Induction of the Hypoxia-Inducible Factor System by Low Levels of Heat Shock Protein 90 Inhibitors

Nadia O. Ibrahim,¹ Torsten Hahn,¹ Corinna Franke,¹ Daniel P. Stiehl,² Renato Wirthner,² Roland H. Wenger,³ and Dörthe M. Katschinski¹

¹Cell Physiology Group, Medical Faculty, Martin Luther University Halle, Halle, Germany and ²Institute of Physiology, University of Zürich, and Center for Integrative Human Physiology, Zürich, Switzerland

Abstract
The heterodimeric hypoxia-inducible factor-1 (HIF-1) is involved in key steps of tumor progression and therapy resistance and thus represents an attractive antitumor target. Because heat shock protein 90 (HSP90) plays an important role in HIF-1α protein stabilization and because HSP90 inhibitors are currently being tested in clinical phase I trials for antineoplastic therapy, we investigated their role as anti-HIF-1α agents. Surprisingly, low-dose (5–30 nmol/L) treatment of HeLa cells with three different HSP90 inhibitors (17-AAG, 17-DMAG, and geldanamycin) increased HIF-1–dependent reporter gene activity, whereas higher doses (1–3 μmol/L) resulted in a reduction of hypoxia-induced HIF-1 activity. In line with these data, low-dose treatment with HSP90 inhibitors increased and high-dose treatment reduced hypoxic HIF-1α protein levels, respectively. HIF-1α protein stabilized by HSP90 inhibitors localized to the nucleus. As a result of HSP90-modulated HIF-1 activity, the levels of the tumor-relevant HIF-1 downstream targets vascular endothelial growth factor (VEGF), prolyl-4-hydroxylase domain protein 3, and vascular endothelial growth factor were increased or decreased after low-dose or high-dose treatment, respectively. Bimodal effects of 17-AAG on vessel formation were also seen in the chick chorioallantoic membrane angiogenesis assay. In summary, these results suggest that dosage will be a critical factor in the treatment of tumor patients with HSP90 inhibitors. (Cancer Res 2005; 65(23): 11094-100)

Introduction
The hypoxia-inducible factor-1 (HIF-1) activates a number of oxygen-regulated genes critically involved in adaptation to hypoxia (1–3). Under normoxic conditions, the von Hippel-Lindau tumor suppressor protein (pVHL) targets the HIF-1α subunit for rapid ubiquitination and proteasomal degradation (4). Binding of the pVHL tumor suppressor protein requires modification of HIF-1α by a family of low-affinity oxygen-dependent prolyl-4-hydroxylase domain proteins (PHD; ref. 5–7). Three HIF-1α–modulating PHDs (i.e., PHD1, PHD2, and PHD3) have been described thus far. HIF-1 is constitutively up-regulated in many cancer types and plays a major role in tumor progression (8–10). HIF-1 elevates vascular endothelial growth factor (VEGF)–dependent tumor angiogenesis, regulates tumor acidosis by increasing carbonic anhydrase IX (CAIX; ref. 11), and mediates the increased glycolytic capacity of tumor cells, known as Warburg effect (12). Moreover, we and others have recently shown that HIF-1 confers resistance against chemotherapy and radiotherapy (13, 14). Because HIF-1 regulates key steps of tumor progression and therapy resistance, it represents an attractive antitumor target. Anti-HIF-1 effects have been described for a variety of agents, including several drugs already in clinical use, such as taxol, topotecan, and the heat shock protein 90 (HSP90)–inhibiting ansamycin derivatives (for review, see refs. 15, 16). The mechanism by which taxol and topotecan destabilize HIF-1α is currently unknown. With regard to ansamycin derivatives, we and others have previously shown that HSP90 is important for hypoxic stabilization of HIF-1α (17–19). In HSP90α-deficient cells, hypoxic stabilization of HIF-1α was significantly delayed compared with wild-type cells (20). A similar effect could be shown by inhibiting HSP90 with the ansamycin derivative geldanamycin, suggesting that HSP90 is required for the rapid hypoxic stabilization of HIF-1α, which otherwise might be degraded by unspecific pathways (20). To inhibit the HIF system, doses in the micromolar range have usually been used (17, 18, 21, 22). Besides HIF-1α, a variety of other classic HSP90 client proteins have been shown to be targeted by ansamycin derivatives, including v-src, FAK, ErbB2, and Akt kinase (23). Because the clinical application of geldanamycin is restricted due to severe side effects, modified ansamycin derivatives like 17-allylamino-17-demethoxygeldanamycin (17-AAG) or the water-soluble 17-demethyl-17-demethoxygeldanamycin (17-DMAG) have been developed (24). The safety of 17-AAG application in humans was shown in three recently published clinical phase I trials (25–27).

Because it is difficult to control the local dose of an i.v. or orally applied drug within the tumor tissue, we have determined the effect of geldanamycin, 17-AAG, and 17-DMAG on the HIF system over a wide dose range. Surprisingly, an increase in HIF-1α protein levels as well as HIF target gene expression was found for all three compounds following application in the low nanomolar range, whereas higher doses efficiently down-regulated the HIF system. These results should be considered when HSP90 inhibitors are to be tested for antitumor therapy.

Materials and Methods

Chemicals. Geldanamycin, 17-DMAG, and 17-AAG were a kind gift of Kosan Biosciences, Inc. (Hayward, CA). All three drugs were dissolved in DMSO to generate 2 mmol/L stock solutions. All further dilutions were prepared in cell culture medium. All other chemicals were obtained from Sigma (Taufkirchen, Germany) or Roth (Karlsruhe, Germany).

Cell lines and cell culture. All cell lines were cultured in DMEM (high glucose) as described previously. Oxygen partial pressures in the hypoxic incubator (Binder, Tuttinglen, Germany) were either 140 mm Hg (20% O2, v/v, normoxia) or 7 mm Hg (1% O2, v/v, hypoxia).

Transient transfections. HeLa and Hep3B cells were transiently transfected with the HIF-dependent firefly luciferase reporter gene

Requests for reprints: Dörthe M. Katschinski, Cell Physiology, Martin Luther University Halle, Magdeburger Str. 2, D-06097 Halle, Germany. Phone: 49-345-557-1374; Fax: 49-345-557-1378; E-mail: dorthe.katschinski@medizin.uni-halle.de.

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construct pEVSVL, containing a total of six HIF-1 DNA-binding sites derived from the *transferrin* gene by the calcium phosphate coprecipitation method as described previously (28). HeLa or Hep3B cells were seeded in 24-well plates at a concentration of 5 × 10^4 per well. One day after seeding, cells were cotransfected with 0.25 µg pEVSVL together with 0.04 µg Renilla luciferase control plasmid pHV-SV40 (Promega, Mannheim, Germany). Cells were treated with HS90 inhibitors dissolved in DMSO or DMSO alone and subsequently exposed to normoxic or hypoxic conditions for 24 hours. Luciferase activities were determined using the dual-luciferase assay kit (Promega). Results were normalized to the solvent-treated normoxic control values, which were arbitrarily defined as 1.

**Protein extraction and immunoblot analyses.** Combined cytoplasmic and nuclear extracts of cultured cells were prepared using 0.4 mol/L NaCl, 10 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA, and 0.1% NP40. Protein concentrations were determined by the Bradford method using bovine serum albumin (BSA) as a standard. For immunoblot analysis, 50 µg cellular protein was electrophoresed through 7.5% SDS-polyacrylamide gels and electrotransferred onto nitrocellulose membranes (Amersham, Freiburg, Germany) by semidyblotting (Bio-Rad, München, Germany). Membranes were stained with Ponceau S (Sigma) to confirm equal protein loading and transfer. HIF-1α, HSP70, HSP90, Akt, HSF-1, and β-actin were detected using the following antibodies: mouse monoclonal anti-HIF-1α IgG1 (Transduction Laboratories, Heidelberg, Germany), mouse monoclonal anti-HSP70 IgG1 (StressGen, Victoria, Canada), rat monoclonal anti-HSP90 IgG2a rabbit (StressGen), rabbit polyclonal anti-Akt (Cell Signalling Technology, Beverly, MA), polyclonal anti-HSF-1 (Santa Cruz Biotechnology, Santa Cruz, CA), and mouse monoclonal anti-β-actin IgG1 (Sigma) followed by the appropriate secondary horseradish peroxidase–conjugated polyclonal antibodies raised in goats (Santa Cruz Biotechnology). Chemiluminescence detection of horseradish peroxidase was done by incubation of the membranes with 100 mmol/L Tris-HCl (pH 8.5), 2.65 mmol/L H₂O₂, 0.45 mmol/L luminol, and 0.625 mmol/L coumaric acid (all purchased from Sigma) for 1 minute followed by exposure to X-ray films (Amersham).

**Hypoxia-inducible factor-1α immunofluorescence.** Cells were fixed with methanol for 5 minutes. The nonspecific binding sites were blocked with 3% BSA in PBS for 30 minutes. The cells were incubated for 1 hour with a mouse monoclonal anti-HIF-1α IgG1 antibody (Transduction Laboratories) diluted 1:100 in PBS followed by a TRITC-coupled secondary anti-mouse (DAKO, Glostrup, Denmark) antibody diluted 1:200 with 3% BSA in PBS. Subsequently, all cells were stained with Hoechst 33258 dye for 5 minutes. After extensive washings with PBS, the slides were mounted and analyzed by fluorescence microscopy (Axioplan 2000, Carl Zeiss Vision, Mannheim, Germany).

**RNA extraction and real-time reverse transcription-PCR.** Hep3B cultures were grown on six-well plates for 24 hours, and total RNA was extracted. First-strand cDNA synthesis was done with 1 µg of RNA using the first-strand synthesis kit from Fermentas (St. Leon-Rot, Germany). Subsequently, mRNA expression levels for CAIX, PHD1, PHD2, and PHD3 were quantified with 1 µL of cDNA reaction by real-time reverse transcription-PCR using a SybrGreen Q-PCR reagent kit (Sigma) in combination with the MX3000P light cycler (Stratagene, Amsterdam, The Netherlands). Initial template concentrations of each sample were calculated by comparison with serial dilutions of a calibrated standard. To verify RNA integrity and equal input levels, ribosomal protein L28 mRNA was determined and data expressed as ratios relative to L28 levels. Primers were as follows: human CAIX (hCAIX) forward, 5'-GGGTTGATCTCTG-GAICTGTGT-3'; hCAIX reverse, 5'-CTTCCTGCTCCTCTCCTAC-3'; PHD2 forward, 5'-GAAGACATGTGCTGTGT-3'; PHD2 reverse, 5'-TTGCT-CTTCGGAAAAATTTCG-3'; PHD3 forward, 5'-aTGAGAAGGCT-GTGTCTTCA-3'; PHD3 reverse, 5'-CTGGATTCACATCTTCTGT-3'; human L28 (hL28) forward, 5'-GCATCTGCAAOTGATGTG-3'; hL28 reverse, 5'-TTGCCTGGACTGATG-3'; chick embryo choroidal membrane assay. Choroidal membrane assays were done as described before (29). In brief, fertilized eggs (purchased from Lohmann Tierzucht, Cuxhaven, Germany) were incubated at 37°C in a humidified atmosphere. The eggs were kept on their sides and turned upside down twice a day. After 3 days, a small hole was drilled through the shell into the air sac (visualized using a strong light source), 2 ml of egg white were aspirated, and a window of ~0.5 cm² was sawed into the side of the egg. The window was sealed with tape, and the eggs were reincubated until day 10, when 17-AAG was applied to the choroidal membrane using the Elvax method. Therefore, ethylene/vinyl acetate copolymer beads (elvax 40L-03, DuPont, Wilmington, DE; kindly provided by C.H. Erbahlé KG, Krefeld, Germany) were extensively washed in ethanol and dried under vacuum. The Elvax beads were then dissolved in methylene chloride at 10% (w/v), and 17-AAG was added to the desired concentration. One drop (40 µL) of this solution was pipetted onto a siliconized glass slide, and the solvent was allowed to evaporate completely. Using forceps, the Elvax disc was carefully lifted from the glass slide and placed onto the choroidal membrane. At day 13, the window was enlarged and the choroidal membrane was documented using a digital camera (Olympus, Hamburg, Germany) coupled to an ocular of a stereomicroscope.

**Results and Discussion**

**Modulation of HIF-1 activity is highly dependent on HS90 inhibitor concentration.** HeLa cells were treated with 17-AAG, 17-DMAG, or geldanamycin at concentrations ranging from 5 mmol/L to 3 µmol/L under normoxic or hypoxic conditions for 4 hours. We first showed the efficiency of the HS90 inhibitors in HeLa cells by determining the concentration of the heat shock transcription factor-1 (HSF-1). HSF-1 mediates the increased expression of HSPs following induction of a heat shock response with HS90 inhibitors as reported previously (30, 31). In nonstressed HeLa cells grown at 37°C, HSF-1 was detected as an ~85-kDa protein by immunoblotting (Fig. 1). After treating HeLa cells for 4 hours at 42°C, however, the HSF-1 appeared as an ~95-kDa protein. This shift in molecular mass most likely corresponds to the phosphorylation of HSF-1 and hence indicates the extent of its activation. A similar shift in molecular mass was observed after treating HeLa cells with increasing concentrations of 17-AAG (Fig. 1). Similar effects were seen after treatment with 17-DMAG and geldanamycin (data not shown), showing that the HS90 inhibitors were fully functional and confer a heat shock–like response.

To explore whether the HS90 inhibitors affect HIF-1 activity, transient transfections were done with a HIF-1–dependent reporter gene. After simultaneous treatment with hypoxia and increasing concentrations (5 mmol/L to 3 µmol/L) of 17-AAG, 17-DMAG, or geldanamycin, luciferase activity was measured as a marker for HIF-1 transcriptional activity (Fig. 2A-C). Hypoxia

![Figure 1](http://example.com/figure1.png)

**Figure 1.** 17-AAG activates a heat shock response in HeLa cells. HeLa cells were treated under normoxic (20% O₂) or hypoxic (1% O₂) conditions with increasing concentrations of 17-AAG as indicated. After 4 hours, the cells were lysed, and HSF-1 activation by phosphorylation was detected by immunoblotting as described in Materials and Methods. Protein extracts derived from HeLa cells incubated at 37°C or 42°C for 4 hours were used as negative and positive controls, respectively, for HSF-1 activation. As a control for equal loading and blotting, β-actin was subsequently determined on the same blot.
Hep3B cells as described above. 

Columns, or geldanamycin. 

A

B

C

D

Figure 2. HSP90 inhibitors affect HIF-1 activity. A-C, HeLa cells were transiently transfected with a HIF-1–dependent reporter gene plasmid as described in Materials and Methods. Subsequently, the cells were treated with the indicated concentrations of 17-AAG (A), 17-DMAG (B), or geldanamycin (GA, C) and exposed to 20% O2 or 1% O2. After 24 hours, the cells were lysed and luciferase activity was measured. Induction factors were normalized to nontreated cells cultured under normoxic conditions. Columns, means of n = 3 independent experiments; bars, ± SE. D, reporter gene assays were done in Hep3B cells as described above. x-axis, concentrations of 17-AAG, 17-DMAG, or geldanamycin. Columns, means of n = 6 independent experiments; bars, ±SE.

alone resulted in a roughly 50-fold induction of HIF-1 activity compared with normoxia. Surprisingly, treatment of HeLa cells with 5 or 30 nmol/L of 17-AAG or geldanamycin resulted in a significant increase of hypoxia-mediated HIF activity. A similar effect was observed after treating the cells with 5 nmol/L 17-DMAG. However, treatment with higher concentrations (i.e., 1-3 μmol/L 17-AAG, 17-DMAG, or geldanamycin), significantly decreased reporter gene activity. To rule out a cell line–specific effect, Hep3B cells were treated with 17-AAG, 17-DMAG, or geldanamycin (Fig. 2D). Like in HeLa cells, low-dose treatment with 5 nmol/L of all three drugs resulted in an increased activity of HIF-1, whereas the activity was decreased after treatment with 3 μmol/L.

Bimodal effect of HSP90 inhibitors on hypoxia-inducible factor-1α protein levels. Exposure of HeLa cells to 1% oxygen for 2 to 24 hours resulted in hypoxic stabilization of HIF-1α (Fig. 3). Hypoxic stabilization was strongest after 4 hours of incubation at 1% O2, whereas at later time points (i.e., 12 and 24 hours), the hypoxic stabilization was less pronounced. Treatment with 5 nmol/L or 3 μmol/L 17-AAG induced a heat shock–like response with increased expression of HSP70 and to a minor extent HSP90. This effect was more pronounced after treatment with 3 μmol/L compared with 5 nmol/L. In addition, the expression of HSPs increased with duration of 17-AAG treatment. In line with the decreased HIF-1 transcriptional activity and as described previously by us and others, treatment with high doses of 17-AAG (3 μmol/L) decreased hypoxic HIF-1α protein accumulation (17, 18, 21, 22). However, consistent with the increased HIF-1 transcriptional activity, treatment with low doses of 17-AAG (5 nmol/L) further increased the hypoxic stabilization of HIF-1α most prominently at early time points and to a lesser extent after 24 hours of incubation. This may be explained by the fact that 17-AAG is accumulating intracellularly with time (32). The affinity of the isolated HSP90 protein for ansamycins has been determined to be in the low micromolar range (33, 34). However, because ansamycins accumulate intracellularly, even nanomolar concentrations may be effective to reach this level inside the cells. In contrast to the treatment with 5 nmol/L 17-AAG, the effect with 3 μmol/L 17-AAG on HIF-1α accumulation as well as the full heat shock–like response were seen at later time points. Decreased levels after treatment with 3 μmol/L, but no increase with 5 nmol/L 17-AAG were seen for the HSP90 client protein Akt, showing that the dose-dependent modulation of HIF-1α is not a general phenomenon of all HSP90 client proteins. The phosphatidylinositol 3-kinase/Akt pathway is involved in HIF-1α stabilization (35). Because Akt was down-regulated after 3 μmol/L 17-AAG treatment, this effect may be involved in the down-regulation of HIF-1α after high-dose 17-AAG treatment and may explain partly the time-dependent and dose-dependent effects on the HIF system. Other signal transduction pathways involved in the stabilization or activation of HIF-1α especially in cancer have also been described to be affected by HSP90 inhibitor treatment. In this respect, it is interesting to note that erbB2 and the Raf/Ras/mitogen-activated protein kinase pathways, which are involved in HIF-1α stabilization and activation, have likewise been described to be affected by HSP90 inhibition (23, 36, 37).

Hypoxia-inducible factor-1α protein induced by heat shock protein 90 inhibitors localizes to the nucleus. We next determined whether HSP90 inhibitors affect the nuclear translocation of hypoxia-induced HIF-1α protein. Therefore, HeLa cells were treated with 1% O2 with or without addition of 5 nmol/L or
3 μmol/L 17-AAG for 4 hours. As shown by indirect immunofluorescence analysis, HIF-1α protein accumulated exclusively within the nucleus under hypoxic conditions. Addition of 5 nmol/L 17-AAG did not affect nuclear translocation of HIF-1α, whereas 3 μmol/L 17-AAG reduced HIF-1α protein levels (Fig. 4). Similar results were obtained by treating HeLa cells with geldanamycin or 17-DMAG (data not shown).

Heat shock protein 90 inhibitors modulate hypoxia-inducible factor–dependent target gene expression involved in hypoxia adaptation. Modulation of HIF-1α protein expression and HIF-1 transcriptional activity by HSP90 inhibitors suggest that these drugs may affect pathways involved in tumor progression. Therefore, we investigated the effects of 17-AAG on the expression of the HIF-1 target gene CAIX (38). CAIX has...
a major role in regulating proton flux, and blockade of CAIX results in increased cell death under severe hypoxia, indicating an important mechanism of hypoxic adaptation (39). Exposure of Hep3B cells for 24 hours at 1% O2 indeed resulted in a strong increase in CAIX mRNA levels compared with cells grown at 20% O2 (Fig. 5). Low-dose (5 nmol/L) 17-AAG enhanced CAIX mRNA expression at 20% O2 and even more pronounced at 1% O2 (P < 0.05), whereas treatment with 3 μmol/L 17-AAG resulted in a significant decrease of CAIX mRNA levels (P < 0.001).

PHD2 and PHD3, which regulate HIFα stability in an oxygen-dependent manner, are HIF target genes themselves (40, 41). A similar regulation as for CAIX by 5 nmol/L 17-AAG was seen for PHD3 mRNA but not PHD2 mRNA (Fig. 5). PHD1 mRNA was barely detectable in these cells (data not shown). Previously, it has been reported that PHD3 is strongly induced by hypoxia, which is mediated by HIF-1 (41). The hypoxic induction of PHD3 limits HIF-1α accumulation during long-time hypoxia and enables rapid destabilization following reoxygenation (42, 43). The increased expression of PHD3 by hypoxia and 5 nmol/L 17-AAG may explain the down-regulation of hypoxic stabilization of HIF-1α after 24 hours and the diminished effect of 5 nmol/L 17-AAG after 24 hours of treatment.

Effects of heat shock protein 90 inhibitors on angiogenesis. Besides metabolic tumor adaptation towards hypoxia, tumor angiogenesis is a major event during tumor progression. A major regulator of tumor angiogenesis is the HIF-1 downstream target VEGF. Therefore, we investigated whether HSP90 inhibitors also induce VEGF synthesis. Consistent with the

Figure 5. Effects of 17-AAG on HIF-1 target gene expression. Hep3B cells were exposed to normoxic (20% O2) or hypoxic (1% O2) conditions without or with the addition of 5 nmol/L or 3 μmol/L 17-AAG for 24 hours. The cells were lysed; total RNA was extracted and reverse transcribed; and the CAIX, PHD2, PHD3, and L28 mRNA levels were quantitated by real-time PCR. Columns, means of n = 3; bars, ± SE.
Figure 6. Effects of 17-AAG on VEGF protein secretion and angiogenesis in the chorioallantoic membrane. HeLa cells (A) or Hep3B cells (B) were exposed to normoxic (20% O₂) or hypoxic (1% O₂) conditions for 24 hours without or with the addition of 5 nmol/L or 3 μmol/L 17-AAG. Subsequently, the supernatants were collected and VEGF protein concentration was estimated by ELISA. Columns, means of n = 6 experiments (HeLa cells) or n = 3 experiments (Hep3B cells); bars, ±SE. C, inert polymer discs containing solvent only (control), 5 nmol/L 17-AAG, or 3 μmol/L 17-AAG were placed onto the chorioallantoic membrane at day 10 of embryonic development as described in Materials and Methods. Pictures were taken 3 days later.

reporter and target gene studies, treatment of HeLa or Hep3B cells with 5 nmol/L 17-AAG increased hypoxia-induced VEGF secretion, whereas treatment with 3 μmol/L significantly reduced VEGF production (Fig. 6A and B).

In two previous studies, the effects of geldanamycin, 17-AAG, or 17-DMAG on angiogenesis were investigated in Matrigel angiogenesis assays (44, 45). In both studies, HSP90 inhibitors decreased the motility response of endothelial cells to VEGF or fibroblast growth factor-2 in a dose-dependent manner. However, in a complex tumor model, the effects of HSP90 inhibitors not only on endothelial cells but also on tumor cells have to be taken into account. Our data suggest that HSP90 inhibitors may increase or decrease the HIF system, including the expression of VEGF in tumor cells, depending on the local concentrations. VEGF acts on endothelial cells as both chemotactic and mitogenic factor via cell-specific receptors. VEGF within a solid tumor is produced in part by endothelial cells, in part by tumor cells, in part by stroma cells, and in part by invading macrophages, mainly as a result of changes in oxygenation (46). To mimic the complexity of the in vivo situation, we thus used the chorioallantoic membrane assay to investigate the effect of HSP90 inhibitors on angiogenesis. Treating chorioallantoic membranes with 5 nmol/L and 3 μmol/L 17-AAG resulted in significant changes compared with the solvent-treated control chorioallantoic membranes. In line with HIF-1 transcriptional activity and VEGF production, 36% (5 of 14 total) of 5 nmol/L 17-AAG-treated eggs showed clear signs of proangiogenesis, and 50% (5 of 10 total) of 3 μmol/L 17-AAG-treated eggs showed signs of deteriorated and decreased vessel formation as illustrated in Fig. 6B. None of the eggs treated with 5 nmol/L or 3 μmol/L 17-AAG showed signs of antiangiogenesis or proangiogenesis, respectively.

Taken collectively, our data show bimodal effects of HSP90 inhibitors on the HIF system, which are highly dose dependent. HIF-1α-stabilizing effects have also been described for other anticancer/chemopreventive agents, like epicatechin gallate and cisplatin (47, 48). These data support the need for the development of more specific inhibitors of the HIF system, because the in vivo effects of pleiotropic HSP90 inhibitors are difficult to predict over a wide dose range.

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