

## HSP90 Inhibitor 17-AAG Selectively Eradicates Lymphoma Stem Cells

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### Abstract

Cancer stem cells (CSC; also called tumor-initiating cells) comprise tumor cell subpopulations that preserve the properties of quiescence, self-renewal, and differentiation of normal stem cells. In addition, CSCs are therapeutically important because of their key contributions toward drug resistance. The hypoxia-inducible transcription factor HIF1 $\alpha$  is critical for CSC maintenance in mouse lymphoma. In this study, we showed that low concentrations of the HSP90 inhibitor 17-AAG eliminate lymphoma CSCs *in vitro* and *in vivo* by disrupting the transcriptional function of HIF1 $\alpha$ , a client protein of HSP90. 17-AAG preferentially induced apoptosis and eliminated the colony formation capacity of mouse lymphoma CSCs and human acute myeloid leukemia (AML) CSCs. However, low concentrations of 17-AAG failed to eliminate highly proliferative lymphoma and AML cells (non-CSCs), in which the AKT-GSK3 signaling pathway is constitutively active. The heat shock transcription factor HSF1 is highly expressed in non-CSCs, but it was weakly expressed in lymphoma CSCs. However, siRNA-mediated attenuation of HSF1 abrogated the colony formation ability of both lymphoma and AML CSCs. This study supports the use of 17-AAG as a CSC targeting agent and, in addition, shows that HSF1 is an important target for elimination of both CSCs and non-CSCs in cancer.

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### Introduction

Molecular chaperone proteins function to ensure the proper conformation of client proteins when cells experience stress or damage (1). Heat shock protein 90 (HSP90) is the most studied and well-known molecular chaperone that facilitates the maturation and stable conformation of several client proteins, including transcription factors hypoxia-inducible factor 1 $\alpha$  (HIF1 $\alpha$ ) and p53, serine/threonine kinases (AKT, Raf-1, and Cdk4), receptor/non-receptor kinases (HER2, EGFR, Src family kinases), and steroid hormone receptors (androgen and estrogen; refs. 2–10). Because many of these client proteins significantly contribute to tumor growth and survival, abrogation of their function with a single inhibitor has been an attractive prospect, making HSP90 an attractive molecular target for drug discovery (11). One of the original and most studied

HSP90 inhibitors is a derivative of the geldanamycin antibiotic, 17-N-allylamino-17-demethoxy geldanamycin (17-AAG; ref. 12). Through reversible binding to the ATP pocket of HSP90, 17-AAG potently disrupts its function, and ultimately induces tumor cell death.

Tumor stem cells from both glioma and acute myeloid leukemia (AML) have been shown to rely on the activity of HIF1 $\alpha$  or 2 $\alpha$ , respectively, for their maintenance (13–15). In particular, human AML CSCs are a rare population of CD34<sup>+</sup> CD38<sup>-</sup> cells, and are thought to be responsible for the resistance of conventional therapies (13, 16, 17). Current efforts for targeting CSCs have focused on disruption of self-renewal (18). However, this approach may be hampered by the quiescent nature of CSCs. Therefore, disrupting genes that are required for both maintaining CSCs in a stem-like state as well as self-renewal might provide a more effective therapy. We have previously reported a strain of T-cell receptor transgenic mice (TGB), which spontaneously developed lymphoma with 100% incidence, due to an insertional mutation of the *Emp2a* gene (19). Using this mouse lymphoma model, we identified that a small subset of cells expressing both c-Kit and Sca1 are lymphoma CSCs, and represent HIF1 $\alpha$  is a target for lymphoma CSC therapy (14).

17-AAG has been shown to target both the bulk population of cells and CSCs present within glioma and AML (13–15, 20). In this regard, we hypothesized that 17-AAG may be more suitable for targeting both lymphoma CSCs and bulk lymphoma cells simultaneously through the disruption of multiple HSP90 client proteins. Unfortunately, acquired

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resistance to 17-AAG has been observed in both glioblastoma and melanoma through reduced expression of NAD(P)H/quinone oxidoreductase 1 (NQO1), an enzyme responsible for activation of 17-AAG. In addition, induction of stress-response proteins, such as HSP27, and HSP70 by 17-AAG plays a large role in resistant cancer cells (21, 22). This induction relies on the transcription factor heat shock factor 1 (HSF1), which regulates expression of HSP70, HSP27, and HSP90 (23–26). Thus, targeting HSF1 in order to abrogate 17-AAG-induced heat shock response has been proposed as an anticancer strategy (27). This concept is supported by reports showing that HSF1 is required for the initiation of both lymphoma and RAS oncogene-induced tumors (28, 29).

In our study, we showed that low concentrations of 17-AAG preferentially eliminate the CSCs of both lymphoma and AML, but leaves the differentiated cancer cells unaffected due to their high levels of HSF1 expression. Knock-down of HSF1 abrogates the colony formation ability of lymphoma and AML CSCs through disrupting HSP90 $\alpha$ -mediated HIF1 $\alpha$  stability. In addition, this provides a new anti-CSC strategy by targeting HSF1 to eliminate aggressive malignancies.

## Materials and Methods

### Mice, cells, and reagents

TGB mice in the B10.BR background were maintained by heterozygous breeding pairs. B10.BR mice were used as recipients in lymphoma cell inoculation, and severe combined immunodeficiency (SCID) mice were used for human AML maintenance. Both lines were purchased from the National Cancer Institute. All mice were kept and used for the tumor implantation and treatment according to the procedures approved by the Unit of Laboratory Animal Facility (ULAM) at University of Michigan.

TGB lymphoma cells were maintained in 1% methylcellulose medium (MethoCult GF, 03434, Stem Cell Technologies). For some *in vitro* experiments, TGB lymphoma cells were transferred from 1% methylcellulose culture to an expansion medium: RPMI1640 medium supplemented with 10% FBS, interleukin (IL)-3, IL-6, and stem cell factor cytokines (R&D Systems), 100  $\mu$ mol/L non-essential amino acids, 10 mmol/L HEPES, and 1 mmol/L sodium pyruvate. Human AML cells were derived from the enlarged spleen tissue of SCID mice injected with aggressive AML CSCs (AML-71) as described previously (14), and maintained in SCID mice via transplantation. Short-term expansion and colony formation assays of the AML CSCs were carried out similarly as for our lymphoma cells, except for a substitution of human cytokines for murine cytokines. Clinical subtypes and genetic characterization of AML cells by examining the appearance of the malignant cells and by using cytogenetics to characterize any underlying chromosomal abnormalities were authenticated by the University of Michigan Comprehensive Cancer Center.

17-AAG was purchased from LC Laboratories. The sources of rabbit polyclonal antibodies to the specific proteins are as follows: HSF1 (4356), HSP90 (4874), HSP70 (4874), phosphor-

S21/S9 GSK3 $\alpha$ / $\beta$  (9331), phosphor-S473AKT (9271), and AKT (9272) were purchased from Cell Signaling. Mouse antibodies to GSK3 $\alpha$ / $\beta$  (0011-A; Santa Cruz) and  $\beta$ -actin (AC-15; Sigma-Aldrich) were used in the studies.

### Isolation of lymphoma CSCs and Colony formation assay

The lymphoma CSCs (c-Kit<sup>+</sup>Sca1<sup>+</sup>) are a rare population in TGB lymphoma tissue and express the surface markers c-Kit and Sca1. The CSCs were sorted out from primary spleen lymphoma tissue of TGB mice by fluorescence-activated cell sorting (FACS) technology, or isolated by auto-MACS microbeads (Miltenyi Biotech) twice, after staining with antibodies to c-Kit and Sca1 (both from Becton Dickinson Biosciences). For the detection of some downstream proteins requiring larger amounts of cells, the c-Kit<sup>+</sup> fraction (c-Kit<sup>+</sup>Sca1<sup>+</sup> subset combined with c-Kit<sup>+</sup> Sca1<sup>-</sup> subset) were used. For colony formation assays, lymphoma cells were seeded in 1% methylcellulose medium containing these murine cytokines in a 12-well plate. After 6 to 8 days of culture, colonies were counted, pooled, and then replated for consecutive rounds. For tumor inoculation and viral infection, the cultured lymphoma cells were expanded in the RPMI1640 medium as described above, 24 hours before these experiments.

### Lentiviral infection and plasmids

The lentiviruses for short-hairpin RNA (shRNA) targeting HSF1 (Sh-HSF1) and scrambled control Sh-RNA were generated in HEK293FT cells in accordance with the lentiviral preparation protocol (Invitrogen). Lentiviruses were prepared in a 150 mm falcon culture dish, and 50 mL of media was collected and centrifuged at 550g for 10 minutes to remove cell debris. Viral particles obtained after centrifugation at 12,857g overnight were suspended with 2 mL of RPMI1640 medium containing 10% FBS, then aliquoted and stored at  $-80^{\circ}$ C. Between 50 and 100  $\mu$ L of concentrated virus per million cells was mixed with 100  $\mu$ L culture medium and used for spinoculation in a U-bottom tube in a mixture containing 9  $\mu$ g/mL polybrene. The spinoculation was carried out at 2,500 rpm, for 2 hours at  $30^{\circ}$ C. After spinoculation, the cell pellet was suspended gently and completely with fresh culture medium, seeded and cultured in 1% methylcellulose for another 5 to 7 days before use.

The sequence of Sh-HSF1 (5-GCTCATTGAGTTCCTGATC-3) has been previously validated (30), and was cloned into a Sh-lentiviral vector as described previously (14). The Sh-HSF1 sequence is shared by mouse and human. The constructs of hypoxia responsive element (HRE) or its mutant (HRE-Mt) EGFP reporter specific for HIF1 $\alpha$  were described previously (14). The full-length coding region of Hsf1 cDNA was amplified by real time-PCR using mouse bone marrow mRNA as template, and cloned into a pcDNA vector with Flag tag fused into the C-terminus of Hsf1.

### Western blot

In general, sorted CSCs or cultured lymphoma cells were lysed with 1% Triton X-100 lysis buffer containing 20 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 40 mmol/L NaF, 1 mmol/L dithiothreitol, protease, and phosphatase inhibitor cocktail

(Sigma). Supernatants of the lysate were used for Western blotting and resolved on a reducing and non-heated (95°C for 3 minutes) denaturing 10% SDS PAGE gel. Transfer membrane was blotted with primary antibody overnight at 4°C with shaking, and was linked with secondary antibody at room temperature for 5 hours.

#### MTT assay

The expanded lymphoma cells were seeded in the RPMI1640 culture medium at a cellular density of  $2.5 \times 10^5$  cells/mL in a 24-well plate. Next, the cells were treated with increasing concentrations of 17-AAG and cultured for 48 hours. At the end of the treatment, the MTT assay for cell viability determination was conducted according to the manufacturer's protocol (Promega) and the plate was read at 490 nm.

#### Real time-PCR

Total RNAs from FACS sorted lymphoma CSCs and non-CSCs (c-Kit<sup>-</sup>Sca1<sup>-</sup>) were extracted using the RNeasy extraction kit (Qiagen). After reverse transcription of RNA to cDNA, RT-PCR using the converted cDNA as template was carried out in triplicate using SYBR Green PCR Master Mix. Following primers were used: Hsf1, F-TTTGACCAGGGCCAGTTTG, R-TTCGGAAGCCATACATGTTGAG; Hif1 $\alpha$ , F-AGTCTAGAGATGCAGCAAGATCTC, R-TCATATCGAGGCTGTGTCGACTGA; Hsp90 $\alpha$ , F-TGTTGCGGTACTACACATCTGC, R-GTCCTTGGTCTCACCTGTGATA; Hsp90 $\beta$ , F-CAAACAAGGAGATTTTCCTCCG, R-GCTGTCCAACCTAGAAGGGTC; Glut1, F-TGTGCTGTGCTCATGACCATC, R-ACGAGGAGCACCGTGAAGAT;  $\beta$ -actin, F-GGCTGTATTCCCCTCCATCG, R-CCAGTTGGTAA-CAATGCCATGT. Fold differences was calculated using the delta Ct method (31) with  $\beta$ -actin as a reference gene.

#### Apoptosis assay

The cultured lymphoma and AML cells, after treatment with 17-AAG for 24 hours, were stained with surface markers of fluorescence-conjugated c-Kit and Sca1 (CD34 and CD38 for AML cells), and then Annexin-V for 15 minutes at room temperature before addition of 5  $\mu$ mol/L DAPI. The apoptosis percentage of these CSCs and differentiated cells were then analyzed by flow cytometry.

#### In vivo treatment of lymphoma with 17-AAG

B10.BR mice were inoculated with  $5 \times 10^5$  lymphoma cells through intraperitoneal injection. Seven days after tumor implantation, the mice were intraperitoneally (i.p.) injected with 17-AAG or vehicle [10% DMSO + 40% Cremophor EL: Ethanol (3:1; v/v) + 50% PBS] every other day for 3 weeks. At the cessation of treatment, mice were monitored up to 80 days after tumor cell injection. To determine the effects of 17-AAG on lymphoma initiation *in vivo*, secondary B10.BR recipient mice were implanted by intraperitoneal injection of  $1 \times 10^5$  lymphoma cells from the spleens of first round mice that had been treated with 17-AAG or vehicle. These mice were followed up until 160 days post tumor cell injection to monitor differences in tumor initiation between the mice.

## Results

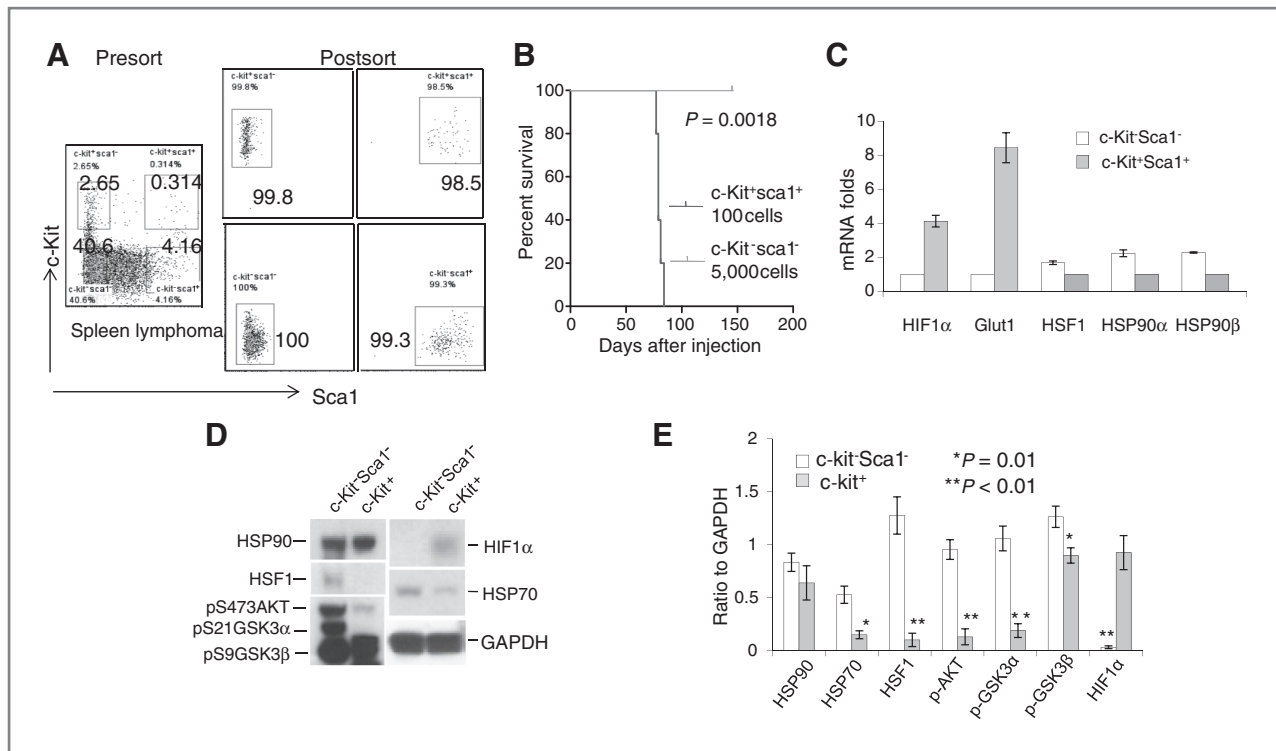
### Distinct features of lymphoma CSCs and bulk lymphoma cells (non-CSCs)

We have previously shown that AKT-GSK3- $\beta$ -catenin signaling is constitutively activated in the TGB lymphoma c-Kit<sup>-</sup>Sca1<sup>-</sup> cells (non-CSCs), and that HIF1 $\alpha$  plays a critical role in the maintenance of the lymphoma c-Kit<sup>+</sup>Sca1<sup>+</sup> cells (CSCs; refs. 14, 19). These lymphoma CSCs can efficiently initiate lymphoma when small numbers are injected into immunocompetent recipient mice. Following tumor initiation, the mice ultimately succumb to the committed, highly proliferative lymphoma cells, which rapidly grow into a tumor mass in the spleen and metastasize into other organs, such as the liver and lung, leading to death within 1 to 2 months post-inoculation (14, 19). These findings prompted us to devise a drug therapy that could effectively target both CSCs and non-CSCs, for the treatment of advanced lymphoma in humans. Because the CSCs and non-CSCs harbor constitutively active HIF1 $\alpha$  and AKT (2 client proteins of HSP90), respectively, we tested the HSP90 inhibitor 17-AAG for the simultaneous targeting of the 2 cell populations.

We first examined the expression levels of chaperones and the transcriptional factor HSF1 in the CSCs and non-CSCs. Using a FACSAria cell sorter (Becton Dickinson), 2 subsets of TGB lymphoma cells (c-Kit<sup>+</sup>Sca1<sup>+</sup>, c-Kit<sup>-</sup>Sca1<sup>-</sup>) were sorted from mouse spleen tissue enlarged with the TGB lymphoma cells (Fig. 1A). As previously described (14), the sorted c-Kit<sup>+</sup>Sca1<sup>+</sup> cells initiated lymphoma with 50-fold lesser number of cells than the sorted c-Kit<sup>-</sup>Sca1<sup>-</sup> subset (Fig. 1B). Quantitative RT-PCR (qRT-PCR) showed that the mRNA levels of HIF1 $\alpha$  and its target Glut1 were 4- and 8-fold higher, respectively, in the c-Kit<sup>+</sup>Sca1<sup>+</sup> cells than the c-Kit<sup>-</sup>Sca1<sup>-</sup> cells. This confirmed the hypothesis that the c-Kit<sup>+</sup>Sca1<sup>+</sup> subset still maintained the unique marker of active HIF1 $\alpha$ . The mRNA levels of chaperones HSP90 $\alpha$ , HSP90 $\beta$ , and HSP70, and HSF1 were approximately 1-fold higher in the c-Kit<sup>-</sup>Sca1<sup>-</sup> than in the c-Kit<sup>+</sup>Sca1<sup>+</sup> cells (Fig. 1C). Consistent with mRNA levels, protein levels of HSP70 were higher in the c-Kit<sup>-</sup>Sca1<sup>-</sup> cells. Strikingly, there were large differences in the protein levels of HSF1, HIF1 $\alpha$ , and the activated status of the AKT-GSK3 pathway between the 2 cell populations (Fig. 1D and E). HSF1 had more than 10-fold higher expression in the c-Kit<sup>-</sup>Sca1<sup>-</sup> population, compared with the c-Kit<sup>+</sup> population. Consistent with our previous results (14, 19), stable HIF1 $\alpha$  was exclusively expressed in the CSCs, while active AKT (pS473AKT) and GSK3 (pS21/9GSK3 $\alpha$ / $\beta$ ) were present in higher amounts in the non-CSCs than CSCs. These distinctive expression patterns for stable HIF1 $\alpha$  and the active AKT-GSK3 pathway supports our initial hypothesis for targeting CSCs and non-CSCs simultaneously with 17-AAG.

### 17-AAG selectively eliminates lymphoma CSCs over non-CSCs

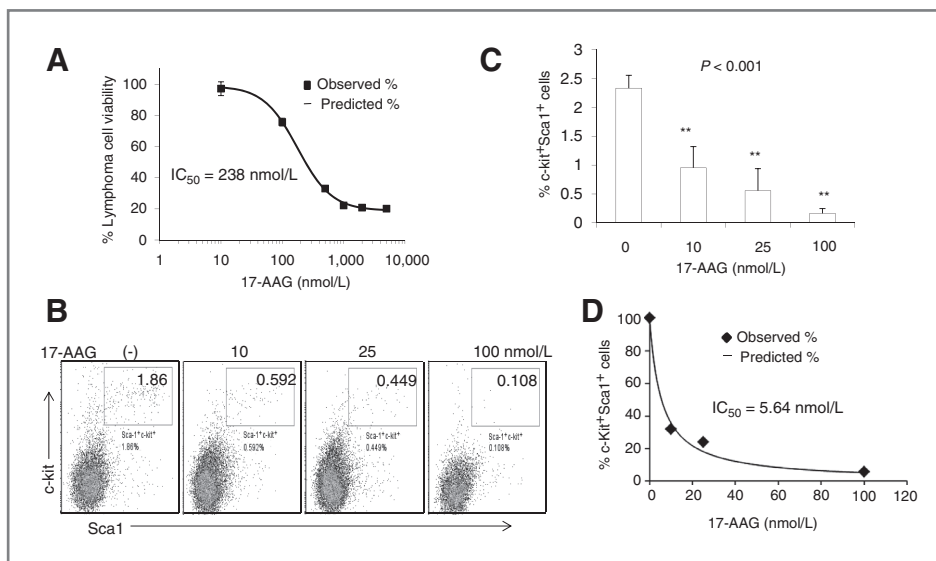
We next examined the ability of 17-AAG to suppress TGB lymphoma growth using the MTT cell viability assay. TGB lymphoma viability was inhibited with 17-AAG, with an IC<sub>50</sub>



**Figure 1.** The distinct features of lymphoma CSCs (c-Kit<sup>+</sup>Sca1<sup>+</sup>) and differentiated cells (c-Kit<sup>-</sup>Sca1<sup>-</sup>). A, lymphoma cells were sorted by FACSaria sorting system (Becton Dickinson). Lymphoma cells freshly isolated from splenic tissues of TGB lymphoma mice were stained with c-Kit and Sca1 and sorted into 4 subsets. B, sorted subsets of c-Kit<sup>+</sup>Sca1<sup>+</sup> and c-Kit<sup>-</sup>Sca1<sup>-</sup> were injected into B10-BR mice and lymphoma-initiating capacity of the 2 subsets was expressed on the basis of the mouse survival rate. C, quantitative RT-PCR showing the relative fold increase in mRNAs of indicated genes in the c-Kit<sup>-</sup>Sca1<sup>-</sup> and c-Kit<sup>+</sup>Sca1<sup>+</sup> subsets; the subset with lower mRNA levels is arbitrarily set to 1. D and E, the levels of indicated proteins in the subsets of c-Kit<sup>-</sup>Sca1<sup>-</sup> and c-Kit<sup>+</sup> (c-Kit<sup>+</sup>Sca1<sup>+</sup> combined with c-Kit<sup>-</sup>Sca1<sup>-</sup>). The relative ratios of bands to GAPDH in E were calculated by densitometry.

of 238 nmol/L (Fig. 2A). In contrast, c-Kit<sup>+</sup>Sca1<sup>+</sup> lymphoma cells treated with 17-AAG were significantly more sensitive to HSP90 inhibition, with an IC<sub>50</sub> of 5.64 nmol/L (Fig. 2B–D). These substantial differences in the IC<sub>50</sub> indicate a profound sensitivity to 17-AAG within the CSC population.

Previous studies have shown that treatment with 17-AAG results in programmed cell death via apoptosis (32). To determine whether 17-AAG induced apoptosis in the CSCs and bulk tumor cells, we stained lymphoma cells with Annexin V, a surface marker of apoptotic cells, after 17-AAG treatment. As



**Figure 2.** 17-AAG eliminates lymphoma CSCs. A, MTT assay determined the IC<sub>50</sub> of 17-AAG against lymphoma cells. Cultured lymphoma cells were treated with increasing concentrations of 17-AAG for 48 hours before MTT dye was added and the assay was conducted. B–D, 17-AAG selectively eliminated the c-Kit<sup>+</sup>Sca1<sup>+</sup> cells in a dose-dependent manner. Forty-eight hours after 17-AAG treatment, the lymphoma cells were stained with antibodies to c-Kit and Sca1 and analyzed by flow cytometry (B). The percentage of c-Kit<sup>+</sup>Sca1<sup>+</sup> cells from triplicate experiments was quantified by flow cytometry (C). The predicted IC<sub>50</sub> of 17-AAG to c-Kit<sup>+</sup>Sca1<sup>+</sup> cells are shown in D.

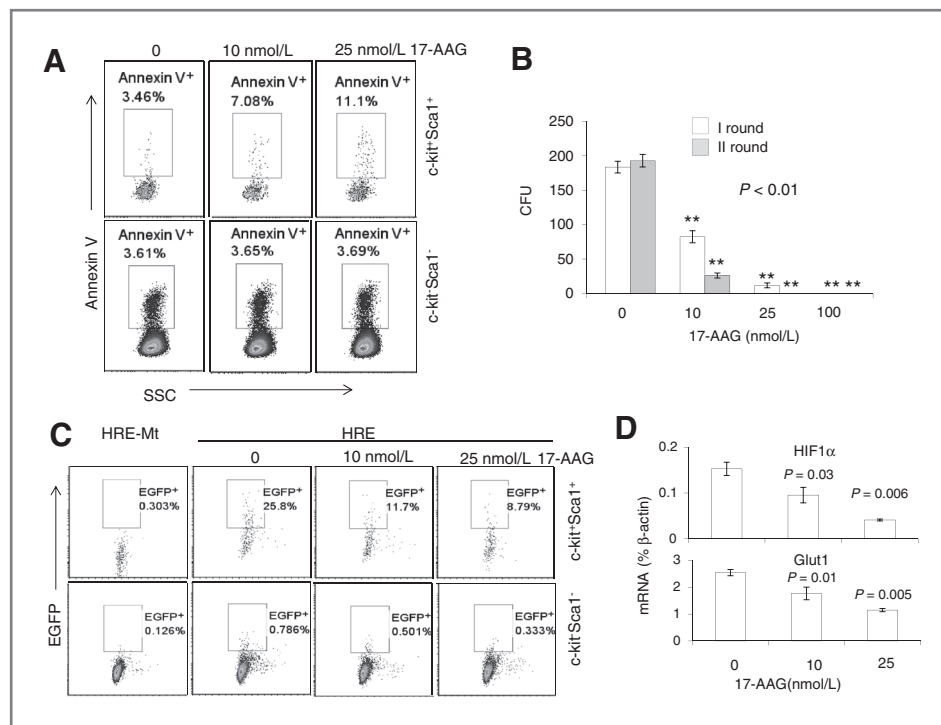


shown in Fig. 3A, low doses of 17-AAG (10 and 25 nmol/L) increased apoptosis of c-Kit<sup>+</sup>Sca1<sup>+</sup> cells by 2- to 3-fold, as compared with the untreated control. However, 17-AAG failed to induce apoptosis of c-Kit<sup>-</sup>Sca1<sup>-</sup> cells at these low concentrations.

As CSCs are best defined by their ability to self-renew and initiate tumor growth rather than by their surface marker expression, functional assays provide a more accurate assessment of drug-mediated therapeutic effects (33). The generation of spheroid colonies has been widely used as a manner to assay the presence of CSCs (34–36). Formation of these colonies is dependent on the residing stem cell population, and is reduced with each stage of differentiation. To assess whether 17-AAG affected CSC self-renewal, we plated lymphoma cells and treated them with increasing concentrations of 17-AAG. As a result, 10 nmol/L 17-AAG reduced the colony formation unit (CFU) of CSCs by more than 50% (Fig. 3B), consistent with the dose necessary for induction of apoptosis. These colonies were then replated in the absence of drug, to observe whether self-renewal was also disrupted. Cells from colonies previously treated with 10 nmol/L 17-AAG were dramatically reduced in their capacity to initiate

secondary colonies, indicating that 17-AAG eliminated the self-renewal ability of the CSCs *in vitro*.

In order to observe whether 17-AAG interfered with HIF1 $\alpha$ 's transcriptional activity, we constructed a lentiviral reporter system that used HIF1 $\alpha$  binding to specific hypoxia-inducible responsive elements (HRE) to control EGFP reporter expression (14). Lymphoma cells were infected with either this reporter, or with a reporter containing a mutant HRE (HRE-Mt) sequences (as a negative control for gating). Without treatment, 25.8% of HRE-EGFP positive cells were present in the c-Kit<sup>+</sup>Sca1<sup>+</sup> subset, whereas only 0.786% were in the c-Kit<sup>-</sup>Sca1<sup>-</sup> subset (Fig. 3C). This is consistent with the specific expression of stable HIF1 $\alpha$  in the CSCs. With treatment, 10 nmol/L 17-AAG reduced HRE-EGFP expression in the CSCs by 2-fold, but had little effect on the HRE-EGFP expression in non-CSCs, showing that 17-AAG specifically suppressed HIF1 $\alpha$  activity in the CSCs. In addition to the reduction of HIF1 $\alpha$  transcriptional activity induced by 17-AAG, the mRNA levels of HIF1 $\alpha$  and its target Glut1 were significantly decreased in a dose-dependent manner (Fig. 3D), indicating that HIF1 $\alpha$  and its target transcription are controlled by HIF1 $\alpha$  stability. These



**Figure 3.** 17-AAG selectively induces CSC apoptosis through inhibition of HIF1 $\alpha$  transcriptional activity. A, 17-AAG preferentially induced the apoptosis of c-Kit<sup>+</sup>Sca1<sup>+</sup> but not c-Kit<sup>-</sup>Sca1<sup>-</sup> cells. Twenty-four hours after 17-AAG treatment, cultured lymphoma cells were stained with an antibody cocktail of c-Kit, Sca1, and then Annexin V. Annexin V<sup>+</sup> cells on gated c-Kit<sup>+</sup>Sca1<sup>+</sup> or c-Kit<sup>-</sup>Sca1<sup>-</sup> subsets were analyzed by flow cytometry. B, 17-AAG dramatically reduced the CFUs of lymphoma CSCs. After treatment with increasing concentrations of 17-AAG, the lymphoma cells were seeded in 1% methylcellulose culture medium, the CFUs were counted, and the colony replating assay was conducted after a 7-day culture. Data shown are means  $\pm$  SD of colony numbers in triplicates and are representative of 3 independent experiments. C and D, 17-AAG suppressed HIF1 $\alpha$  transcriptional activity. The lymphoma cells were infected with a lentivirus containing HRE-EGFP or HRE-Mt (Mt, HIF1 binding site mutated) sequences. After a 7-day culture in 1% methylcellulose medium, the infected cells were treated with 17-AAG for 24 hours. The EGFP positive cells in the 2 subsets, relative to the negative control gate of HRE-Mt, were analyzed by flow cytometry. After 17-AAG treatment, the lymphoma cells were subjected to mRNA extraction and quantitative RT-PCR using specific primers for HIF1 $\alpha$  and Glut1 (D). The percentage of mRNA relative to  $\beta$ -actin is shown. All data presented in this figure have been repeated at least twice.

results show that 17-AAG specifically eliminates the CSCs through inhibition of the HSP90-mediated mRNA expression and transcriptional activity of HIF1 $\alpha$  at concentrations, which fail to eliminate the more differentiated c-Kit<sup>-</sup>Sca1<sup>-</sup> lymphoma cells.

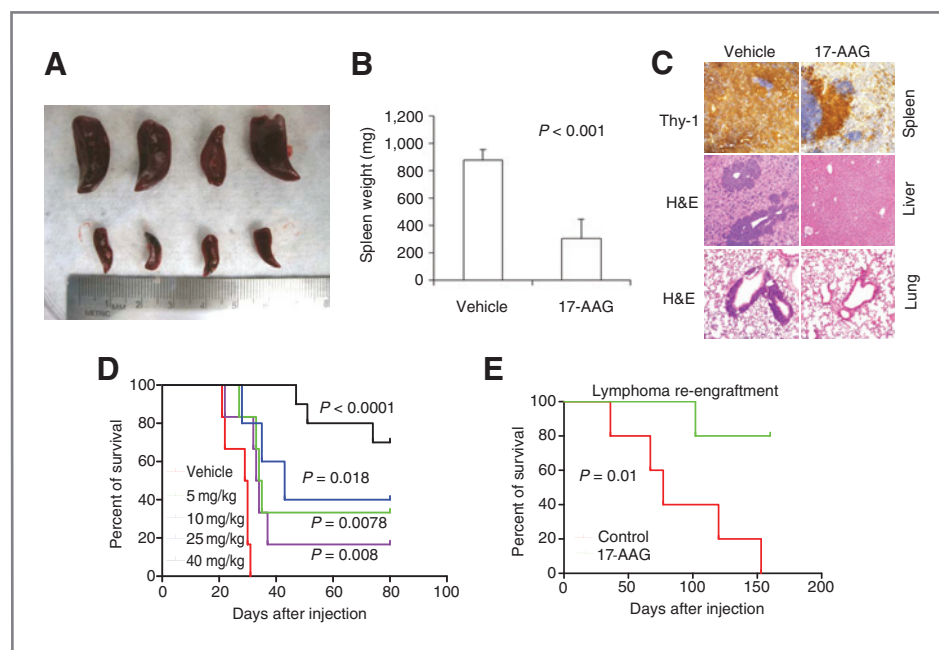
#### 17-AAG eradicates lymphoma initiation *in vivo*

To investigate whether HSP90 inhibition can reduce lymphoma initiation *in vivo*, we injected  $5 \times 10^5$  TGB lymphoma cells i.p. into recipient B10.BR mice. Seven days after injection, different doses of 17-AAG (5–40 mg/kg) or vehicle control were given to mice every other day for 3 weeks. Upon cessation of treatment, 4 mice from the 40 mg/kg 17-AAG or control group were euthanized and their spleens weighed for indication of lymphoma. Vehicle-treated mice exhibited enlarged spleens enriched in lymphoma cells that stained positive for Thy-1, a marker for T lymphoma cells (Fig. 4A–C). In addition, the lymphoma cells had metastasized into liver and lung tissue, as typically seen with this cancer (19). In contrast, spleens from mice that had received 17-AAG were dramatically smaller, with less infiltrating lymphoma cells in the spleen, and a lower metastatic spread into other organs, compared with the vehicle-treated control (Fig. 4C). In addition, 17-AAG-treated mice survived for significantly longer compared with mice that had received vehicle alone, showing the effectiveness of 17-AAG in suppressing the lymphoma *in vivo* (Fig. 4D).

A hallmark of CSC function is their ability to serially reestablish tumors in recipient mice. This CSC trait reflects their capacity for self-renewal *in vivo*, and provides a method for detecting chemical agents that can disrupt this process. To examine whether 17-AAG treatment impaired the self-renewal ability of TGB lymphoma CSCs *in vivo*,  $1 \times 10^5$  cells obtained from either 40 mg/kg 17-AAG- or vehicle-treated spleens were i.p. injected into 5 healthy secondary recipient B10.BR mice. All of the mice injected with vehicle-treated spleen cells eventually succumbed to lymphoma. In contrast, only 1 mouse that received 17-AAG-treated spleen cells died over the course of the study (Fig. 4E). These data show that 17-AAG effectively impairs the self-renewal of the lymphoma CSCs *in vivo*.

#### Knockdown of HSF1 abrogates the colony formation capacity of lymphoma CSCs

To understand the mechanism underlying 17-AAG resistance in the non-CSCs, we investigated the effect of silencing HSF1 on the growth of CSCs and non-CSCs, as HSF1 was more highly expressed in the differentiated population. When we treated the lymphoma cells with 50 nmol/L 17-AAG, a dose capable of killing nearly all of the CSCs, we found that the bulk differentiated cells were largely unaffected, as protein levels of pS473AKT, pS21GSK3 $\alpha$ , and pS9GSK3 $\beta$  remained unchanged (Fig. 5A). Coinciding with reports in the literature, we also observed an increase in HSP70 in reaction to HSP90 inhibition with 100 nmol/L 17-AAG. However, 100 nmol/L 17-AAG could



**Figure 4.** 17-AAG suppresses lymphoma growth in lymphoma-transplanted congenic recipient mice. A–D, B10.BR mice were inoculated with  $5 \times 10^5$  cultured lymphoma cells through intraperitoneal injection. Seven days after implantation, randomly grouped mice were treated with different doses of 17-AAG as indicated, every other day for 3 weeks. After the mice were monitored up to 80 days post tumor cell injection, 4 of the surviving mice from 40 mg/kg or vehicle control group were sacrificed to measure the spleen sizes (A) and weights (B); 2 mice from the 25 mg/kg or control group were subjected to identification of lymphoma by an antibody to the tumor T-cell marker Thy-1 and to metastasis examination by H&E staining (C). Dark purple cells are metastatic lymphoma cells. D, the survival rate was evaluated and data shown are representative of 2 independent experiments. E, 17-AAG reduced the self-renewal ability of lymphoma CSCs. B10.BR mice were implanted by intraperitoneal injection with  $1 \times 10^5$  splenic cells from mice previously treated with vehicle or 17-AAG, and monitored for 160 days. The survival rate was evaluated after 160 days post spleen cell inoculation.

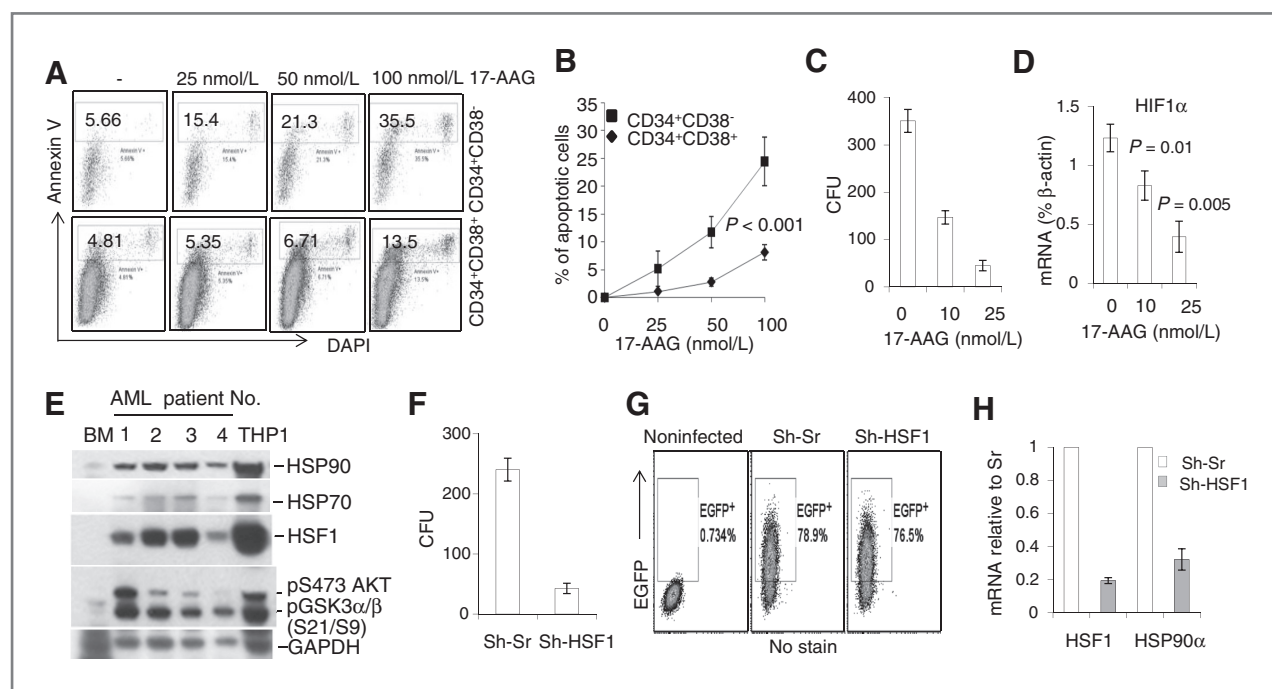


(14). To see whether 17-AAG also selectively targeted AML CSCs, AML cells were treated with increasing concentrations of 17-AAG. A dose of 25 nmol/L 17-AAG increased the percentage of apoptotic CSCs ( $CD34^+CD38^-$ ) nearly 3-fold, compared with vehicle-treated CSCs. In contrast, 25 nmol/L 17-AAG had little effect on the induction of apoptosis in the non-CSCs ( $CD34^+CD38^+$ ; Fig. 6A and B). Although a higher dose (100 nmol/L) of 17-AAG induced apoptosis in both CSCs and non-CSCs, CSCs initiated apoptosis to a higher extent (35.5%) than non-CSCs (13.5%; Fig. 6A and B). Similar to lymphoma CSCs, a dramatically low dose (10 nmol/L) of 17-AAG greatly reduced the capacity for AML CSCs to form colonies (Fig. 6C). This colony reduction could be attributable to the inhibition of HSP90-mediated HIF1 $\alpha$  stability by 17-AAG, as the mRNA levels of HIF1 $\alpha$  were correspondingly reduced by 17-AAG treatment (Fig. 6D). Compared with normal mouse bone marrow (BM) cells, the AML cells also highly expressed HSF1, phosphorylated AKT, and GSK3, in a similar manner as our TGB lymphoma cells, indicating that AML cells possibly respond to 17-AAG treatment via a similar mechanism as the lymphoma cells (Fig. 6E). Accordingly, knockdown of HSF1 by Sh-HSF1 silencer abrogated the colony formation capacity of the AML CSCs, as compared with the Sh-Sr (Fig. 6F). Finally, to

verify that the reduction in colony formation in AML CSCs after knockdown of HSF1 was through a reduction in HSP90, we sorted the lentivirus-infected AML cells by gating on EGFP-positive cells (Fig. 6G) and determined the mRNA levels of HSF1, as well as its target gene HSP90 $\alpha$ . Knockdown of HSF1 dramatically reduced the mRNA levels of HSP90 $\alpha$  (Fig. 6H), verifying the requirement for functional HSP90 $\alpha$  and HSF1 in the maintenance of the AML CSCs. Taken together, these results show that low doses of 17-AAG selectively eliminate AML CSCs, possibly through the loss of HSP90-mediated HIF1 $\alpha$  activity. In addition our results show that a functional HSF1 is required for AML CSC maintenance.

## Discussion

T-Cell lymphoma (represented by our TGB mouse model) and AML are fast-growing aggressive tumors that are difficult to treat, and have a high relapse rate for patients in remission. Therefore, discovery of new therapies capable of eliminating CSCs are needed to block tumor recurrence. In this study, both CSCs derived from lymphoma and AML were eliminated through 17-AAG mediated HSP90 inhibition. However, the bulk population of lymphoma cells, which



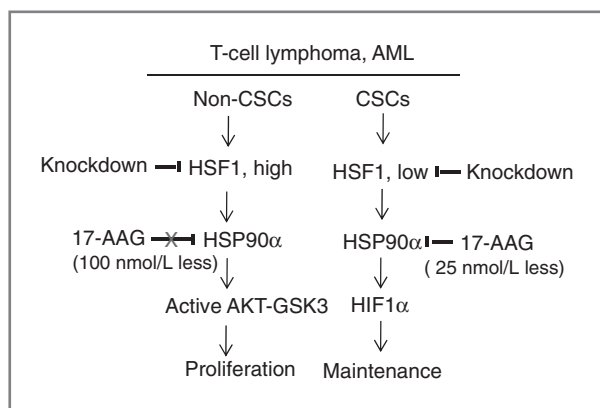
**Figure 6.** The effects of 17-AAG and HSF1 knockdown on AML CSCs. A–D, low doses of 17-AAG preferentially induced apoptosis and eliminated colony formation of AML CSCs ( $CD34^+CD38^-$ ). After 24-hour treatment with increasing concentrations of 17-AAG, AML-71 cells were stained first with antibodies for CD34, CD38, and then with antibody for Annexin V and dye DAPI. The percentage of Annexin V<sup>+</sup> cells in the gated  $CD34^+CD38^-$  and  $CD34^+CD38^+$  populations were analyzed by flow cytometry. The nuclear dye DAPI stained dead cells. The apoptotic cells in 17-AAG-treated or untreated cells from triplicate experiments are shown in B. The CFUs after 17-AAG treatment are shown in C. Data shown are the means  $\pm$  SD of the colony numbers in triplicate plates and are representative from at least 3 independent experiments. The percentage of mRNA levels of HIF1 $\alpha$  relative to  $\beta$ -actin is shown in D. E, the expression patterns of human AML cells. Compared with normal mouse bone marrow (BM) cells, the 4 AML cells from patients highly expressed chaperones, HSF1, and the active AKT-GSK3 pathway as TGB lymphoma cells as determined by Western-blot. THP-1, human AML cell line. F–H, knockdown of HSF1 abrogated colony formation of AML CSCs. After lentiviral spinoculation, portions of AML cells were seeded directly in triplicate in 1% methylcellulose medium for CFUs assays (F), and the remaining cells were cultured for 48 hours to detect the EGFP expression (G) and determine the relative mRNA levels of HSF1 and HSP90 $\alpha$  to scrambled control (Sr), which was arbitrarily set as 1, in sorted EGFP-positive cells expressing Sh-HSF1 or Sh-Sr (H). All data presented in this figure have been repeated at least twice.



highly express HSF1, are resistant to 17-AAG treatment. As summarized in our model (Fig. 7), low expression of HSF1 in CSCs renders them more susceptible to 17-AAG, as at physiological conditions, the low amount of HSF1 likely fails to induce HSP90 $\alpha$  transcription in a sufficiently fast manner to compensate for the loss of HSP90 $\alpha$ . Thus, 17-AAG-mediated HSP90 inhibition abrogates the function of its client HIF1 $\alpha$  in CSC. In contrast, high amounts of HSF1 in non-CSCs confers greater resistance to 17-AAG, as the high levels of HSF1 protein compensate for HSP90 inhibition by stimulating HSP90 $\alpha$  expression at physiological conditions. Thus, the knockdown of HSF1 eliminates the HSF1–HSP90 $\alpha$  axis, which supports the activation of AKT–GSK3 pathway that is essential for non-CSC proliferation.

The chaperone HSP90 is required for the proper folding of its client proteins that play a critical role in cancer proliferation and survival, such as HIF1 $\alpha$ , AKT, and HER2 (3, 4, 38–40). Because HSP90 expression is controlled by the homotrimer of HSF1 and/or the heterotrimer of HSF1 and HSF2 (30, 41, 42), successful HSP90 inhibition by 17-AAG naturally depends on the levels and activation of HSF1. Deletion of HSF1 in chicken B lymphocyte revealed that HSP90 $\alpha$  expression is inducible and controlled by HSF1 (37), which is consistent with our results showing knockdown of HSF1 reduced the levels of HSP90 $\alpha$ . HSP90 $\beta$  is constitutively expressed and does not respond to the HSF1 deletion (37). Although both of the chaperone isoforms are capable of binding their client proteins (43), whether one plays more of an essential role in their client's function over the other remains undefined. In terms of our CSC models, we predicate that HSP90 $\alpha$  plays a critical role in the maintenance of HIF1 $\alpha$  function.

Low doses of 17-AAG inhibit the maintenance and self-renewal of lymphoma CSCs through blockade of HIF1 $\alpha$  activity, as evidenced by the loss of HIF1 $\alpha$  transcription



**Figure 7.** A schematic diagram depicting the molecular mechanism underlying the resistance of non-CSCs and the sensitivity of CSCs to 17-AAG. High levels of HSF1 confer 17-AAG resistance to non-CSCs by ensuring HSP90 $\alpha$  expression and chaperone-mediated activation of the AKT–GSK3 pathway, which is essential for non-CSC proliferation. In contrast, low levels of HSF1 render CSCs sensitive to 17-AAG, as reduced levels of HSF1 are inadequate to compensate for HSP90 $\alpha$  inhibition by 17-AAG, thus leading to the loss of HSP90 $\alpha$ -mediated HIF1 $\alpha$  maintenance of CSCs. Knockdown of HSF1 disrupts both CSC and non-CSC activities without a need of HSP90 inhibition by 17-AAG.

activity and the reduced expression of HIF1 $\alpha$  and its target gene *Glut1*. In addition, this mechanism could be effective in AML CSCs, as HIF1 $\alpha$  expression was reduced by 17-AAG treatment, and the expression patterns for the active AKT–GSK3 pathway, HSF1, and HIF1 $\alpha$  were similar to that in our TGB lymphoma cells (14). Further, loss of HIF1 $\alpha$  activity following 17-AAG treatment induced apoptosis at concentrations that were more than 20-fold lower than the IC<sub>50</sub> required for the elimination of the differentiated non-CSCs. This further illustrates the dependence of lymphoma CSCs on HIF1 $\alpha$  for survival. In addition, significantly lower concentrations of 17-AAG were able to largely eliminate colony formation capacity of both lymphoma CSCs and AML CSCs, indicating an impairment of self-renewal *in vitro*. Although mice treated with 17-AAG had prolonged survival, a few mice still succumbed to lymphoma. This is not surprising, given that high expression of HSF1 in the rapidly proliferative tumor bulk can provide resistance to 17-AAG, showing the need for combination of conventional therapies with CSC-targeting agents. However, consistent with this self-renewal blockage *in vitro*, residual lymphoma cells present in spleens from 17-AAG-treated mice had a reduced ability for secondary tumor initiation, illustrating a direct inhibitory effect of 17-AAG on the lymphoma CSC's self-renewal *in vivo*.

The selective elimination of mouse lymphoma CSCs and human AML CSCs by low doses of 17-AAG provided validation for its potential therapeutic role in targeting CSCs. 17-AAG has been used to treat various types of hematological malignancies in clinical phase I trials. However, only partial responses were seen with 17-AAG therapy (44). In addition, the high doses of 17-AAG used in advanced solid tumors caused serious adverse events, including intestinal and liver toxicity. These reports emphasize the limitations seen with using high doses of 17-AAG (45).

We revealed that the high expression of HSF1 in non-CSCs of both lymphoma and AML helps promote resistance to 17-AAG and maintain their aggressive proliferation. Similarly, the low expression of HSF1 in TGB lymphoma CSCs, and possibly also in AML CSCs, is essential for CSC maintenance through regulation of HSP90 $\alpha$  and their client proteins, such as HIF1 $\alpha$ . Therefore, targeting HSF1 may also efficiently eradicate advanced lymphoma and AML, in addition to potentially reducing recurrence. These results are supported by reports showing that genetic deletion of HSF1 prevented initiation of oncogene-induced cancers and tumor suppressor p53 null-mediated lymphoma (28, 29). Efforts to develop HSF1 inhibitors are ongoing (46). Combined with a HSF1 inhibitor, 17-AAG may be a powerful therapy for eradicating hematological cancers via a broad inhibition of transcriptional factors that are required for the proliferation of differentiated cancer cells, and the maintenance of CSCs. In addition, the low dose of 17-AAG used for the elimination of these CSCs shows that it alone has the potential to prevent relapse or, in combination with conventional therapy, to treat relapsed or refractory hematological cancers. In addition, using a low dose of 17-AAG to target CSCs may lessen toxicity arising from off target effects, as many of the

signaling pathways used by CSCs are shared with their normal counterparts (47). Finally, our work, together with reports from glioma (20) and chronic myeloid leukemia (48), further extend the strategy of HSP90 inhibition as a means for eliminating tumorigenic stem cells.

In summary, our studies show that low doses of 17-AAG selectively target lymphoma CSCs through HSP90 $\alpha$ -mediated HIF1 $\alpha$  inhibition. This selectivity is likely attributable to the expression status of HSF1 in the CSCs and non-CSCs subsets. Knockdown of HSF1 abrogates the activity of the 2 subsets, providing important considerations for future drug development in patients with malignant lymphoma/leukemia. The regulatory mechanisms among HSF1, HSP90 $\alpha$ , and HIF1 $\alpha$  that are required for the maintenance of the highly proliferative state of differentiated cancer cells and the quiescent state of CSCs is worthy of further investigation, for future development of cancer therapy.

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## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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