# Inhibition of Neuroblastoma Xenograft Growth by Hsp90 Inhibitors

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**Abstract.** *Background: Advanced-stage neuroblastomas are often resistant to chemotherapy. Heat shock protein (Hsp) 90 is a molecular chaperone that maintains the stability of important signal transduction proteins. We have previously reported that geldanamycin (GA), an Hsp90 inhibitor, decreases Raf-1 and Akt protein expressions and induces apoptosis in neuroblastoma cells. We sought to determine the in vivo effects of Hsp90 inhibitor compounds on human neuroblastomas. Materials and Methods: Human neuroblastoma (LAN-1 and SK-N-SH) xenografts (4-mm$^3$ tumor implants) were placed in the flanks of athymic nude mice. The mice received either Hsp90 inhibitors (17-AAG or EC5) or vehicle (control). The tumor dimensions were measured twice weekly. Proteins were extracted for Western immunoblotting. Results: Hsp90 inhibitor compounds significantly blocked both LAN-1 and SK-N-SH neuroblastoma growth in vivo. Drug-treated tumors showed decreases in Raf-1 and cleaved PARP expressions. Conclusion: Hsp90 inhibitors may prove to be important novel therapeutic agents for patients with advanced-stage neuroblastoma who fail to respond to current treatment regimens.*

Neuroblastoma is the most common extracranial solid tumor of childhood, accounting for 15% of cancer-related deaths (1). Despite recent advances in multi-modality treatment protocols, the advanced-stage tumors remain aggressive and frequently resistant to chemotherapeutic regimens with an overall 5-year survival rate of only 30-40%. Altered cellular responses to apoptosis are thought to play an important role in drug therapy, in high-risk neuroblastomas (2). Therefore, novel drug therapy, targeting cellular signal transduction pathways regulating the apoptotic cascade, may be crucial in the treatment of drug-resistant neuroblastomas.

Heat shock protein (Hsp) 90 is a ubiquitously-expressed molecular chaperone that is required for the conformational maturation and stability of a range of client proteins. Hsp90 clients include key mediators of signaling cascades that regulate cellular proliferation as well as blocking apoptotic signaling (3). Inhibition of Hsp90 results in simultaneous disruption of several key signaling pathways, thus providing a potential target for new chemotherapeutic drugs. Geldanamycin (GA), a benzoquinone ansamycin antibiotic, is an Hsp90 inhibitor that binds to the N-terminal ATP-binding pocket of Hsp90; it inhibits ATP binding and ATP-dependent Hsp90 chaperone activity (4, 5) and directs the proteasomal degradation of Hsp90 clients (6). Recent studies have shown that Hsp90 is in an "activated" state in tumor cells which, therefore, exhibit high-affinity binding to Hsp90 inhibitor drugs. This property then allows for specific accumulation in tumors and not in normal tissues, where Hsp90 is in a "latent" or inactive state (7). Taken together, this suggests that Hsp90 represents a selective anticancer drug target and Hsp90 inhibition can lead to degradation of key oncogenic proteins.

We recently reported that treatment with GA increased apoptosis in neuroblastoma cells *in vitro* by inhibition of the Raf-1 and Akt pathways (8). However, the effects of Hsp90 inhibition on human neuroblastoma growth *in vivo* have not been described. A GA-derived Hsp90 inhibitor, 17-allylamino-demethoxygeldanamycin (17-AAG), which lacks the clinical toxicity of GA (9), has been shown to demonstrate potent antitumor activity in other preclinical models (10, 11), and is currently in phase II clinical trials. Therefore, the purpose of this study was to determine the effects of 17-AAG and another novel ansamycin Hsp90 inhibitor, EC5, on the growth of human neuroblastoma xenografts in athymic nude mice.

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## Materials and Methods

*Reagents and antibodies.* The ansamycin Hsp90 inhibitor drugs, 17-AAG and EC5, were synthesized by Conforma Therapeutics (San Diego, CA, USA) as previously described (12). Anti-Raf-1 and Hsp90 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-poly ADP-ribose polymerase (PARP) antibody was purchased from Cell Signaling (Beverly, MA, USA). Anti-β-actin was obtained from Sigma (St. Louis, MO, USA). All secondary antibodies against mouse, rabbit and goat IgG were purchased from Santa Cruz.

*In vivo experiments.* The human neuroblastoma cell line, SK-N-SH, was purchased from the American Type Culture Collection (Manassas, VA, USA), and LAN-1 was a gift from Dr. Robert C. Seeger (University of Southern California, Los Angeles, CA, USA). First, xenografts were established in athymic nude mice (Harlan Sprague Dawley, Indianapolis, IN, USA) by injecting SK-N-SH cells ($1x10^7$ cells per injection) into the subcutaneous flanks. Once the xenografts had been established, the tumor implants (4-$mm^3$) were then transferred into the bilateral flanks of male athymic nude mice. One week later, the mice were randomized into two experimental groups (three to five mice/group): group 1, (control) receiving vehicle solution alone; group 2, receiving intraperitoneal injections of 17-AAG (60 mg/kg/day) three consecutive days per week. The dosage of 17-AAG study was chosen based on previous studies (3, 12).

For the second set of experiments, the effects of 17-AAG on xenografts established from another neuroblastoma cell line, LAN-1, were assessed. Lastly, the apoptotic effects of 17-AAG and EC5 (40 mg/kg/day) on LAN-1 neuroblastomas were determined. For all the experiments, the drug treatments were delivered by daily intraperitoneal injections three consecutive days per week. Tumor growth was assessed biweekly by measuring the two greatest perpendicular tumor dimensions with vernier calipers (Mitutoyo, Tokyo, Japan). The mice were weighed weekly. The tumor volumes were calculated as follows: tumor volume ($mm^3$) = [tumor length (mm) x tumor width $(mm)^2$]/2. At sacrifice, the tumors were resected, weighed and snap-frozen in liquid nitrogen for storage at –70˚C.

*Western blot analysis.* The tumor sections were lysed with buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5 mM NP40, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 25 μg/ml each of aprotinin, leupeptin and pepstatin A on ice. The lysates were centrifuged at 15,000 xg for 30 min at 4˚C. After the protein concentrations had been determined, equal amounts of proteins (100 μg) were resolved on NuPAGE Novex 4-12% Bis-Tris Gel (Invitrogen, Carlsbad, CA, USA) and electrophoretically transferred to immunoblot polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA). The membranes were incubated overnight at 4˚C in a blocking solution (Tris-buffered saline containing 5% non-fat dried milk and 0.05% Tween 20), followed by a 3-h incubation with primary antibodies, washed three times in Tris-buffered saline containing 1% non-fat dried milk and 0.05% Tween 20 and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. After three additional washes, the immune complexes were visualized by the enhanced chemiluminescence (ECL) detection system (Amersham, Piscataway, NJ, USA). Densitometric analyses were performed using Kodak 1D Image Analysis Software Version 3.6.

*Statistical analysis.* The tumor size was analyzed using analysis of variance for a two-factor experiment with repeated measures on time. The two factors were Hsp90 inhibitors and day. The first-order autoregressive covariance was used for a covariance structure. All effects were assessed at the 0.05 level of significance and all interactions of the effects were assessed at the 0.15 level of significance as the experiment-wise error rates. Fisher's least significant difference procedure was used for multiple comparisons with 0.005 as the comparison-wise error rate. The average tumor weight of two tumors from each animal was analyzed using the Kruskal-Wallis test. Data analysis was conducted using PROC MIXED with LSMEANS option and Satterthwaite approximation for the denominator degrees of freedom in SAS®, Release8.2 [R1].

## Results

*17-AAG inhibits SK-N-SH neuroblastoma growth.* To examine whether Hsp90 inhibitors block neuroblastoma tumor growth *in vivo*, the effects of 17-AAG were first determined. The mean tumor volume in either treatment group was not different before drug treatment when compared to the control group (day 1; $p$ value $>0.755$). As shown in Figure 1A, 17-AAG significantly inhibited the growth of SK-N-SH neuroblastoma xenografts by treatment day 21 when compared to control mice receiving the vehicle alone. This result corroborates our previous report demonstrating inhibition of human neuroblastoma cell growth by GA *in vitro* (8).

Depletion of the Hsp90 client proteins Raf-1 and Akt, as a consequence of GA treatment, has been demonstrated in various cell lines such as MCF-7 breast cancer cells, CHP100 neuroepithelioma cells and NIH3T3 mouse fibroblast cells (13, 14). As shown in Figure 1B and 1C, 17-AAG treatment also reduced the levels of Raf-1 and Akt expressions in SK-N-SH xenografts.

*17-AAG inhibits LAN-1 neuroblastoma growth.* To ensure that the *in vivo* inhibitory effect of Hsp90 inhibitors was not unique to SK-N-SH neuroblastomas, which express low levels of N-myc, the effects of 17-AAG on another human neuroblastoma xenograft established from the LAN-1 cell line with high N-myc expression were next examined. Similar to the results noted with SK-N-SH xenografts in Figure 1, LAN-1 tumor growth was also markedly inhibited by treatment with 17-AAG when compared to the control group (Figure 2A). As shown in Figure 2B and 2C, LAN-1 neuroblastoma xenografts also showed reduction in Raf-1 and Akt expressions after 17-AAG treatment.

*EC5 and 17-AAG induce apoptosis in neuroblastoma xenografts.* Similar to the results noted with 17-AAG (Figure 1 and Figure 2), EC5 also significantly inhibited LAN-1 tumor growth when compared to the control group (Figure 3A). Consistent with their inhibitory effects on

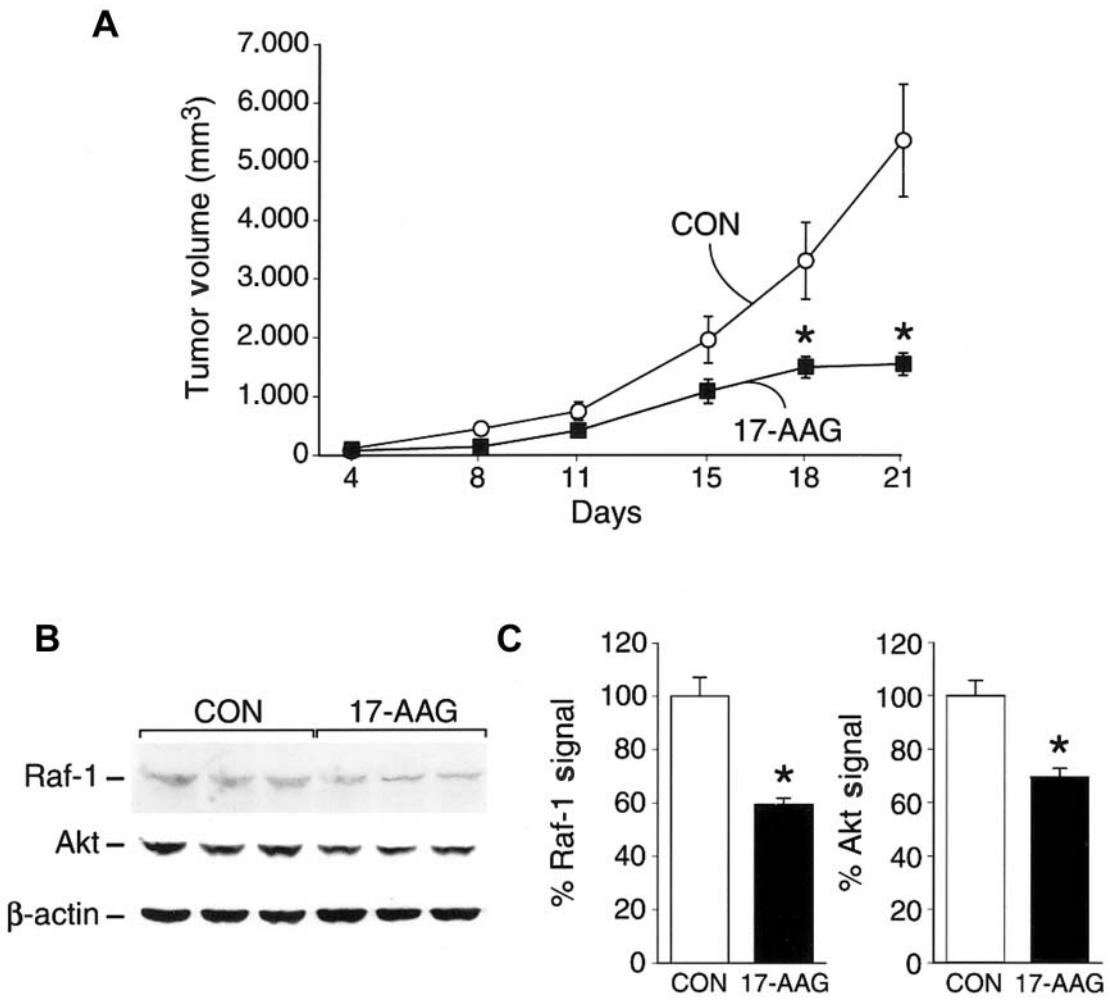


Figure 1. *(A) Tumor volumes in nude mice bearing SK-N-SH human neuroblastoma during treatment with 17-AAG. (*=p<0.05 vs. control). (B) Western immunoblots demonstrate depletion in Raf-1 and Akt expressions in 17-AAG-treated group. β-actin shows relative equal protein loading. Three representative tumors from each group are shown. (C) Percentage of protein measurement of Raf-1 and Akt are presented. The values were normalized with the expression of β-actin. (*=p<0.05 vs. control)*

tumor volume, tumor weight in both 17-AAG- and EC5-treated groups was also markedly less when compared to the control group at the time of sacrifice (Figure 3B). Interestingly, there was no significant difference between the two drugs used on neuroblastoma tumor growth inhibition. All mice tolerated intraperitoneal injections of either compound without obvious systemic toxicities; however, a slight weight loss (12%) was noted in mice receiving EC5 (data not shown).

To determine whether the antitumor effect of 17-AAG or EC5 was associated with apoptosis, the LAN-1 tumors were harvested at sacrifice and protein extracted to determine the cleavage products of PARP, a marker of cells undergoing apoptosis. Treatment with both 17-AAG and EC5 increased the protein expression of cleaved PARP, suggesting an induction of apoptosis in LAN-1 tumors (Figure 3C and 3D). Taken together, these results further confirm that treatment with Hsp90 inhibitor drugs reduced the growth rate of neuroblastomas, irrespective of the N-myc amplification status.

## Discussion

Little progress has been made in improving the overall prognosis of patients with advanced-stage neuroblastomas (15). In this report, the effects of the Hsp90 inhibitors 17-AAG, which is currently in phase II clinical trials (16), and EC5 on LAN-1 and SK-N-SH human neuroblastoma xenografts were examined. It was found that both Hsp90 inhibitors were effective in blocking neuroblastoma tumor

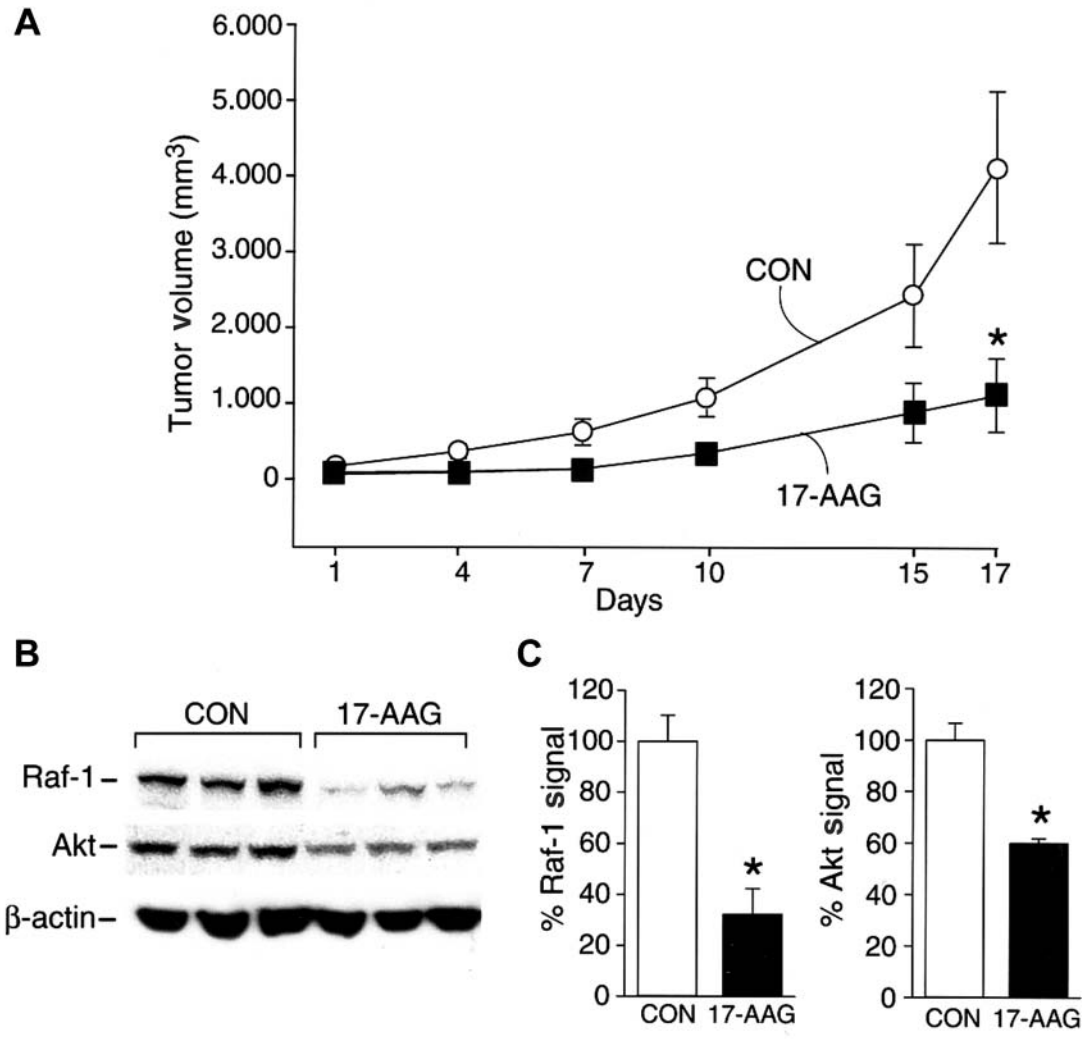


Figure 2. *(A) Treatment with 17-AAG resulted in inhibition of LAN-1 neuroblastoma growth (*=p<0.05 vs. control). (B) Western immunoblots showed reduction in Raf-1 and Akt protein levels in the 17-AAG-treated group. β-actin showed relative equal protein loading. (C) Percentages of Raf-1 and Akt protein measurements as in B (*=p<0.05 vs. control).*

growth *in vivo*. Furthermore, Hsp90 inhibitors induced apoptosis in neuroblastoma xenografts and decreased the expression of Raf-1 and Akt protein levels. Our results are consistent with the findings of other *in vivo* experiments, where 17-AAG resulted in the inhibition of prostate cancer xenografts (10), and the EC5 compound decreased the growth of head and neck squamous cell carcinoma xenografts (12).

Hsp90 is a critical component of the multi-chaperone complexes that regulate the activity of a wide range of signal transduction proteins within cells (17). Hsp90 clients include key components of the mitogenic signaling pathways (such as the Raf-1/MAPK pathway) that drive cell cycle progression, as well as survival signaling pathways (such as the PI3-K/Akt pathways) that inhibit apoptosis (3, 8). In addition, Hsp90 clients include proteins that contribute to important functions in growth signaling, evasion of apoptosis, sustained angiogenesis, tissue invasion and metastasis (18). Thus, the ability of Hsp90 inhibitors to simultaneously destabilize many oncoproteins in multiple signaling pathways and block key features of malignancy is likely to be a major factor in their effectiveness against cancer cells.

GA and 17-AAG have been shown to destabilize Hsp90-associated client proteins by increasing their degradation *via* the ubiquitin-proteasome pathway (19, 20). Recently, 17-AAG has completed phase I clinical trials as the first in-class Hsp90 inhibitor (18, 21). In an attempt to identify useful markers for monitoring the activity of Hsp90 inhibitors in pediatric cancer patients enrolled in clinical

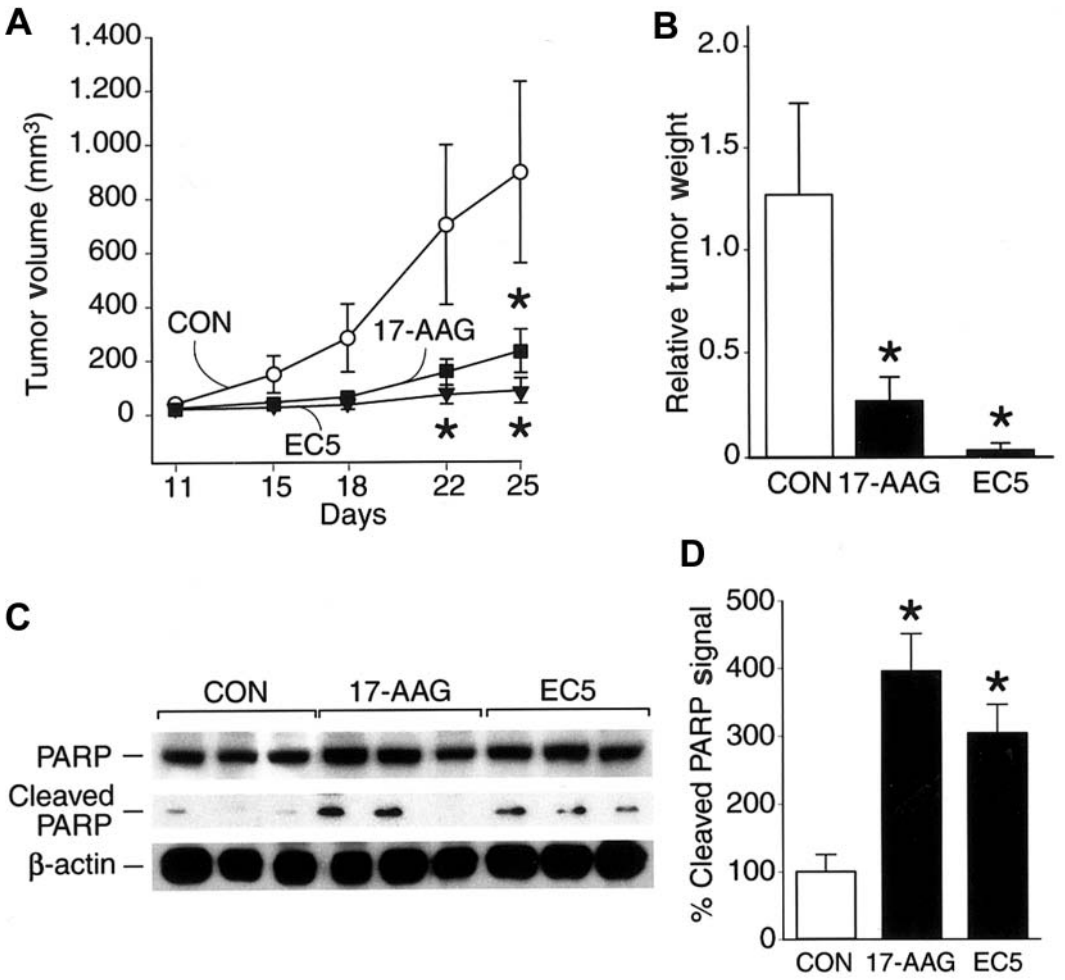


Figure 3. *(A) Growth inhibition of LAN-1 neuroblastoma xenografts with 17-AAG and EC5 treatment (*=p<0.05 vs. control). (B) Effect of treatment with 17-AAG and EC5 on tumor weight of LAN-1 human neuroblastoma xenografts. (*=p<0.05 vs. control). (C) Western blots showed induction in cleaved PARP expression with 17-AAG or EC5 treatment. β-actin showed relative equal protein loading. (D) Percentage of cleaved PARP protein measurements as in C (*=p<0.05 vs. control).*

trials, we evaluated the effect of Hsp90 inhibitors on two client proteins that are important in childhood solid tumors, Akt and Raf-1. Because of its role in anti-apoptotic signaling, Akt was selected as a potential marker of the activity of Hsp90 inhibitors. Akt functions as a critical protein in the activation of various survival pathways (22). Depletion of Raf-1 expression with GA treatment has been described in various cell lines, including the murine neuroblastoma N2A (23). Consistent with these reports, we also found that treatment with 17-AAG and EC5 led to reduction of Raf-1 and Akt protein expressions in human neuroblastoma xenografts in athymic nude mice. Moreover, increased cleavage products of PARP suggest that the tumor growth inhibitory actions of Hsp90 inhibitors may involve regulation of apoptotic pathways. Our future studies will involve further elucidation of this potential mechanism of action of Hsp90 inhibitors on neuroblastomas.

17-AAG has some limitations as a therapeutic agent due to its poor solubility (24); therefore, we also examined the effects of another analog, EC5, a novel ansamycin-based compound designed to stabilize the drug-target interaction for increased effectiveness (12) on neuroblastoma growth. In our study, both EC5 and 17-AAG significantly inhibited neuroblastoma growth. Additionally, the antitumor actions of these compounds were equally effective against high (LAN-1) as well as low (SK-N-SH) N-myc-expressing neuroblastomas. This finding is particularly important for potential clinical applications since aggressive tumor behavior refractory to current treatment modalities is associated with a spectrum of neuroblastoma N-myc amplification.

In conclusion, we found that 17-AAG and another ansamycin Hsp90 inhibitor, EC5, blocked human neuroblastoma growth *in vivo*. Our study suggests that targeting Hsp90, an important signaling protein stabilizer, could prove to be a novel therapy in advanced-stage neuroblastomas.

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