Heat Shock Protein 90 Inhibition Depletes TrkA Levels and Signaling in Human Acute Leukemia Cells

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Abstract

Nerve growth factor (NGF) induces autophosphorylation and downstream progrowth and prosurvival signaling from the receptor tyrosine kinase TrkA. Overexpression or activating mutation of TrkA has been described in human acute myeloid leukemia cells. In the present study, we show the chaperone association of TrkA with heat shock protein 90 (hsp90) and the inhibitory effect of the hsp90 inhibitor, 17-DMAG, on TrkA levels and signaling in cultured and primary myeloid leukemia cells. Treatment with 17-DMAG disrupted the binding of TrkA with hsp90 and the cochaperone cdc37, resulting in polyubiquitylation, proteasomal degradation, and depletion of TrkA. Exposure to 17-DMAG inhibited NGF-induced p-TrkA, p-AKT, and p-ERK1/2 levels, as well as induced apoptosis of K562, 32D cells with ectopic expression of wild-type TrkA or the constitutively active mutant ΔTrkA, and of primary myeloid leukemia cells. Additionally, 17-DMAG treatment inhibited NGF-induced neurite formation in the rat pheochromocytoma PC-12 cells. Cotreatment with 17-DMAG and K-252a, an inhibitor of TrkA-mediated signaling, induced synergistic loss of viability of cultured and primary myeloid leukemia cells. These findings show that TrkA is an hsp90 client protein, and inhibition of hsp90 depletes TrkA and its progrowth and prosurvival signaling in myeloid leukemia cells. These findings also support further evaluation of the combined activity of an hsp90 inhibitor and TrkA antagonist against myeloid leukemia cells. Mol Cancer Ther; 9(8); OF1–II. ©2010 AACR.

Introduction

TrkA is a transmembrane, glycosylated receptor tyrosine kinase, which is encoded by the NTRK1 gene (1). Binding of TrkA to its ligand, nerve growth factor (NGF), induces autophosphorylation and activation of TrkA (1). TrkA mediates NGF-induced signaling for differentiation in neuronal cells, e.g., neurite formation and sympathetic neuron-like phenotype in PC-12 cells (2, 3). Complete NGF withdrawal or pharmacologic inhibition of TrkA activity attenuates p-TrkA levels and ERK1/2 and AKT activity in PC-12 cells (2). Besides involvement in tumors of neuronal origin, Trk mutations and translocations have been reported in breast and pancreatic cancer cells as well as in lymphoma and multiple myeloma cells (4–10). A TrkA mutation conferring ligand-independent progrowth and prosurvival activity has been documented in AML (11, 12). In this mutation, a 75-amino acid deletion of TrkA was identified, also designated as ΔTrkA. This mutation is strongly leukemogenic and transforms hematopoietic stem cells by activating the phosphoinositide-3-kinase/mTOR pathways (11, 12). A recent study has shown that AML cells coexpress at least one or more isoforms of the Trk receptors (13). Here, a retrovirus-mediated coexpression of TrkA and its ligand NGF in 32D cells resulted in leukemia when the cells were transplanted into mice (13). TrkA mRNA and protein expression has been shown to be highly upregulated in human AML expressing AML1-ETO (14). CD34+ cells expressing AML-EETO were shown to respond to NGF and interleukin 3 stimulation by expanding in liquid culture (14). Additionally, recent studies have shown the role of neurotrophin-induced TrkA signaling in non- Hodgkin lymphoma and diffuse large B-cell lymphoma cells (15).

Heat shock protein 90 (hsp90) is an abundantly expressed and stress-inducible, homodimeric, ATP-dependent molecular chaperone (16). Hsp90 forms the core of a super chaperone machine, which is required for maintaining a number of signaling protein kinases and transcription factors, known as hsp90 client proteins, into their functionally mature and active conformation (16, 17). ATP binding to the hydrophobic NH2 terminus pocket also alters hsp90 conformation, promoting the interaction of hsp90 with a set of cochaperones, e.g., p23 and cdc37, that fold the metastable signaling client proteins into their active conformation (16–19). In transformed cells, hsp90 client oncoproteins include several unmutated and mutated protein kinases, e.g., Bcr-Abl,
FLT-3, c-KIT, c-Raf, and AKT (16, 17, 20–24). The hsp90 antagonist geldanamycin and its more soluble analogue, 17-DMAG, bind to the NH₂ terminus ATP-binding pocket of hsp90, replacing the nucleotide and inhibiting the chaperone function of hsp90 (16, 17, 25–27). Binding of 17-DMAG to hsp90 shifts it from a refolding chaperone complex to one that promotes the degradation of client proteins (16, 17, 28). The misfolded client protein is then directed to a covalent linkage with polyubiquitin by an E3 ubiquitin ligase, and is subsequently degraded by the 26S proteasome (16, 17, 28). Thus, 17-DMAG treatment promotes polyubiquitylation and proteasomal degradation of the misfolded hsp90 client proteins, including Bcr-Abl, FLT-3, c-Raf, CDK4, and c-Kit (20–24, 29). Recently, among the Trk receptor family members, TrkB mediated activation of TrkB was shown to interact with hsp90 in retinal ganglion cells (30). Additionally, in tumor cells, brain-derived neurotrophic factor–mediated activation of TrkB was shown to be dependent on hsp90 (31). In the present study, we show that TrkA is an hsp90 client protein, and treatment with 17-DMAG depletes the levels and signaling mediated by TrkA in cultured and primary human myeloid leukemia cells. Furthermore, cotreatment with 17-DMAG and a TrkA antagonist was noted to exert synergistic activity against cultured and primary human myeloid leukemia cells.

Materials and Methods

Cell culture

Human chronic myeloid leukemia (CML)-BC K562 cells were obtained from American Type Culture Collection and maintained in culture in RPMI medium containing 10% fetal bovine serum, Minimal Essential Medium Non-essential Amino Acids (MEM-NEAA), and penicillin-streptomycin (21, 24). HS-5 cells were obtained from American Type Culture Collection and maintained in DMEM containing 10% fetal bovine serum, 1% MEM-NEAA, and 1% penicillin-streptomycin. Cocultures of HS-5 and leukemic cells were carried out as described previously (32, 33). The rat pheochromocytoma PC-12 cells were obtained from American Type Culture Collection and maintained in F-12K medium supplemented with 10% fetal bovine serum, 5% horse serum, MEM-NEAA, and penicillin-streptomycin. 32D cells ectopically overexpressing wild-type TrkA (32D/wtTrkA) or mutant TrkA (32D/ΔTrkA) were created and maintained in culture, as previously described (14, 15). Human cancer cell lines obtained from American Type Culture Collection were maintained according to guidelines (available online at http://www.atcc.org and http://www.aacr.org). Logarithmically growing cells were used for all experiments.

Reagents and antibodies

17-DMAG was obtained from the National Cancer Institute and Kosan Biosciences (South San Francisco, CA). K-252a, an inhibitor of TrkA signaling (34, 35), was purchased from Calbiochem. Monoclonal anti-TrkA antibody was purchased from Santa Cruz Biotechnology. p-TrkA, p-AKT, and AKT antibodies were purchased from Cell Signaling Technology. Antibodies for c-Raf were obtained from BD Biosciences. Ubiquitin antibody was obtained from Covance. ERK1/1 and p-ERK1/2 antibodies were obtained from Invitrogen.

Primary leukemia blasts

Primary AML and CML cells were obtained with informed consent as part of a clinical protocol approved by the Institutional Review Board of the Medical College of Georgia. As previously described, bone marrow and/or peripheral blood samples were collected in heparinized tubes, and mononuclear cells were separated using Lymphoprep (Axis- Shield), as previously described (36). Cells were counted prior to their use in experiments.

Immunoprecipitation of TrkA, hsp90, and immunoblot analyses

Following the designated treatments, cells were lysed in lysis buffer [20 mmol/L Tris (pH 8), 150 mmol/L sodium chloride, 1% NP40, 0.1 mol/L sodium fluoride, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 2.5 μg/mL leupeptin, and 5 μg/mL aprotinin] for 30 minutes on ice, and the lysate was cleared by centrifugation, as previously described (24). Cell lysates were incubated with the hsp90 or TrkA monoclonal antibody for 1 hour at 4°C. Washed protein G agarose beads were then added and incubated overnight at 4°C. The immunoprecipitates were washed three times with lysis buffer and proteins were eluted with SDS sample-loading buffer prior to immunoblotting analyses with specific antibodies against hsp90, TrkA, anti-cdc37, or anti-ubiquitin antibodies (24).

Western analyses of proteins

Western analyses were done using specific antisera or monoclonal antibodies according to previously reported protocols, and horizontal scanning densitometry was done on Western blots as previously described (24, 27).

Reverse transcription-PCR to detect TrkA mRNA levels

Primers were designed to detect wild-type TrkA and ΔTrkA. These primers were: TrkA forward, 5′-TCCC-GGCCAGTGTCAGCTG-3′, and TrkA reverse, 5′-AGGATGGGTGTCCTCGGGTTGAA-3′. Following drug treatments, total RNA was isolated using TRIzol reagent (Invitrogen). Two micrograms of total RNA was reverse transcribed with a Superscript First-strand Synthesis Kit (Invitrogen). The resulting cDNA was used to amplify the 326-bp wtTrkA or the 101-bp ΔTrkA by PCR. Primers designed against β-actin were used as an internal loading control. These primers were β-actinfor, 5′-CTACATG-AGCTGCGTGG-3′, and β-actinrev, 5′-AAGGAAG-GCTGAAAGATGTC-3′. The resulting PCR products were separated on a 1% agarose gel and imaged with a UV transilluminator.
Apoptosis assessment by Annexin V/propidium iodide staining and assessment of nonviable cells by propidium iodide staining

After drug treatments, cells were washed with PBS, re-suspended in 100 μL of Annexin V staining solution (containing Annexin V-FITC conjugate and propidium iodide in a HEPES buffer). Annexin V-FITC was obtained from BD PharMingen. Following incubation at room temperature for 15 minutes, cells were analyzed by flow cytometry using BD FACSCalibur (24, 27). Alternatively, following exposure to drugs, cells were washed free of drugs and stained with propidium iodide. The percentage of nonviable cells was determined by flow cytometry. Synergism, defined as a more than expected additive effect, was assessed using the median dose-effect of Chou-Talalay and the combination index (CI) for each drug combination was obtained using the commercially available software CalcuSyn (Biosoft). CI < 1, CI = 1, and CI > 1 represent synergism, additivity, and antagonism between two agents, respectively. CI values between 0.1 and 0.3 represents strong synergism, 0.3 to 0.7 represents synergism, and 0.7 to 0.9 represents moderate to slight synergism. Fa or the fraction affected by the treatments is the percentage of apoptotic cells.

Immunofluorescent staining and confocal microscopy

K562 cells were exposed to 17-DMAG and fixed with 4% paraformaldehyde for 10 minutes. Following this, the slides were blocked with 3% bovine serum albumin for 30 minutes and incubated with anti-TrkA and anti-ubiquitin antibody (Enzo Lifesciences International, Inc.). After washing three times with PBS, the slides were incubated in anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 594 secondary antibodies (Molecular Probes, Invitrogen) for 1 hour at 1:3,000 dilution. After washing three times with PBS, the cells were counterstained using Vectashield mountant containing 4′,6-diamidino-2-phenylindole and imaged using Zeiss LSM510 confocal microscope (Carl Zeiss), as previously described (27).

Figure 1. Treatment with 17-DMAG depletes the levels and induces proteasomal degradation of TrkA. A, K562 cells and TF-1 cells were treated with the indicated concentrations of 17-DMAG for 8 h. Following this, total cell lysates were harvested and probed for TrkA and β-actin by Western blot analyses. B, 32D/wtTrkA and 32D/ΔTrkA cells were treated with the indicated concentrations of 17-DMAG for 8 or 24 h. Following this, total cell lysates were harvested and probed for TrkA and β-actin by Western blot analyses. C, K562 cells were treated with the indicated concentrations of 17-DMAG for 24 h. Following this, total RNA was extracted and reverse transcription-PCR analysis was done for TrkA. The levels of β-actin served as the loading control. D, K562 cells were treated with the indicated concentrations of 1.0 μmol/L of 17-DMAG and/or 100 nmol/L bortezomib for 4 h. Following this, total cell lysates were immunoblotted for determining TrkA, c-Raf, and β-actin levels. E, K562 cells were exposed to the indicated concentrations of 17-DMAG for 48 h and the percentage of apoptotic cells was determined by Annexin V/propidium iodide staining followed by flow cytometry.
Statistical analysis
Significant differences between the values obtained in a population of leukemia cells treated under different experimental conditions were determined using Student’s t test.

Results

17-DMAG depletes the protein levels and induces proteasomal degradation of TrkA in human leukemia cells
We first determined the effects of 17-DMAG on the levels of TrkA in cultured CML blast crisis K562 and acute myeloid leukemia TF-1 cells. Figure 1A shows that treatment with 17-DMAG dose-dependently decreased the levels of unglycosylated and glycosylated forms of TrkA (11). We next determined the effects of exposure to 17-DMAG for 8 or 24 hours on the myeloid progenitor cell line 32D overexpressing either wild-type (32D/wtTrkA) or mutant (32D/ΔTrkA) TrkA (Fig. 1B). Similar to K562, treatment with 17-DMAG dose-dependently depleted the levels of wild-type and mutant TrkA in 32D cells, although 17-DMAG was more potent and effective in depleting the mutant versus the wild-type TrkA (Fig. 1B). We next determined the effects of 17-DMAG on the mRNA levels of TrkA in K562 cells. Treatment of K562 cells with 17-DMAG did not alter the mRNA levels of TrkA, suggesting that

Figure 2. 17-DMAG inhibits the chaperone association of TrkA with hsp90 and promotes polyubiquitylation of TrkA. A, K562 cells were treated with 1.0 μmol/L of 17-DMAG for the indicated time intervals. Following this, total cell lysates were harvested and TrkA was immunoprecipitated. The immunoprecipitates were resolved by SDS-PAGE and immunoblotted for hsp70, hsp90, and TrkA. B, K562 cells were treated with 1.0 μmol/L of 17-DMAG for the indicated exposure intervals. Following this, TrkA immunoprecipitates were resolved by SDS-PAGE and immunoblotted with anti-cdc37 and anti-TrkA to assess binding of cdc37 to TrkA. C, K562 cells were treated with 1.0 μmol/L of 17-DMAG for the indicated time intervals. Following this, TrkA was immunoprecipitated from the total cell lysates and the immunoprecipitates were resolved by SDS-PAGE. Polyubiquitylation of TrkA was determined by immunoblot analysis using an anti-ubiquitin antibody. D, K562 cells were exposed to DMSO or 0.25 μmol/L of 17-DMAG for 24 h. Cells were then fixed, permeabilized, and immunostained for TrkA (green) and ubiquitin (red). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). The slides were imaged with a LSM 510 meta confocal microscope using a 63×/1.2W lens.
the effect of 17-DMAG in depleting TrkA was posttranscriptional (Fig. 1C). Consistent with the observation that inhibition of hsp90 directs the hsp90 client oncoproteins to proteasomal degradation (16, 17, 28), we also determined that cotreatment with the proteasome inhibitor bortezomib restored 17-DMAG–mediated depletion of TrkA and c-Raf levels in K562 cells (Fig. 1D). This suggested a chaperone association of TrkA with hsp90 in human leukemia cells that is disrupted by treatment with 17-DMAG. Finally, we show that treatment of K562 cells with 17-DMAG results in a dose-dependent increase in apoptosis, which likely ensues as a consequence of the abrogation of chaperone association of hsp90 with prosurvival signaling proteins including c-Raf and AKT (Fig. 1E).

**Figure 3.** Treatment with 17-DMAG and/or K252a attenuates NGF-mediated autophosphorylation of TrkA and downstream signaling in K562 cells and 32D/wtTrkA cells. A, K562 and 32D/wtTrkA cells were serum-starved for 2 h, then treated with 100 ng/mL of NGF and/or 1.0 μmol/L of 17-DMAG for 12 min. Following this, Western blot analysis of p-TrkA, p-AKT, and p-ERK1/2 was done on total cell lysates. The levels of β-actin served as the loading control. B, 32D cells expressing vector, wt/TrkA, or ΔTrkA were exposed to the indicated concentrations of 17-DMAG for 48 h and the percentage of apoptotic cells were assessed by Annexin V/propidium iodide staining, followed by flow cytometry. C, TF-1 and K562 cells were exposed to the indicated concentrations of K-252a for 48 h and the percentage of apoptotic cells were determined by Annexin V/propidium iodide staining followed by flow cytometry. D, K562 and 32D/wtTrkA cells were serum-starved for 2 h and treated with 100 ng/mL of NGF alone or cotreated with 100 ng/mL of NGF and 17-DMAG or 150 nmol/L of K-252a for 12 min. Following this, Western blot analysis of p-TrkA was done on total cell lysates. The levels of β-actin served as the loading control. E, K562 cells were exposed to 100 nmol/L of 17-DMAG and the indicated concentrations of K-252a for 48 h and the percentage of apoptotic cells was measured by Annexin V and propidium iodide staining followed by flow cytometry. Isobologram analysis was done to obtain CI values for each fraction using CalcuSyn software.

17-DMAG inhibits the chaperone association of TrkA with hsp90, promoting the polyubiquitylation of TrkA

Treatment using an hsp90 inhibitor is known to decrease the chaperone association of client proteins with hsp90, along with a simultaneous increase in binding to hsp70. As shown in Fig. 2A, treatment with 17-DMAG led to a time-dependent decrease in binding of TrkA with hsp90 and a reciprocal increase in the binding of TrkA to hsp70 (Fig. 2A). We next determined the effects of...
17-DMAG on the association of TrkA with hsp90 cochaperone cdc37, which is involved in the loading of kinase client proteins onto hsp90 (18, 19, 35). Figure 2B shows that, in K562 cells, following treatment with 17-DMAG for an interval as short as 1 hour, TrkA binding to cdc37 was reduced, with a further decline in binding of TrkA to cdc37 after 2 hours. Treatment with 17-DMAG also inhibited the association of hsp90 with the cochaperone p23 (data not shown). We next determined whether inhibition of chaperone association of hsp90 with TrkA would induce polyubiquitylation of TrkA. Treatment with 17-DMAG increased the intracellular levels of polyubiquitylated TrkA within 2 hours without a reduction in the total TrkA levels (Fig. 2C). The effects of 17-DMAG on the intracellular localization of TrkA was determined by immunofluorescence microscopy. In untreated K562 cells, TrkA was predominantly localized to the cell surface membrane (Fig. 2D, top row). In contrast, following treatment with 0.25 μmol/L of 17-DMAG, the cell surface expression of TrkA was decreased (Fig. 2D, bottom row). Taken together, these results indicate that 17-DMAG treatment inhibits the chaperone association of TrkA with hsp90, followed by polyubiquitylation, proteasomal degradation, and reduced membrane localization of TrkA.

**Treatment with 17-DMAG and/or K-252a attenuates the NGF-mediated autophosphorylation of TrkA and downstream signaling**

NGF is known to bind TrkA and induces downstream signaling involving autophosphorylation of TrkA (pY490), AKT, and ERK1/2 (2, 4, 5). To determine the effects of hsp90 inhibition on NGF-induced signaling, K562 cells were treated with NGF alone or with the combination of NGF and 17-DMAG. NGF treatment induced rapid autophosphorylation of TrkA and increased p-AKT and ERK1/2 in both K562 and 32D cells with endogenous and exogenous expression of TrkA, respectively (Fig. 3A). Cotreatment with 17-DMAG inhibited the NGF-mediated increase in p-TrkA, p-AKT, and p-ERK1/2 (Fig. 3A). The decline in p-TrkA and p-AKT levels was more pronounced than in p-ERK1/2 levels. Previous studies have shown that 32D cells expressing ΔTrkA survive and grow in interleukin 3–depleted culture conditions, as well as exhibiting increased levels of phosphorylation of Y490 on TrkA, p-ERK1/2, and p-AKT and induce AML in mice (11, 12). In the present study, we observed that treatment with 17-DMAG induced significantly more apoptosis of 32D cells expressing either wild-type TrkA or ΔTrkA than 32D cells transfected with vector alone (Fig. 3B).

We next determined the effects of 17-DMAG and/or TrkA-specific signaling inhibitor K-252a in human leukemia cells. As shown in Fig. 3C, treatment with K-252a induced a dose-dependent increase in the apoptosis of TF-1 compared to treatment with K562 cells. We then determined the effect of inhibiting TrkA signaling in K562 and 32D/wtTrkA cells. As previously reported, whereas exposure to K-252a inhibited NGF-induced p-TrkA levels (34, 35), cotreatment with 17-DMAG and K-252a produced a further decline in the NGF-induced phosphorylation of TrkA (Fig. 3D). A similar effect of 17-DMAG and K-252a cotreatment was also observed on p-AKT levels (data not shown).

**Table 1. Isobologram analysis to determine synergistic interaction between 17-DMAG and K-252a**

<table>
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<tr>
<th>17-DMAG (nmol/L)</th>
<th>K-252a (nmol/L)</th>
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<th>CI</th>
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<td>0.263</td>
<td>0.883</td>
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<tr>
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<td>0.615</td>
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<tr>
<td>100</td>
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<td>150</td>
<td>0.413</td>
<td>0.487</td>
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<tr>
<td>100</td>
<td>175</td>
<td>0.39</td>
<td>0.590</td>
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NOTE: CI < 1.0 corresponds to synergistic interactions. K562 cells were exposed to 100 nmol/L of 17-DMAG and varying concentrations of K-252a. The percentage of apoptotic cells resulting from the cotreatment (Fa) by the drug combination is presented.

**Figure 4.** Treatment with 17-DMAG results in the induction of apoptosis in K562 cells and K562/HS-5 cocultures. A, K562 cells were cultured in the presence or absence of HS-5 cells and exposed to indicated concentrations of 17-DMAG for 48 h. The percentage of apoptotic cells was assessed by Annexin V and propidium iodide staining, followed by flow cytometry. B, K562 cells were cultured in the presence or absence of HS-5 cells and treated with the indicated concentrations of 17-DMAG for 8 h. The resulting cell pellets were lysed and the expression of TrkA was assessed by immunoblotting. The levels of β-actin served as a loading control.
shown). Consistent with these observations, combined treatment with K-252a and 17-DMAG exerted a superior antiapoptotic effect against K562 cells (Fig. 3E; Table 1). Analysis of the dose-effect relationship for 17-DMAG (100 nmol/L) and K-252a (50–175 nmol/L) in K562 cells was done according to the median dose-effect method of Chou and Talalay. Following this, the CI values were calculated using the percentage of apoptotic cells (fraction affected or Fa) by the cotreatment of the two agents. As can be observed, the combined treatment of 17-DMAG and K-252a results in a synergistic increase in the fraction of apoptotic cells (Fa ranging from 0.2 to 0.4) with the CI values ranging from 0.8 to 0.4, respectively. These observations suggest that, as compared with each agent alone, cotreatment with K-252a and 1-DMAG more potently abrogates TrkA-mediated survival signaling and induces cell death of human leukemia cells.

**Activity of 17-DMAG is not affected by coculture with bone marrow stromal cells**

Coculture with the HS-5 bone marrow stromal cells and NGF produced by these cells has been shown to promote the survival of TrkA expressing leukemia cells (33, 37, 38). We next determined whether 17-DMAG would induce the apoptosis of leukemia cells cocultured with HS-5 cells. Our findings show that 17-DMAG treatment induced similar rates of apoptosis in K562 cells with or without coculture with HS-5 cells (Fig. 4A). Additionally, treatment with 17-DMAG attenuated the levels of TrkA to a similar extent in K562 cells with or without coculture with bone marrow stromal cells (Fig. 4B).

**Treatment with 17-DMAG attenuates the levels of TrkA and inhibits NGF-mediated differentiation of PC-12 cells**

PC-12 cells differentiate and form neurites following exposure to NGF and TrkA-induced signaling (39, 40). We next determined the effect of 17-DMAG on TrkA levels and NGF-mediated neurite formation and differentiation in PC12 cells. As shown in Fig. 5A, treatment with 17-DMAG dose-dependently decreased the levels of TrkA with a concomitant decline in c-Raf levels, a known hsp90 client protein. Additionally, treatment with 17-DMAG inhibited NGF-induced neurite formation and differentiation of PC-12 cells (Fig. 5B). Collectively, these data show that 17-DMAG abrogates NGF-induced, TrkA-mediated signaling for differentiation in cells derived from the neuroectoderm, in addition to inhibiting progrowth and prosurvival signaling in myeloid leukemia cells.

**17-DMAG attenuates TrkA levels and NGF-induced signaling in primary CML and AML cells**

We next determined the effects of 17-DMAG on the levels of TrkA and NGF-induced p-AKT and p-ERK1/2 levels in primary CML and AML cells. Peripheral blood mononuclear cells from three primary AML samples and four CML samples were treated with 17-DMAG for 24 hours. 17-DMAG treatment depleted TrkA levels to varying extents in primary CML and AML mononuclear cells (Fig. 6A and B). As was noted in the cultured leukemia cells, exposure to NGF rapidly increased the phosphorylation of TrkA, AKT, and ERK1/2 in the primary AML and CML cells. The effect on a representative sample of each primary cell type is shown in Fig. 6C. Cotreatment
with 17-DMAG attenuated NGF-induced levels of p-TrkA, p-AKT, and p-ERK1/2 (Fig. 6C). The inhibitory effect of 17-DMAG on NGF-induced p-TrkA levels was pronounced. Furthermore, cotreatment with K-252a and 17-DMAG resulted in synergistic loss of viability in the three primary AML samples, with the CIs ranging from 0.001 to 0.5 (Fig. 6D; Table 2), whereas the lethal effects of the combination were subadditive in the primary CML mononuclear cells (data not shown). This suggests that in primary CML cells, survival signaling is predominantly mediated by BCR-ABL and less by TrkA. The findings also indicate that targeting TrkA-mediated prosurvival signaling by 17-DMAG sensitizes primary AML cells to K-252a.

Discussion

Here, we report for the first time that the chaperone association of TrkA with hsp90 is inhibited by treatment with 17-DMAG. This leads to the depletion of TrkA and inhibition of downstream signaling through p-AKT and p-ERK1/2, resulting in apoptosis of myeloid leukemia cells with endogenous or ectopic expression of the unmutated TrkA or constitutively active ΔTrkA. These findings are consistent with a recent report demonstrating that TrkAI and its oncogenic alternative TrkAIII splice variant exhibit geldanamycin-sensitive interactions with hsp90 in human neuroblastoma cells (41). However, in our studies, we also show that the geldanamycin analogue 17-DMAG, which is clinically active against human AML (42), simultaneously reduced the binding of TrkA to hsp90 and cdc37. The latter is an hsp90 cochaperone associated with the loading of client protein kinases to the hsp90 chaperone complex (18, 19). Reduced binding of TrkA to hsp90 and cdc37 was associated with a concomitant increase in the binding of TrkA to hsp70, resulting in polyubiquitylation and proteosomal degradation of TrkA. Following NGF treatment, it has been shown that the monoubiquitylation of TrkA is
involved in endosomal sorting and trafficking (43). In contrast, polyubiquitlitation of TrkA leads to its degradation by the proteasome. Although following NGF treatment, lysosomes might also be involved in the degradation of polyubiquiltated TrkA (40, 43), our studies show that 17-DMAG treatment-mediated degradation of TrkA is primarily through the proteasome. This is supported by the observation that cotreatment with 17-DMAG and bortezomib causes the accumulation of TrkA in the detergent-insoluble fraction (Fig. 1D; data not shown). Collectively, these observations indicate that TrkA is a bona fide hsp90 client protein and is degraded by the proteasome following inhibition of hsp90 function with 17-DMAG.

The role of neurotrophins and their receptors in promoting the growth and survival of tumors of neuronal and nonneuronal origin is well-established (3, 4, 11–14). For example, the Trk family of receptors is expressed not only in neuroblastoma, but also in the solid tumors, lymphoma, and leukemia (4–10). In neuroblastoma, TrkB/brain-derived neurotrophic factor expression has been correlated with resistance to DNA-damaging agents by activating the prosurvival phosphoinositide-3-kinase/AKT pathway (44). TrkA expression has also been implicated in leukemogenesis, thereby highlighting the need for targeting TrkA for the therapy of myeloid leukemia (3, 4, 11–14). Here, we show that 17-DMAG treatment inhibited activated TrkA and its downstream signaling through p-AKT and p-ERK1/2, resulting in the apoptosis of cultured and primary human AML and CML cells. In primary and cultured myeloid leukemia cells, 17-DMAG also inhibited NGF-induced p-TrkA and downstream p-AKT and p-ERK1/2 levels. Similar effects were also observed from 17-DMAG in mouse myeloid 32D cells overexpressing wild-type TrkA or the mutant ΔTrkA. 17-DMAG treatment caused more depletion of ΔTrkA compared with wtTrkA, associated with more apoptosis of 32D-ΔTrkA versus 32D-wtTrkA cells. This is consistent with the observations that, for maintaining their active conformation, the mutant forms of some of the oncoprotein kinases, e.g., BCR-ABL and FLT-3, were more dependent on their chaperone association with hsp90, and hence, more susceptible to depletion following treatment with an hsp90 inhibitor (20, 22, 45). In addition, 17-DMAG was effective in inducing the apoptosis of K562 cells with or without coculture with bone marrow stromal HS-5 cells. This is important because NGF produced by HS-5 cells is known to improve the survival of AML cells, as well as inhibit apoptosis induced by chemotherapeutic agents (33, 38). Coculture of non-Hodgkin’s lymphoma cells with HS-5 cells also resulted in the activation of the NF-κB pathway, thereby promoting the survival of lymphoma cells (32). Therefore, the ability of 17-DMAG to induce the apoptosis of myeloid leukemia cells regardless of coculture with HS-5 cells suggest that 17-DMAG treatment might override this resistance mechanism in human myeloid leukemia cells.

Following treatment with NGF, rat adrenal pheochromocytoma PC-12 cells produced neurite projections as phenotypic markers of differentiation (2, 39). Treatment with the TrkA-specific inhibitor K-252a inhibits NGF-induced neurite extensions of PC-12 cells (34, 35). We observed that 17-DMAG treatment depleted TrkA and c-Raf, inhibited NGF-induced p-TrkA, p-AKT, and p-ERK1/2 levels, and also inhibited NGF-induced neurite formation and differentiation in PC-12 cells. Whether NGF and TrkA mechanistically regulate not only growth and survival but also the differentiation arrest of myeloid leukemia cells has not been elucidated, and was not the focus of the present study. Our findings also show that treatment with K-252a and 17-DMAG alone inhibited NGF-induced p-TrkA, p-AKT, and p-ERK1/2 levels in myeloid leukemia cells. Importantly, cotreatment with 17-DMAG and K-252a exerted synergistic lethal activity against cultured and primary myeloid leukemia cells. Although the precise mechanistic basis of this synergy is not clear, it might be due to a greater attenuation of p-TrkA and its downstream signaling, or due to attenuation mediated by 17-DMAG of the other collateral survival signaling proteins, e.g., NF-κB and Pim1 (46, 47). These findings suggest that combined treatment with an hsp90 inhibitor and a TrkA-specific inhibitor would be

## Table 2. Isobologram analysis for determining synergistic interaction between 17-DMAG (100 nmol/L) and K-252a (50–200 nmol/L) was done using CalcuSyn software

<table>
<thead>
<tr>
<th>17-DMAG (nmol/L)</th>
<th>K-252a (nmol/L)</th>
<th>Fa</th>
<th>CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML #1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>50</td>
<td>0.390</td>
<td>0.042</td>
</tr>
<tr>
<td>100</td>
<td>100</td>
<td>0.386</td>
<td>0.064</td>
</tr>
<tr>
<td>100</td>
<td>125</td>
<td>0.385</td>
<td>0.075</td>
</tr>
<tr>
<td>100</td>
<td>175</td>
<td>0.504</td>
<td>0.001</td>
</tr>
<tr>
<td>100</td>
<td>200</td>
<td>0.428</td>
<td>0.009</td>
</tr>
<tr>
<td>AML #2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>50</td>
<td>0.494</td>
<td>0.415</td>
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<tr>
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<td>0.582</td>
<td>0.150</td>
</tr>
<tr>
<td>100</td>
<td>125</td>
<td>0.555</td>
<td>0.273</td>
</tr>
<tr>
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<td>150</td>
<td>0.614</td>
<td>0.143</td>
</tr>
<tr>
<td>100</td>
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<td>0.614</td>
<td>0.167</td>
</tr>
<tr>
<td>100</td>
<td>200</td>
<td>0.663</td>
<td>0.096</td>
</tr>
<tr>
<td>AML #3</td>
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<td></td>
<td></td>
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<tr>
<td>100</td>
<td>50</td>
<td>0.382</td>
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<tr>
<td>100</td>
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<td>0.517</td>
<td>0.664</td>
</tr>
</tbody>
</table>

**NOTE:** CI < 1.0 represents a synergistic interaction of the two drugs. The percentage of cells affected by the combination (Fa) of the two agents is presented.
a promising novel therapy for myeloid leukemia that shows oncogenic “addiction” to the activating mutation or overexpression of TrkA, an hsp90 client protein, as well as nononcogenic addiction to the heat shock response (48).

Disclosures of Potential Conflicts of Interest

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