]. It is conceivable, therefore, that induction of HSP70 production would provide an effective means of blocking apoptotic JNK signaling.

In the present study, a search for novel compounds that block JNK-induced cell death was conducted. Screening for small-molecule inhibitors of sympathetic neuron apoptosis induced by nerve growth factor (NGF) withdrawal led to the identification of a novel compound, AEG3482. AEG3482 blocked p75NTR- and NRAGE-induced apoptosis of the PC12 neuronal cell line and attenuated apoptotic JNK signaling. The suppression of JNK signaling and apoptosis by AEG3482 was mediated through induced HSP70

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expression that occurred via an HSF1-dependent pathway. Loss-of-function experiments, in which HSP70 levels were reduced by RNAi knockdown, confirmed that HSP70 accumulation was required for AEG3482-mediated suppression of JNK signaling. Collectively, these studies demonstrate that AEG3482 is a potent antiapoptotic compound that blocks the JNK signaling pathway through increased expression of HSP70.

Results

The Synthetic Compound AEG3482 Inhibits NGF Withdrawal-Induced Death in SCG Neurons

Survival of neonatal primary sympathetic neurons is dependent on trophic support provided by NGF (reviewed in [26]). To identify novel pharmacological inhibitors of neuronal apoptosis, a high-throughput screen was developed to identify compounds that block NGF withdrawal-mediated death of primary sympathetic neurons. Over 17,000 natural, synthetic, and semisynthetic compounds were analyzed for their ability to inhibit NGF withdrawal-induced apoptosis. One of the most promising compounds to emerge from this screen was an imidazothiadiazole sulfonamide of 281 D, which was designated AEG3482 (Figure 1A). Survival of sympathetic neurons withdrawn from NGF was strongly enhanced in the presence of AEG3482, with an EC_{50} of ~20 μM (Figure 1B).

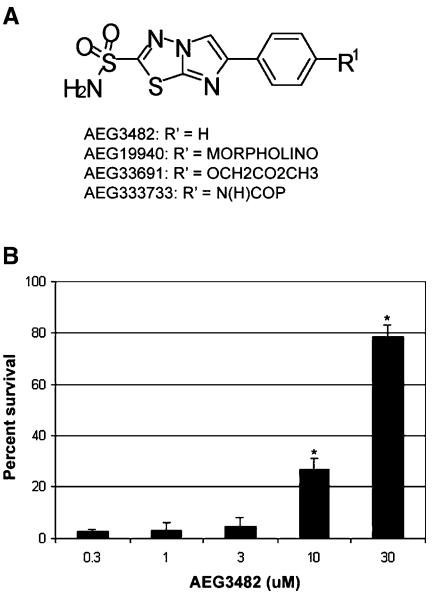

Figure 1. The Synthetic Compound AEG3482 Inhibits NGF Withdrawal-Induced Cell Death

(A) Molecular structure of AEG3482 and its analogs. The compound AEG3482 was isolated in a high-throughput screen for novel inhibitors of NGF withdrawal-induced death of sympathetic neurons. Substitutions for position R^1 are shown at the left.

(B) AEG3482 inhibits NGF withdrawal-induced death of SCG neurons. Cultures of rat SCG neurons (see Experimental Procedures) initially maintained in 10 ng/ml NGF were withdrawn from NGF and maintained in media containing AEG3482 at the concentrations indicated. After 2 days in AEG3482, cell viability was assayed by using an MTS assay. (*$p < 0.01$ relative to no AEG3482). Results are normalized to survival in 10 ng/ml NGF and represent the mean +/− standard deviation of an experiment performed in triplicate (*$p < 0.01$ relative to no AEG3482).

AEG3482 Inhibits p75NTR- or NRAGE-Induced Apoptosis of PC12 Cells

We have previously shown that adenoviral-mediated overexpression of p75NTR, or its cytosolic interactor, NRAGE, leads to extensive JNK-dependent apoptosis of PC12 cells and primary cortical neurons [14–16]. Because the signaling pathways that lead to p75NTR- or NRAGE-induced cell death have been unambiguously established in PC12 cells, this system was employed to determine the mechanism of action of AEG3482. We previously produced an adenovirus that drives NRAGE expression via a doxycycline-inducible element (constitutive NRAGE expression is cytotoxic; see [16] for details), and, for the experiments described below, a PC12 subline ($PC12^{rtTA}$) that stably expresses the doxycycline-activated transcription factor, rtTA, was used.

$PC12^{rtTA}$ cells were infected with adenoviruses encoding p75NTR (Adp75), NRAGE (AdNRG), or, as a control, LacZ (AdLacZ), and they were concurrently exposed to increasing concentrations of AEG3482 for a period of 40 hr. Cell death was assessed by using an LDH release assay. Overexpression of NRAGE or p75NTR led to extensive death of $PC12^{tTA}$ cells, which was strongly attenuated by cotreatment with AEG3482 in a dose-dependent manner (Figures 2A and 2B). Treatment with 40 μM AEG3482 reduced p75NTR- or NRAGE-induced cell death by greater than 90%. At the highest concentration tested (80 μM), the compound effectively inhibited apoptosis, but it also exerted a slight toxic effect in cells infected with the LacZ control. These results indicated that the $PC12^{tTA}$ overexpression paradigm provided a convenient and biochemically tractable system for analyzing the mechanism of action of AEG3482.

AEG3482 Inhibits p75NTR- or NRAGE-Mediated JNK Activation

Activation of JNK is necessary for the induction of p75NTR- or NRAGE-initiated caspase cleavage and cell death [14, 16]. Therefore, the effect of AEG3482 on the activity of JNK was assessed by analyzing alterations in the phosphorylation level of the JNK target, c-Jun, and the cleavage status of caspase-3. Figure 2C shows immunoblots of lysates of $PC12^{rtTA}$ cells that were infected with AdNRG, or the control virus, AdLacZ, in the presence of increasing concentrations of AEG3482. In the absence of the compound, NRAGE expression resulted in robust c-Jun phosphorylation and caspase-3 cleavage. Levels of c-Jun protein were also increased, presumably due to the auto-activation of c-Jun transcription by phosphorylated c-Jun protein [32]. Treatment with AEG3482 strongly attenuated each of these effects; a significant decrease in c-Jun phosphorylation and caspase-3 cleavage was detectable at 10 μM AEG3482 and was virtually complete at 40 μM. Figure 2D shows that similar results were obtained in cells infected with Adp75 and treated with increasing concentrations of AEG3482. Thus, the suppression of JNK signaling and caspase-3 cleavage mediated by AEG3482 occurred with a dose dependency similar to the antiapoptotic activity of AEG3482.

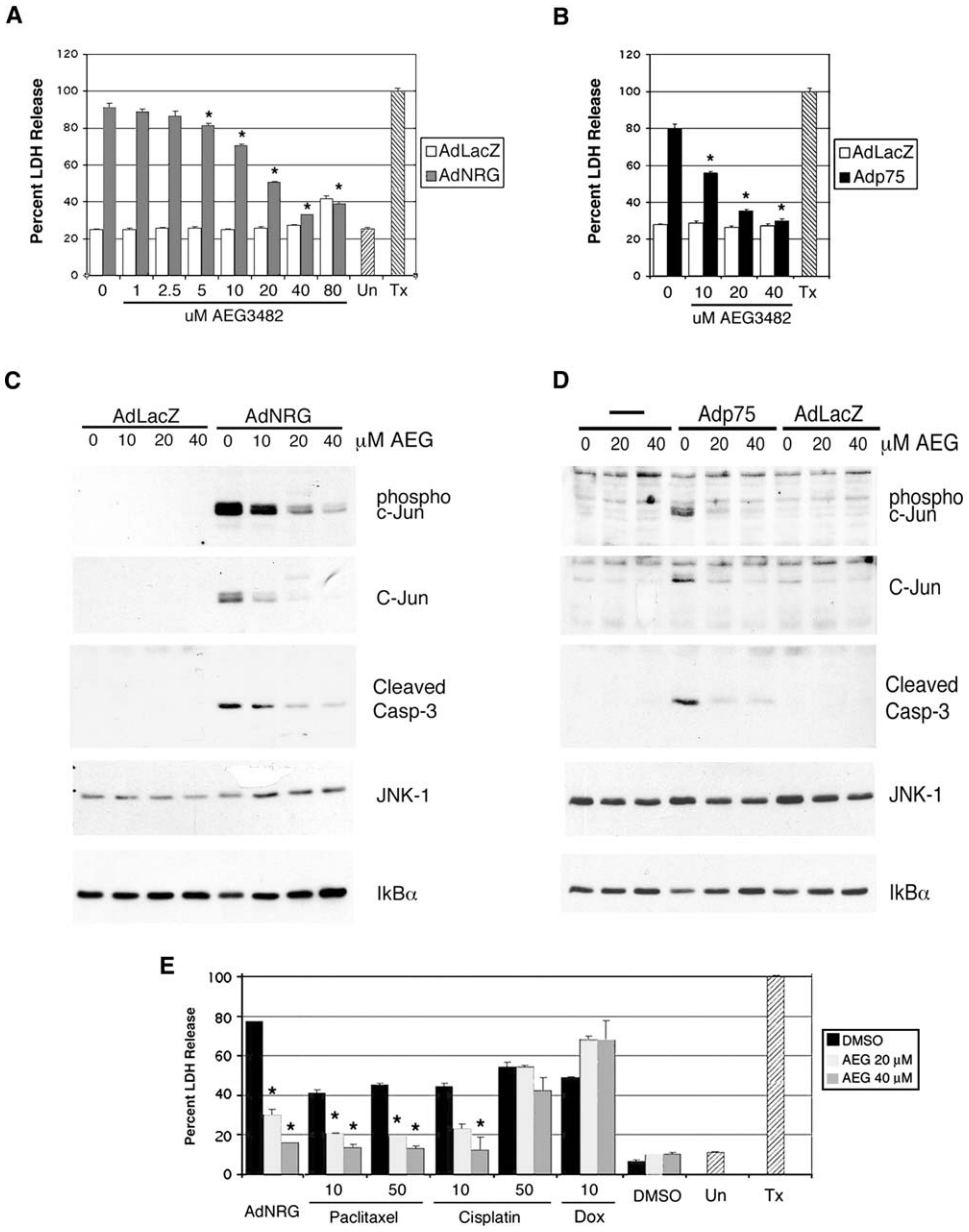

Figure 2. AEG3482 Inhibits JNK-Dependent Cell Death

(A and B) AEG3482 inhibits (A) NRAGE- or (B) p75NTR-mediated cell death. PC12^{rtTA} cells were infected with recombinant adenoviruses expressing full-length NRAGE (AdNRG) at 5 multiplicity of infection (MOI), full-length p75NTR (Adp75) at 50 or 100 MOI, or the control protein β-galactosidase (AdLacZ) at 5 MOI in (A) and 50 MOI in (B). At the time of infection, the cells were treated with an increasing concentration of AEG3482 or with vehicle alone, as indicated. After 40 hr, cells were assayed for death by using the lactate dehydrogenase (LDH) assay; untreated cells (Un) and cells treated with 1% Triton X-100 (Tx) were used to delineate the output range of the assay. Results are normalized relative to those obtained with 1% Triton X-100 and represent the mean ± standard deviation of a representative experiment performed in triplicate (*$p < 0.005$ relative to no AEG3482).

(C and D) AEG3482 inhibits NRAGE- and p75NTR-induced JNK activation. PC12^{rtTA} cells were infected with (C) AdNRG at 5 MOI, with (D) Adp75 at 50 MOI, or with AdLacZ at an equivalent MOI for each experiment, as indicated. At the time of infection, the cells were treated with increasing concentrations of AEG3482 or with vehicle alone, as indicated. After 30 hr of infection, cells were lysed, normalized for protein content, and analyzed for levels of phospho-Jun, total c-Jun, cleaved caspase-3, JNK, IkBα, (C) NRAGE, and (D) p75NTR, by immunoblotting.

(E) Cells were infected with AdNRG at 5 MOI or treated with paclitaxel, cisplatin, or doxorubicin (Dox) at the indicated micromolar concentrations. At the time of treatment, AEG3482 or vehicle was added to the cultures, as indicated. After 40 hr, cells were assayed for death by using the LDH assay. Results are normalized relative to those obtained with 1% Triton X-100 (Tx) and represent the mean ± standard deviation of a representative experiment performed in triplicate (*$p < 0.01$ relative to 0 μM AEG3482 for each insult).

Experiments in (A)–(D) were performed three times, and that in (E) was performed twice, with essentially identical results.

AEG3482 Inhibits PC12 Cell Death Induced by Paclitaxel and Cisplatin

The ability of AEG3482 to inhibit apoptosis in response to a variety of other insults, including the DNA damaging agent cisplatin, the microtubule disruptor paclitaxel, and doxorubicin, a topoisomerase inhibitor, was investigated. Previous studies demonstrate that cell death induced by paclitaxel is dependent on JNK activation [33–35], and Figure 2E shows that apoptosis induced by low (10 μM) and high (50 μM) concentrations of paclitaxel is effectively reduced by AEG3482. Cisplatin-induced apoptosis has been reported to be JNK dependent when used at low concentrations (10 μM), but it is JNK independent at concentrations >25 μM [36]; consistent with this, AEG3482 protected cells exposed to 10 μM, but not 50 μM, cisplatin (Figure 2E). Doxorubicin-induced apoptosis is JNK independent [35], and AEG3482 treatment did not offer any protection against doxorubicin-induced cell death. Collectively, these results are consistent with the hypothesis that AEG3482 inhibits apoptosis by blocking the JNK pathway.

AEG3482 Treatment Induces Expression of HSP70

The structure of AEG3482 does not resemble previously identified kinase inhibitors, and AEG3482 has no effect on JNK activity in vitro (data not shown). The conclusion that AEG3482 does not directly suppress JNK activity was supported by the observation that the JNK inhibitory effects of AEG3482 occurred in cells subjected to extended (i.e., 24 hr), but not short-term (i.e., 1 hr), incubation with the compound (Figure 3A). These results suggested that AEG3482 suppresses apoptotic signaling through alternate means, possibly by enhancing transcription and translation of an endogenous factor that inhibits JNK activity. Several studies have established that heat shock protein 70 (HSP70) can bind and inhibit JNK [27–31], and the possibility that AEG3482 promotes HSP70 production and thereby inhibits the JNK signaling pathway was therefore explored.

To address this, uninfected PC12^{rtTA} cells were exposed to AEG3482 for 18 hr, then lysed and assessed for HSP70 levels by immunoblot. Figure 3B shows that AEG3482 treatment does indeed result in a large increase in cellular HSP70 levels. To test if the accumulation of HSP70 induced by AEG3482 reflected increased steady-state levels of its mRNA, RNA isolated from PC12 cells treated with increasing concentrations of AEG3482 for 18 hr was subjected to semiquantitative rtPCR. Figure 3C shows that AEG3482 treatment increased levels of HSP25 and HSP70 mRNA, but had no effect on HSP90 or actin mRNA levels. HSP protein levels were also assessed in PC12^{rtTA} cells infected with either AdLacZ, Adp75, or AdNRG and, at the same time, exposed to AEG3482. Figure 3D shows that treatment of PC12^{rtTA} cells with increasing concentrations of AEG3482 resulted in a robust increase in HSP70 and HSP25, but not HSP40, in cells infected with either LacZ or with Adp75. Cells infected with AdNRG showed enhanced expression of HSP70 even in the absence of AEG3482. However, treatment of cells infected with AdNRG pushed HSP70 levels substantially higher that those observed with AdNRG alone.

Transcriptional Regulation of HSP70 and HSP25, but Not HSP40, Is Mediated by the Heat Shock Factor 1 Transcription Factor

In unstimulated cells, HSF1 is maintained in an inactive, latent state through an interaction with its binding partner, HSP90. Induction of cell stress results in the interaction of HSP90 with misfolded proteins and disrupts its association with HSF1, allowing it to bind and activate HSP70 and HSP25 promoters [36]. The specific effect of AEG3482 on HSP25 and HSP70 production led us to hypothesize that AEG3482 binds HSP90, causing it to release HSF1, which then binds and activates HSP70 and HSP25 promoters. In initial experiments, the activity of HSP70 transcriptional reporter constructs transfected into PC12 cells was found to be stimulated by AEG3482 (data not shown). To specifically address whether HSF1 plays a role in this response, the effects of AEG3482 on mouse embryonic fibroblasts (MEFs) derived from either wild-type or HSF1 null mice were examined [37]. Figure 4A shows that transcriptional activity of the HSP70 promoter reporter construct was strongly induced by AEG3482 in wild-type MEFs, but that it remained at baseline in MEFs lacking HSF1. Expression of endogenous HSP proteins showed corresponding changes in the MEFs, with strong induction of HSP70 and HSP25 in the wild-type cells, but not in MEFs lacking HSF1 (Figure 4B).

These data indicate that AEG3482 mediates the production of heat shock proteins through an HSF1-dependent pathway, and they are consistent with the hypothesis that AEG3482 directly binds HSP90. To test this directly, AEG3482 was chemically crosslinked to Sepharose beads and used as an affinity reagent in pull-down experiments. Beads were incubated with purified HSP90 and washed extensively, and bound proteins were then eluted and analyzed by SDS-PAGE and immunoblotting. Figure 4C shows that AEG3482 beads, but not control beads, bound HSP90, and that this interaction was strongly attenuated by incubation with excess unbound AEG3482. Collectively, these data indicate that AEG3482 directly binds HSP90, thereby facilitating HSF1-dependent expression of HSP70 and HSP25.

AEG3482 Inhibition of JNK Activity Is Mediated by the Induction of HSP70

Previous studies have shown that HSP70, but not HSP25, is capable of blocking JNK activation [27–31], and we therefore focused on the functional interaction between AEG3482, HSP70 induction, and JNK pathway inhibition. We first compared the cellular effects of three analogs of AEG3482, which differed from the parental compound in a single functional group (Figure 1). One of these compounds (AEG19940) induced HSP70 production, whereas two others (AEG33691 and AEG33733) did not (Figure 5A). Compounds that induced HSP70 production (AEG3482 and AEG19940) were effective in preventing Adp75- or AdNRG-induced cell death (Figure 5B) and reduced Jun phosphorylation (Figure 5C), whereas the other compounds had no effect in these assay. Thus, by using these four related compounds, a strong correlation emerged between the induction of HSP70, the inhibition of JNK activity, and cell death.

Loss-of-function experiments were then used to establish a direct causal link between the induction of

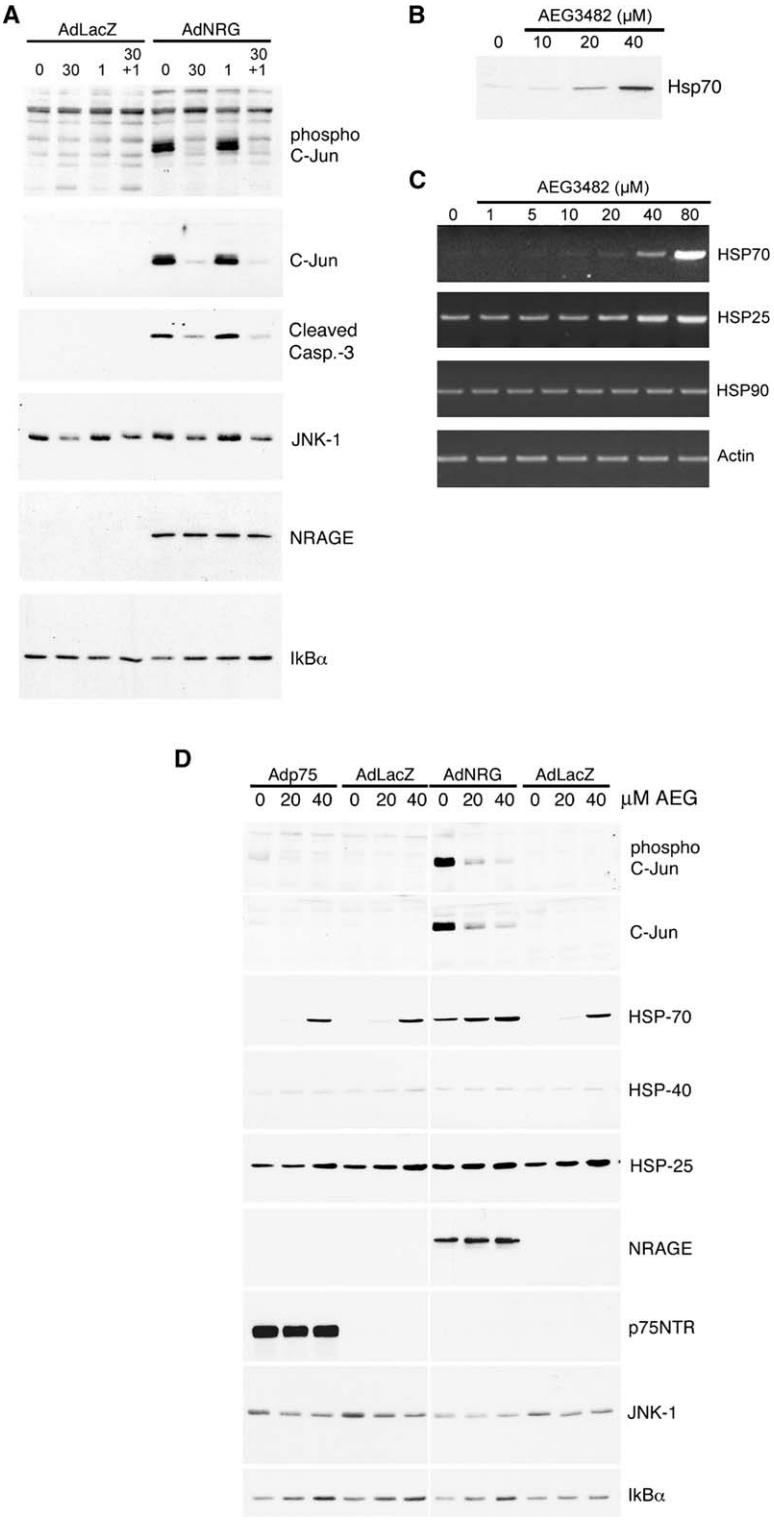

Figure 3. AEG3482 Treatment Induces Expression of HSP70

(A) PC12 cells were infected with 5 MOI AdNRG (NRG) or AdLacZ (LacZ), as indicated. Cells were left untreated (0), or they were treated with 40 μM AEG3482 at the time of infection (30), 1 hr before lysis (1), or at both times (30 + 1). Cells were lysed 30 hr after infection, normalized for protein content, and analyzed for levels of phospho-Ser^{63} c-Jun and total c-Jun, total JNK, IkBα, and NRAGE by immunoblotting.

(B) PC12 cells were treated with the indicated concentrations of AEG3482 for 18 hr and then lysed, normalized for protein content, and analyzed for levels of HSP70 by immunoblotting. Lysate from untreated cells is indicated by "0."

(C) RNA isolated from PC12^{rtTA} cells treated with increasing concentrations of AEG3482 for 18 hr was analyzed by rtPCR with primers specific to HSP70, HSP25, HSP90, or actin, as indicated. RNA from untreated cells is indicated by "0." Experiments in (A) and (B) were performed three times, and that in (C) was performed twice, with essentially identical results.

(D) PC12^{rtTA} cells were infected with AdNRG at 5 MOI, Adp75 at 50 MOI, or the control virus AdLacZ at 50 MOI. At the time of infection, the cells were treated with increasing concentrations of AEG3482 or with vehicle alone, as indicated. After 30 hr of infection, cells were lysed, normalized for protein content, and analyzed for levels of c-Jun phosphorylation, HSP70, HSP25, HSP40, NRAGE, p75NTR, JNK-1, and IkBα, as indicated.

HSP70 by these compounds and their JNK inhibitory activity. For this, RNA interference directed against HSP70 was used to block its accumulation after treatment with AEG3482. The expectation was that, if induction of HSP70 by the compounds was required for JNK inhibition, then blocking the accumulation of HSP70 should reduce the JNK inhibitory effect. Figure 6A shows that transfection with HSP70 siRNA modestly attenuated expression of HSP70 induced by AEG3482. The relatively poor HSP70 knockdown likely reflects the low transfection efficiencies that can be achieved in PC12^{rtTA} cells. Despite this, a small, but consistent, attenuation in the ability of AEG3482 to block c-Jun phosphorylation was observed in cells transfected with HSP70 RNAi, consistent with the hypothesis that HSP70 induction is required for this effect.

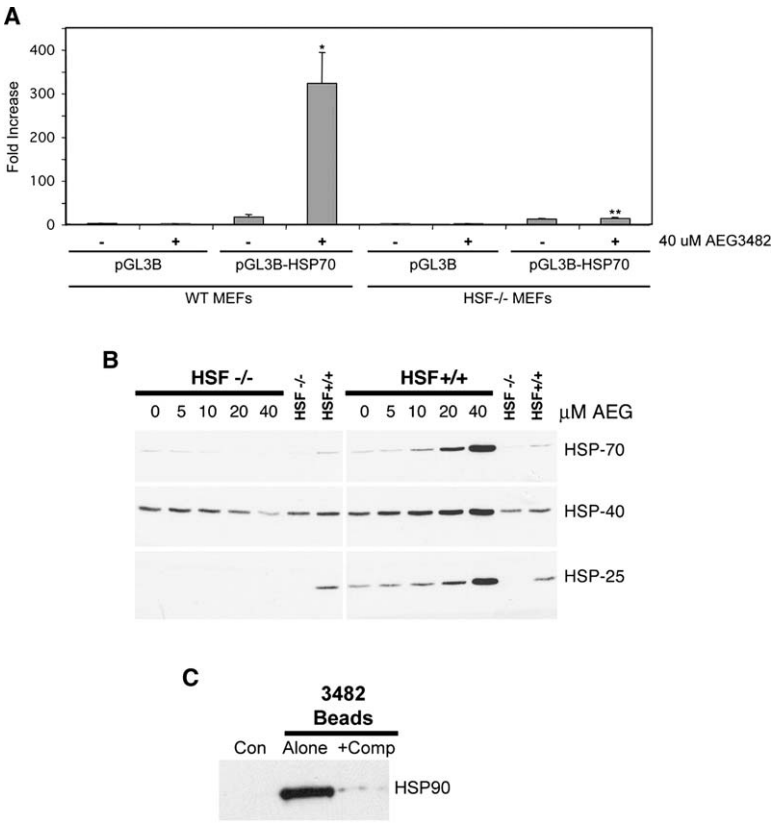

Figure 4. AEG3482 Binds HSP90 and Causes HSF1-Dependent Expression of HSP70 and HSP25

(A) Wild-type or HSF1 null MEFs were transfected with pGL3B-HSP70, an HSP70 promoter reporter construct, or parental vector (pGL3B), then treated with 40 μM AEG3482 for 24 hr and analyzed for luciferase content as described in the Experimental Procedures. Results represent the mean fold increase over untreated wild-type MEFs transfected with plasmid pGL3B +/− standard deviation of experiments performed in triplicate (*$p < 0.005$ relative to no AEG3482; **$p < 0.005$ relative to wild-type MEFs treated with 40 μM AEG3482).

(B) Wild-type or HSF1 null MEFs were treated with increasing concentrations of AEG3482 for 24 hr, and cells were lysed, normalized for protein content, and analyzed for levels of HSP70, HSP40, and HSP25, as indicated.

(C) AEG3482-conjugated Sepharose beads were incubated with purified HSP90 for 2 hr and washed, and levels of bound HSP90 were analyzed by immunoblot. Con = control beads, Alone = AEG3482 beads alone, Comp = AEG3482 beads + competing AEG3482 (at 80 μM).

Experiments in (A)–(C) were performed three times with identical results.

To establish a firmer causal link between HSP70 induction and JNK suppression, and to circumvent the problem of low-transfection efficiency of the PC12^{rtTA} cells, a method was developed to assess JNK pathway activation, specifically in the RNAi-transfected subpopulation of PC12^{rtTA} cells, by using a mammalian expression vector driving production of a fusion protein containing GST fused to amino acids 2–79 of c-Jun. We anticipated that the GST-c-Jun fusion protein would be readily recoverable from lysates, and that its phosphostatus would provide a sensitive read-out that reflects the level of JNK pathway activation in the subpopulation of cells that were transfected. Although overall transfection efficiencies are low in PC12^{rtTA} cells, the GST-c-Jun fusion plasmid and the HSP70 siRNA would be efficiently cotransfected, thereby providing an accurate assessment of the effect of HSP70 depletion on JNK signaling. To validate the GST-c-Jun fusion protein as an in vivo reporter of JNK activity, PC12^{rtTA} cells transfected with GST-c-Jun were treated with TNF or exposed to hyperosmotic shock, two stimuli commonly used to activate the JNK signaling pathway [2]. GST-c-Jun was then recovered from lysates, and its phosphostatus was determined by immunoblotting. Figure 6B shows that both TNF and hyperosmotic shock cause a dramatic increase in phosphorylation of GST-jun which is readily detected by immunoblot. Thus, the phosphostatus of the GST-jun fusion protein provided a sensitive read-out that reflects the level of JNK pathway activation in transfected cells.

We then asked if HSP70 accumulation was required to block NRAGE-induced JNK activity. AEG3482 treatment itself significantly increased expression of the GST-c-Jun fusion construct, complicating the interpretation of results (data not shown). As an alternative, AEG19940 was used since it robustly induced HSP70 production (see Figure 5) without altering GST-jun expression levels. Figure 6C shows that in cells transfected with control siRNA, infection with AdNRG dramatically increased phosphorylation of GST-c-Jun, whereas AEG19940 strongly inhibited this effect. In cells transfected with RNAi directed against HSP70, the ability of AEG19940 to block GST-jun phosphorylation was almost completely lost, indicating that HSP70 accumulation plays a crucial role in the JNK inhibition elicited by this series of compounds.

AEG3482 and Geldanamycin Differ in Their Mechanism of Action

Geldanamycin is a benzoquinone ansamycin that binds to the ATP binding pocket of HSP90 (reviewed in [38–40]. To determine if AEG3482 also occupies the HSP90 binding pocket, we compared AEG3482 and geldanamycin for their ability to block binding of HSP90 to γATP-Sepharose. Figure 7A shows that purified HSP90 readily binds γATP-Sepharose, and that this binding is strongly inhibited in the presence of free ATP. Geldanamycin also blocks association of HSP90 with γATP-Sepharose, consistent with previous results [41]; in contrast, AEG3482 does not decrease the amount of HSP90 bound to γATP-Sepharose, but rather increased their association.

By occupying the ATP binding pocket, geldanamycin is thought to block the chaperone function of HSP90 and

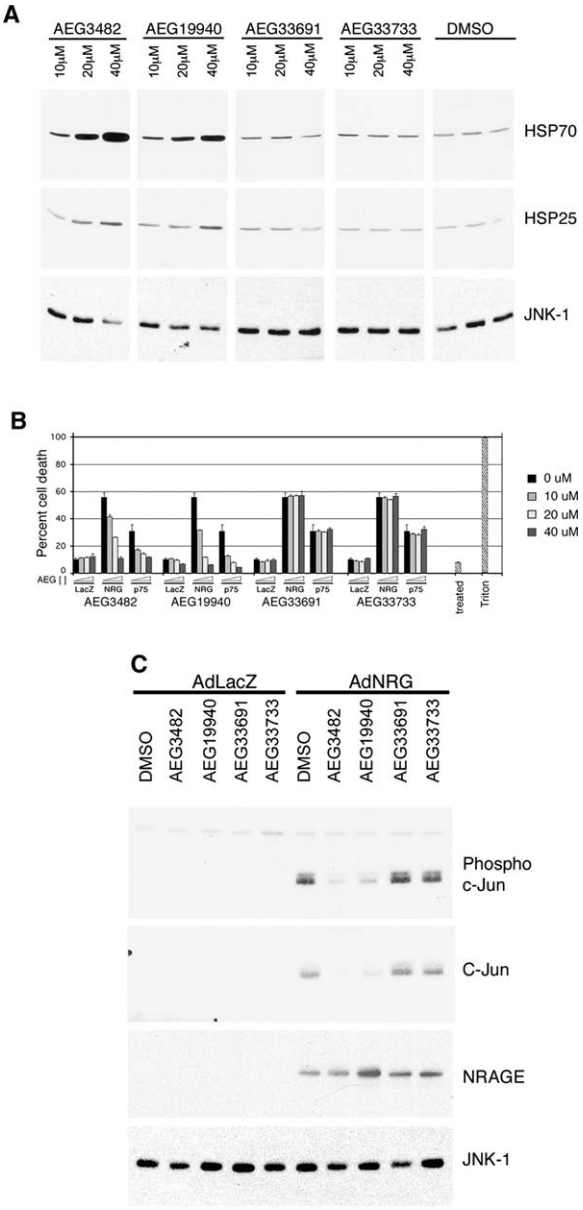

Figure 5. AEG3482 Analogs Inhibit JNK Activation and Induce Expression of HSP70

(A) PC12^{rtTA} cells were infected with 5 MOI AdNRG, 50 MOI Adp75, or 50 MOI of the control virus AdLacZ. At the time of infection, the cells were treated with increasing concentrations of AEG3482; with its analogs, AEG19940, AEG33691, and AEG33733; or with vehicle alone, as indicated. After 30 hr of infection, cells were lysed, normalized for protein levels, and analyzed for levels of HSP70, HSP25, and JNK-1, as indicated.

(B) PC12^{rtTA} cells were treated as described above, and after 40 hr of infection, they were assayed for death by using the lactate dehydrogenase (LDH) assay. Results are normalized relative to those obtained with 1% Triton X-100 and represent the mean ± standard deviation of a representative experiment performed in triplicate (*$p < 0.025$ relative to 0 for each AEG concentration).

(C) PC12^{rtTA} cells were infected with 50 MOI LacZ or 5 MOI AdNRG50, and, at the time of infection, they were treated with 40 μM AEG3482; with its analogs, AEG19940, AEG33691, and AEG33733; or with vehicle alone, as indicated. After 30 hr of infection, cells were lysed, normalized for protein levels, and were analyzed for phosphorylated c-Jun, total c-Jun, NRAGE, and JNK-1, as indicated.

Experiments in (A)–(C) were performed twice, with essentially identical results.

therefore reduce the stability and activity of HSP90 client proteins. We therefore compared the effects of AEG3482 and geldanamycin on Akt, a pro-survival kinase that is an HSP90 client protein. Figure 7B shows that in PC12 cells, geldanamycin treatment lead to a dramatic reduction in levels of total and phosphorylated Akt, consistent with previous results [42], whereas AEG3482 significantly enhanced pAkt levels and reduced total Akt only slightly at the highest concentrations tested.

Discussion

The present study indicates that the antiapoptotic activity of AEG3482 and its analogs arises from their ability to block activation of the JNK pathway. Structurally, AEG3482 does not resemble known kinase inhibitors, and its time course of action suggested that it may function by facilitating production of an endogenous JNK pathway antagonist. HSP70 is an endogenous inhibitor of JNK activity [27–31], and our data show that AEG3482 is a potent inducer of HSP70 production and demonstrate that HSP70 accumulation is required for the effect of AEG3482 on JNK signaling. To our knowledge, this compound is the first shown to inhibit JNK activation through a mechanism involving induced production of HSP70.

HSPs are highly conserved proteins that are induced by a wide variety of chemical and physiological stimuli (reviewed in [43]). It is well established that HSPs play crucial protective roles in stress responses, and that they can suppress apoptosis induced by heat shock, chemotherapeutic agents, nutrient withdrawal, ionizing radiation, or TNF [43]. Induction of HSPs with sublethal stresses gives rise to stress tolerance, and, in several models, HSP70 has been identified as being the main HSP responsible for resistance to future insults [29, 44–47]. The protective nature of HSP70 and other HSPs was originally attributed exclusively to their role as molecular chaperones that prevented stress-induced protein misfolding and aggregation and accelerated refolding [26]. However, recent findings have demonstrated that, in addition to this function, HSP70 suppresses apoptosis by directly inhibiting components of the JNK signaling pathway [27–31]. This inhibition involves direct binding of HSP70 to JNK [28]. The precise HSP70-JNK binding domains have not been identified, but available data suggest that HSP70 binds JNK at, or close to, the docking groove where interactions with both JNK targets and activators occur. It is thus likely that the HSP70-JNK association attenuates the interaction of JNK with upstream MKKs and/or downstream targets [28]. Inhibition of JNK by HSP70 does not appear to be directly related to its chaperone function since HSP70 mutants that lack chaperone function still inhibit JNK; furthermore, HSP70 can inhibit JNK activation even in the absence of stress-induced protein damage [27, 28, 30].

Our data show that AEG3482 exposure results in the accumulation of HSP25 and HSP70 mRNA and protein in PC12 cells. Transcriptional regulation of these HSPs normally requires the action of the HSF1 transcription factor, and we therefore asked if AEG3482 induces HSF1 activity. Transcriptional reporter assays demonstrated

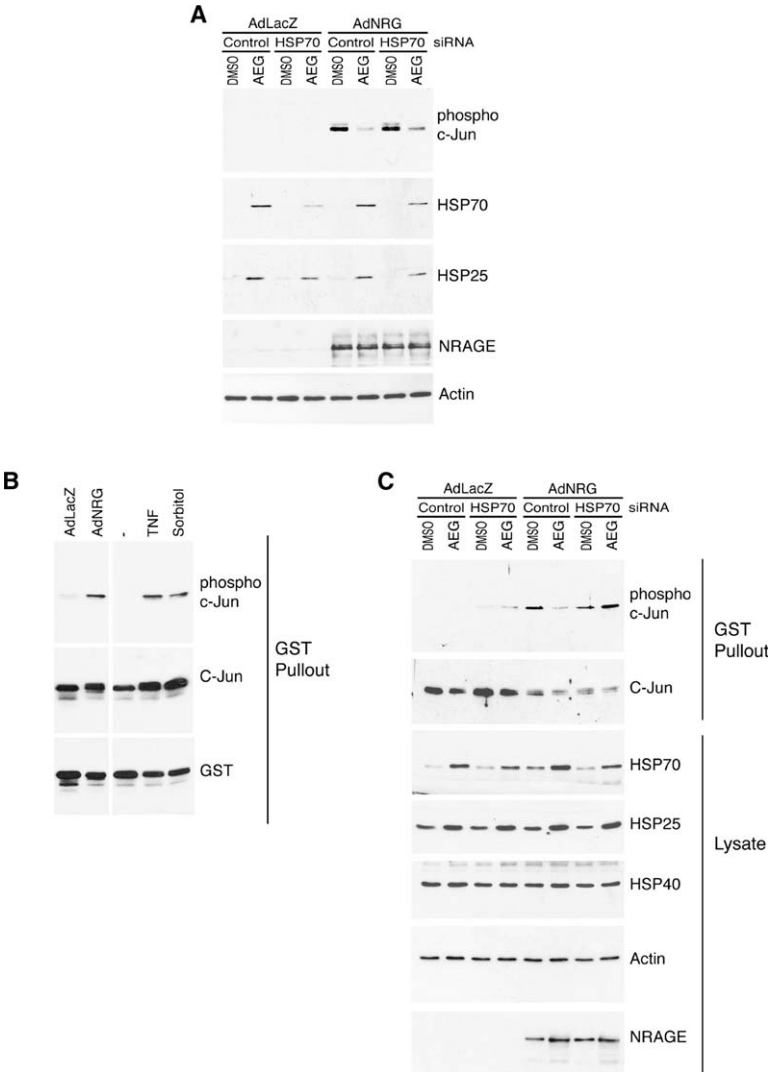

Figure 6. AEG3482 Inhibits JNK through Induced Expression of HSP70

(A) PC12^{rtTA} cells were transfected with RNAi directed against HSP70 or with control RNA, then 24 hr later, they were infected with 5 MOI AdNRG or 50 MOI of the control virus AdLacZ. At the time of infection, the cells were treated with 40 μM AEG19940 or with a vehicle control, as indicated. After 30 hr, cells were lysed, normalized for protein levels, and analyzed for phosphorylated Jun, HSP70, HSP25, NRAGE, and actin levels.

(B) PC12^{rtTA} cells were transfected with an expression construct driving GST-jun. After 24 hr, cells were infected with 5 MOI AdNRG or 50 MOI of the control virus AdLacZ or were left uninfected. After an additional 24 hr, uninfected cells were left untreated or were exposed to 10 ng/ml TNF or 300 mM sorbitol for 15 min. Cells were then lysed and normalized for protein levels, and GST-jun was recovered by glutathione pullout and analyzed for phosphorylation by using an antibody directed against c-Jun pSer63 by immunoblotting. Equivalent pullout of GST-jun was confirmed by immunoblotting with an antibody directed against c-Jun and GST.

(C) PC12^{rtTA} cells were cotransfected with an expression construct driving GST-jun and with RNAi directed against HSP70 or with control RNA, as indicated. After 24 hr, cells were infected with 5 MOI AdNRG or 50 MOI of the control virus AdLacZ. At the time of infection, the cells were treated with 40 μM AEG19940 or with a vehicle control, as indicated. After 30 hr, cells were lysed and normalized for protein levels, and GST-jun was recovered by glutathione pullout and analyzed for phosphorylation by using an antibody directed against c-Jun pSer63 by immunoblotting. Equivalent pullout of GST-jun was confirmed by immunoblotting with an antibody directed against total Jun. Lysate levels of HSP70, HSP25, HSP40, NRAGE, and actin were determined by immunoblot.

Experiments in (A) and (C) were performed three times, and the experiment in (B) was performed twice, all with essentially identical results.

that AEG3482 does indeed activate HSF1 transcriptional activity, and comparison of wild-type MEFs with those lacking HSF1 established that AEG3482 induces HSP25 and HSP70 production through an HSF1-dependent pathway. HSF1 is normally maintained in a latent form by virtue of its association with HSP90 [48], and we hypothesized that an association of AEG3482 with HSP90 releases HSF1 and thereby facilitates HSP25 and HSP70 transcription. Consistent with this, we found that purified HSP90 directly binds AEG3482 in pullout assays.

HSP90 is composed of three main domains. The C-terminal domain contains the HSP90 dimerization site as well as docking sites for various cochaperones. The central domain contains a large hydrophobic surface that is involved in the binding of HSP90 client proteins, and the N-terminal region contains the molecule's ATPase domain (reviewed in [40]). Unlike other chaperones, most known client proteins of HSP90 are involved in the regulation of survival and growth (reviewed in [38]). Geldanamycin binds the ATP binding pocket of HSP90, and this leads to allosteric changes in HSP90 that result in release of HSF1 and subsequent expression of HSP25 and HSP70 [38–40, 48] Because this mechanism is similar to that which we propose for AEG3482 and its analogs, it is reasonable to expect that the cellular effects of AEG3482 and geldanamycin treatment will be similar. However, although scattered reports indicate that geldanamycin does confer protection to cells from protein-damaging stress in vitro and in vivo, the majority of studies have shown that geldanamycin is cytotoxic and kills a variety of normal and transformed cells, including PC12 cells (reviewed in [38, 49]. The toxic effect of geldanamycin is due to the fact that geldanamycin not only releases bound HSF1, but, by occupying the ATP binding pocket, blocks HSP90 chaperone activity and reduces the stability and activity of HSP90 client

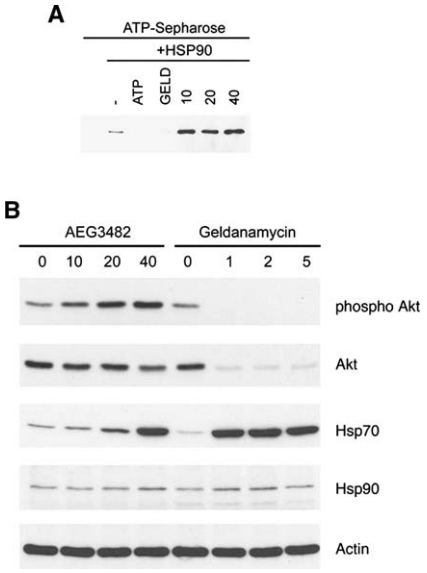

Figure 7. AEG3482 and Geldanamycin Differ in Their Mechanism of Action

(A) Purified HSP90 protein was preincubated with ATP, geldanamycin (1 μM), or with the indicated concentrations of AEG3482 (indicated in μM), and it was then mixed with γATP-Sepharose beads. Bound HSP90 was eluted, and HSP90 content was determined by immunoblotting.

(B) PC12 cells were exposed to increasing concentrations of AEG3482 and geldanamycin (indicated in μM) for 16 hr, lysed, and analyzed by immunoblotting for levels of phosphorylated and total Akt, HSP70, HSP90, and actin, as indicated.

proteins that include Akt and Raf [42, 50–54]. There are major structural and mechanistic differences between geldanamycin and AEG3482, and it appears unlikely that AEG3482 binds to the N-terminal ATP binding domain of HSP90, as does geldanamycin. We have shown that AEG3482 does not occupy the ATP binding pocket of HSP90 and demonstrated that, unlike geldanamycin, AEG3482 does not reduce levels of Akt or block its phosphorylation, but may instead enhance Akt phosphorylation. An enhancement of AKT and Raf activity is observed during the misfolded protein response; misfolded proteins interact with the peptide binding domains of HSP90 and cause the release of HSF1, and our working hypothesis is that AEG3482 interacts with a portion of the peptide binding domain of HSP90, and that this facilitates HSF1 release while retaining HSP90 chaperone activity.

In conclusion, we have identified a class of compounds that inhibit JNK activation by inducing production of HSP proteins. We anticipate that AEG3482 and its analogs will serve as useful tools for basic research, and we believe that they have therapeutic potential for the treatment of acute and chronic neurological disorders.

Experimental Procedures

Materials

AEG3482, AEG19940, AEG33691, AEG33733, and AEG40011 were produced at Aegera Therapeutics. Antibodies directed against JNK1 and IkBα were purchased from Santa Cruz Biotechnology; those against c-Jun, phospho-c-Jun, and cleaved caspase-3 were purchased from Cell Signaling Technology; those against HSP25, HSP40, and HSP70 were purchased from Stressgen; and that against actin was purchased from ICN. Antibodies directed against NRAGE and p75NTR have been previously described [16, 55]. The $PC12^{rtTA}$ cell line was purchased from Clontech. HSP70-specific small interfering RNAs consisted of a "SmartPool" mixture, which was purchased from Dharmacon. Recombinant adenoviruses driving expression of β-galactosidase, p75NTR, and NRAGE have been previously described [56]).

Cell Culture, Infection, Transfection, and Immunoblotting

Sympathetic neurons were maintained and deprived of NGF as previously described [57, 58]. $PC12^{rtTA}$ cells were maintained and infected as previously described [16, 55]; for NRAGE, 1 μg/ml doxycycline was added to all plates at the time of infection. Wild-type and HSF1 nullizygous, immortalized mouse embryonic fibroblasts (MEFs) were maintained in DMEM containing 10% fetal calf serum, 1 μM β-mercaptoethanol, 1% nonessential amino acids, 1% L-glutamine, 1% antimycotic solution, and 1% sodium pyruvate (all from GIBCO-BRL). Sympathetic neuron survival was assessed by using the MTS assay according to the manufacturer's instructions (Promega). Analysis of PC12 cell death was determined by using a lactate dehydrogenase (LDH) assay (Roche) as per the manufacturer's instructions. Transfections with plasmids or RNAi were performed by using Lipofectamine 2000. Immunoblotting was performed as previously described [16, 55].

Detection of Endogenous Phosphorylation of GST-c-Jun

The cDNA region corresponding to amino acids 2–79 of human c-Jun was cloned into a mammalian GST expression vector [59], and this was used to transfect PC12 cells. Lysates of transfected cells were prepared and incubated with 20 μl glutathione-conjugated beads (Pharmacia) for 1 hr at 4ºC. Beads were washed three times, resuspended in Lammeli sample buffer, and incubated at 100ºC for 5 min. The level of GST-c-Jun phosphorylation was assessed by immunoblotting with a phospho-c-Jun-specific antibody.

RT-PCR

PC12 cells were treated with increasing concentrations of AEG3482 for 18 hr, and mRNA was isolated with RNEasy Mini kits (Qiagen). cDNA was generated with the Omniscript RT kit (Qiagen) and random hexamers (Roche) as primers. PCR was performed by using primer pairs directed against rat HSP70, HSP25, and actin (primer sequences and PCR conditions are available upon request).

Transcriptional Assays

MEFs were transfected with pGL3B-HSP70 or with the corresponding parental vector. AEG3482 (40 μM) was added to the cells the next day, and cells were harvested 48 hr after transfection. Transcriptional assays were performed by using a luciferase assay system purchased from Promega.

ATP-Sepharose Interaction Assays

These were performed essentially as described in [41]. Purified HSP90 protein (1 μg) was preincubated with ATP, geldanamycin, or AEG3482 in 200 μl incubation buffer (10 mM Tris [pH 7.5], 50 mM KCl, 5 mM $MgCl_2$, 2 mM DTT, 20 mM Na_2MoO_4, and 0.01% NP-40) for 10 min at room temperature. ATP-Sepharose beads (25 μl) were added, and the reactions were incubated for 30 min at 30ºC. After thorough washing with Be ice-cold incubation buffer, bound proteins were eluted in sample buffer, and HSP90 content was determined by immunoblotting.

Statistical Analysis

For quantitation, each condition was performed in triplicate or quadruplicate, and results were analyzed by multiple analysis of variance with statistical probabilities assigned by using the Tukey test for multiple comparisons.

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