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HSP90 inhibitor 17-AAG selectively eradicates lymphoma stem cells

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Abstract

Cancer stem cells (CSCs, also called tumor initiating cells) comprise tumor cell subpopulations that preserve the properties of quiescence, self-renewal and differentiation of normal stem cells. CSCs are also therapeutically important because of their key contributions to drug resistance. The hypoxia inducible transcription factor HIF1α is critical for CSC maintenance in mouse lymphoma. Here we show that low concentrations of the HSP90 inhibitor 17-AAG eliminate lymphoma CSCs in vitro and in vivo by disrupting the transcriptional function of HIF1α, 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 it also shows that HSF1 is an important target for elimination of both CSCs and non-CSCs in cancer.

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 1α (HIF1α) 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) (2–10). As many of these client proteins significantly contribute to

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Conflicts of interest
The authors declare no competing financial interests.

Author contributions
B.N., H.L. performed lymphoma cell culture, apoptosis and CFU assays, flow cytometry, in vivo animal experiments, and writing the manuscript. Y.W. performed FACS analysis and the isolation, culture, CFU assays of lymphoma and AML cells, and conducted the data analysis and experimental design. D.S. participated in data interpretation and supervised students and project. Y. L. performed the molecular cloning, Western blot, and conducted the data analysis and experimental design. Y.W., D.S. and Y. L. supervised the project and wrote the manuscript.

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) (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 HIF1α or 2α, respectively, for their maintenance (13–15). In particular, human AML CSCs are a rare population of $CD34^{+}CD38^{-}$ 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 HIF1α 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 better suit targeting both lymphoma CSCs and bulk lymphoma cells simultaneously through the disruption of multiple HSP90 client proteins. Unfortunately, acquired 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 suggested as an anticancer strategy (27). This concept is supported by reports demonstrating that HSF1 is required for the initiation of both lymphoma and RAS oncogene–induced tumors (28, 29).

In our study, we demonstrate 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. Knockdown of HSF1 abrogates the colony formation ability of lymphoma and AML CSCs through disrupting HSP90α-mediated HIF1α stability. This also provides a new anti-CSC strategy by targeting HSF1 to eliminate aggressive malignancies.

Materials and Methods

Mice and Cells and Reagents

TCR transgenic B line (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) supplemented with murine cytokines (R&D Systems): 10

ng/mL recombinant IL-3 and IL-6, and 100 ng/mL stem cell factor. For some in vitro experiments, TGB lymphoma cells were transferred from 1% methylcellulose culture to an expansion medium: RPMI 1640 medium supplemented with 10% FBS, IL-3, IL-6, and SCF cytokines, 100 μM non-essential amino acids, 10 mM HEPES, and 1 mM sodium pyruvate. Human AML cells were derived from the enlarged spleen tissue of SCID mice injected with aggressive AML CSCs (AML-71) as described before (14), and maintained in SCID mice via transplantation. Short term expansion and colony formation assays of the AML CSCs were carried out as done similarly for our lymphoma cells, except for a substitution of human cytokines for murine cytokines. AML cells' clinical subtypes and genetic characterization performed by examining the appearance of the malignant cells and by using cytogenetics to characterize any underlying chromosomal abnormalities were authenticated by 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 GSK3α/β (9331), phosphor-S473AKT (9271), AKT (9272) were purchased from Cell Signaling. Mouse antibodies to GSK3α/β (0011-A, Santa Cruz) and β-actin (AC-15, Sigma) were used in the studies.

Isolation of lymphoma CSCs and Colony formation assay

The lymphoma CSCs (c-Kit$^+$Sca1$^+$) 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 BD Bioscience). For the detection of some downstream proteins requiring larger amounts of cells, the c-Kit$^+$ fraction (c-Kit$^+$Sca1$^+$ subset combined with c-Kit$^+$ Sca1$^-$ 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 6–8 days of culture, colonies were counted, pooled, and then replated for consecutive rounds. Afterwards, the cultured lymphoma cells were used for the colony formation assay performed in 1% methylcellulose medium. For tumor inoculation and viral infection, the cultured lymphoma cells were expanded in RPMI1640 medium described above, 24 hours before these experiments.

Lentiviral Infection and Plasmids

The lentiviruses for small hairpin (Sh) RNA targeting HSF1 (Sh-HSF1) and scrambled control Sh-RNA were generated in HEK293FT cells according to the lentiviral preparation protocol (Invitrogen). Lentiviruses were made in a 150 mm falcon culture dish and 50 ml media was collected and centrifuged at 550*g* for 10 min to remove cell debris. Viral particles obtained after centrifugation at 12,857*g* overnight were suspended with 2 ml of RPMI 1640 medium containing 10% FBS, aliquoted, and stored at −80°C. Fifty to one hundred microliters of concentrated virus per million cells was mixed with 100 μl culture medium and used for spinoculation in a U-bottom tube in a mixture containing 9 μg/ml polybrene. The spinoculation was carried out at 2,500 rpm, 2 hours, and 30°C. After spinoculation, the cell pellet was suspended gently and completely with fresh culture medium, seeded and cultured in 1% methylcellulose for another 5–7 days before use.

The sequence of Sh-HSF1 (5-GCTCATTCAGTTCCTGATC-3) has been previously validated (30), and was cloned into a Sh-lentiviral vector as described before (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 HIF1α were described before (14). Full length coding region of Hsf1 cDNA was amplified by RT-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

Generally, sorted CSCs or cultured lymphoma cells were lysed with 1% Triton X-100 lysis buffer containing 20mM Tris-HCl, pH7.4, 150mM NaCl, 40mM NaF, 1mM DTT, protease and phosphatase inhibitor cocktail (Sigma). Supernatants of the lysate were used for Western-blots and resolved on a reducing and non-heated (95°C, 3 min) 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 hrs.

MTT ([3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide]) Assay

The expanded lymphoma cells were seeded in the RPMI 1640 culture medium at a cellular density of 2.5×10^5 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 performed according to the manufacture'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$^-$Sca1$^-$) were extracted using the RNeasy extraction kit (Qiagen). After reverse transcription of RNA to cDNA, real-time PCR using the converted cDNA as template was performed in triplicate using SYBR Green PCR Master Mix. Following primers were used: Hsf1, F-TTTGACCAGGGCCAGTTTG, R-TTCGGAAGCCATACATGTTGAG; Hif1α,F-AGTCTAGAGATGCAGCAAGATCTC, R-TCATATCGAGGCTGTGTCGACTGA; Hsp90α, F-TGTTGCGGTACTACACATCTGC, R-GTCCTTGGTCTCACCTGTGATA; Hsp90β, F-CAAACAAGGAGATTTTCCTCCG, R-GCTGTCCAACTTAGAAGGGTC; Glut1, F-TGTGCTGTGCTCATGACCATC, R-ACGAGGAGCACCGTGAAGAT; β-Actin, F-GGCTGTATTCCCCTCCATCG, R-CCAGTTGGTAACAATGCCATGT. Fold differences was calculated using the delta Ct method (31) and β-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, CD38 for AML cells), and then Annexin-V for 15 minutes at room temperature before 5μM of DAPI was added. 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 5×10^5 lymphoma cells through intraperitoneal injection. Seven days following tumor implantation, the mice were I.P. injected with 17-AAG or vehicle (10% DMSO + 40% Cremophor EL:Ethanol (3:1) (v/v) + 50 % PBS) every other day for three weeks. At the cessation of treatment, mice were monitored up to 80 days post 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 1×10^5 lymphoma cells from the spleens of first round mice that had been treated with 17-AAG or vehicle. These mice were followed up to 160 days post tumor cell injection to monitor differences in tumor initiation between the mice.

Results

The distinct features of lymphoma CSCs and bulk lymphoma cells (non-CSCs)

We have previously demonstrated that AKT-GSK3-β-catenin signaling is constitutively activated in the TGB lymphoma c-Kit$^-$Sca1$^-$ cells (non-CSCs), and that HIF1α plays a critical role in the maintenance of the lymphoma c-Kit$^+$Sca1$^+$ cells (CSCs) (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 1–2 months post-inoculation (14, 19). These findings prompted us to devise a drug therapy which 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 HIF1α and AKT (two client proteins of HSP90), respectively, we tested the HSP90 inhibitor 17-AAG for the simultaneous targeting of the two cell populations.

We first examined the expression levels of chaperones and the transcriptional factor HSF1 in the CSCs and non-CSCs. Using a BD FACSAria cell sorter, two subsets of TGB lymphoma cells (c-Kit$^+$Sca1$^+$, c-Kit$^-$Sca1$^-$) were sorted from mouse spleen tissue enlarged with the TGB lymphoma cells (Fig.1A). As we previously described (14), the sorted c-Kit$^+$Sca1$^+$ cells initiated lymphoma with 50-fold less number of cells than the sorted c-Kit$^-$Sca1$^-$subset (Fig.1B). Quantitative real time-PCR showed that the mRNA levels of HIF1α and its target Glut1 were 4 and 8-fold higher respectively in the c-Kit$^+$Sca1$^+$ cells than c-Kit$^-$Sca1$^-$ cells. This confirmed that the c-Kit$^+$Sca1$^+$ subset still maintained the unique marker of active HIF1α. The mRNA levels of chaperones HSP90α, HSP90β and HSP70, and HSF1 were about one-fold higher in the c-Kit$^-$Sca1$^-$ than c-Kit$^+$Sca1$^+$ cells (Fig.1C). Consistent with mRNA levels, protein levels of HSP70 were higher in the c-Kit$^-$Sca1$^-$ cells. Strikingly, there were large differences in the protein levels of HSF1, HIF1α, and the activated status of AKT-GSK3 pathway between the two cell populations (Fig.1D, E). HSF1 had over 10-fold higher expression in the c-Kit$^-$Sca1$^-$ population, compared to the c-Kit$^+$ population. Consistent with our previous results (14, 19), stable HIF1α was exclusively expressed in the CSCs, while active AKT (pS473AKT) and GSK3 (pS21/9GSK3α/β) were present in higher amounts in the non-CSCs than CSCs. These distinctive expression patterns for stable HIF1α 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 IC50 of 238 nM (Fig. 2A). In contrast, c-Kit$^+$Sca1$^+$ lymphoma cells treated with 17-AAG were significantly more sensitive to HSP90 inhibition, with an IC50 of 5.64 nM (Fig. 2B, C, D). These substantial differences in the IC50 indicates 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 if 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 shown in Fig.3A, low doses of 17-AAG (10 and 25 nM) increased apoptosis of c-Kit$^+$Sca1$^+$ cells by 2 to 3-fold, as compared to untreated control. However, 17-AAG failed to induce apoptosis of c-Kit$^-$Sca1$^-$ 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 if 17-AAG affected CSC self-renewal, we plated lymphoma cells and treated them with increasing concentrations of 17-AAG. As a result, 10 nM 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 if self-renewal was also disrupted. Cells from colonies previously treated with 10 nM 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 if 17-AAG interfered with HIF1α's transcriptional activity, we constructed a lentiviral reporter system that utilized HIF1α 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^{+}Sca1^{+} subset, while only 0.786% were in the c-Kit^{-}Sca1^{-} subset (Fig.3C). This is consistent with the specific expression of stable HIF1α in the CSCs. With treatment, 10nM 17-AAG reduced HRE-EGFP expression in the CSCs by 2-fold, but had little effect on the HRE-EGFP expression in non-CSCs, demonstrating 17-AAG specifically suppressed HIF1α activity in the CSCs. In addition to the reduction of HIF1α transcriptional activity induced by 17-AAG, the mRNA levels of HIF1α and its target Glut1 were also significantly decreased in a dose-dependent manner (Fig.3D), indicating HIF1α and its target transcription are controlled by HIF1α stability. These results show that 17-AAG specifically eliminates the CSCs through inhibition of the HSP90-mediated mRNA expression and transcriptional activity of HIF1α at concentrations which fail to eliminate the more differentiated c-Kit^{-}Sca1^{-} lymphoma cells.

17-AAG abrogates lymphoma initiation in vivo

To investigate whether HSP90 inhibition can reduce lymphoma initiation in vivo, we injected 5×10^{5} TGB lymphoma cells intraperitoneally (I.P.) into recipient B10.BR mice. Seven days after injection, different doses of 17-AAG (5 to 40 mg/kg) or vehicle control were given to mice every other day for three weeks. Upon cessation of treatment, four 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,B,C). The lymphoma cells had also metastasized into liver and lung tissue, as typically seen with this cancer (19). In contrast, spleens from mice which had received 17-AAG were dramatically smaller, with less infiltrating lymphoma cells in the spleen, and a lower metastatic spread into other organs, as compared to the vehicle-treated control (Fig.4C). In addition, 17-AAG treated mice survived significantly longer compared to mice which had received vehicle alone, demonstrating 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 if 17-AAG treatment impaired the self-renewal ability of TGB lymphoma CSCs in vivo, 1×10^{5} cells obtained from either 40 mg/kg 17-AAG- or vehicle-treated spleens were I.P. injected into five healthy secondary recipient B10.BR mice. All of the mice injected with vehicle-treated spleen cells eventually succumbed to lymphoma. In contrast, only one mouse that received 17-AAG-

treated spleen cells died over the course of the study (Fig.4E). These data demonstrate 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 nM 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, pS21GSK3α, and pS9GSK3β remained unchanged (Fig.5A). Coinciding with reports in the literature, we also observed an increase in HSP70 in reaction to HSP90 inhibition with 100nM 17-AAG. However, 100 nM 17-AAG could reduce the levels of these phosphorylated proteins in addition to reducing HSP90 and HSF1. This dramatic loss in cell viability was in part due to drug toxicity, as all of our controls (AKT, GSK3α, GSK3β and β-actin) were sharply decreased and cell death was observed. This result suggests that 17-AAG does not affect HSP90-mediated AKT activation if HSF1 protein levels are very high, as high levels of HSF1 could maintain or further increase the transcription of HSP90 or HSP70 to compensate for HSP90 inhibition.

To observe the effect of HSF1 on the growth of CSCs and non-CSCs, we made a hair-pin (Sh) RNA targeting HSF1. In HEK293 cells, the Sh-HSF1 silencer specifically knocked down ectopically expressed HSF1, but not Lef1, by up to 80% within 24 hours after co-transfection (Fig. 5B). Nearly half (48.1 %) of the TGB lymphoma cells were infected after transfection with the Sh-HSF1 lentivirus, as determined by the expression of an EGFP reporter within the Sh-RNA construct (Fig. 5C). Quantitative RT-PCR revealed that Sh-HSF1 knocked down approximately 80% of the endogenous HSF1 mRNA in sorted EGFP-positive cells, relative to the mRNA levels of sorted cells expressing scrambled Sh-RNA (Sh-Sr) (Fig. 5C,D). With the knockdown of HSF1, the mRNA of one of its target genes, HSP90α (37), was correspondingly reduced. The HSF1 silencer significantly inhibited cell growth and eliminated the colony formation capacity of the CSCs without the need of 17-AAG (Fig. 5E), demonstrating that HSF1 is positioned upstream of HSP90 and can control the level of chaperone activity. This effect was not seen with the Sh-Sr, as it failed to affect colony formation of the CSCs. This result shows that the low levels of HSF1 are required for maintenance of the CSCs.

HSP90 inhibition, via 17-AAG treatment or HSF1 knockdown, eliminates AML CSCs

In order to determine if 17-AAG treatment could selectively eliminate hematopoietic CSCs other than our lymphoma CSCs, we used a mouse model of human AML that has been previously established (14). Similar to our lymphoma cells, AML CSCs stably express HIF1α for their maintenance (14). To see if 17-AAG also selectively targeted AML CSCs, AML cells were treated with increasing concentrations of 17-AAG. Twenty five nanomolar 17-AAG increased the percentage of apoptotic CSCs ($CD34^+CD38^-$) nearly 3-fold, as compared to vehicle treated CSCs. In contrast, 25 nM 17-AAG had little effect on the induction of apoptosis in the non-CSCs ($CD34^+CD38^+$) (Fig. 6A, B). Although a higher dose (100nM) 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, B). Similar to lymphoma CSCs, a dramatically low dose (10nM) 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 HIF1α stability by 17-AAG, as the mRNA levels of HIF1α were correspondingly reduced by 17-AAG treatment (Fig. 6D). Compared to 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, suggesting that AML cells

likely 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 to the Sh-Sr (Fig. 6F). Lastly, 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 HSP90α. Knockdown of HSF1 dramatically reduced the mRNA levels of HSP90α (Fig. 6H), verifying the requirement for functional HSP90α 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 HIF1α activity. In addition our results demonstrate 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 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 HSP90α transcription in a fast enough manner to compensate for the loss of HSP90α. Thus, 17-AAG mediated HSP90 inhibition abrogates the function of its client HIF1α in CSC. In contrast, high amounts of HSF1 in non-CSCs confers to them more resistance to 17-AAG, as the high levels of HSF1 protein compensate for HSP90 inhibition by stimulating HSP90α expression at physiological conditions. Thus, the knockdown of HSF1 eliminates the HSF1-HSP90α 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 HIF1α, 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 HSP90α expression is inducible and controlled by HSF1 (37), which is consistent with our results showing knockdown of HSF1 reduced the levels of HSP90α. HSP90β is constitutively expressed and does not respond to the HSF1 deletion (37). Although both of the two 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 HSP90α plays a critical role in the maintenance of HIF1α function.

Low doses of 17-AAG inhibit the maintenance and self-renewal of lymphoma CSCs through blockade of HIF1α activity, as evidenced by the loss of HIF1α transcription activity and the reduced expression of HIF1α and its target gene Glut1. This mechanism could also be effective in AML CSCs, as HIF1α expression was reduced by 17-AAG treatment, and the expression patterns for the active AKT-GSK3 pathway, HSF1 and HIF1α were similar to our TGB lymphoma cells (14). Loss of HIF1α activity following 17-AAG treatment also induced apoptosis at concentrations more than 20-fold lower than the IC50 required for the viability maintenance of the differentiated non-CSCs. This further illustrates the dependence of lymphoma CSCs on HIF1α 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. While 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, demonstrating 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. Likewise, the low expression of HSF1 in TGB lymphoma CSCs, and likely also in AML CSCs, is essential for CSC maintenance through regulation of HSP90α and their client proteins, such as HIF1α. Therefore, targeting HSF1 may also efficiently eradicate advanced lymphoma and AML, in addition to potentially reducing recurrence. These results are supported by reports demonstrating genetic deletion of HSF1 prevented initiation of both oncogene-induced cancers and in tumor suppressor p53 null-mediated lymphoma (28, 29). Efforts to develop HSF1 inhibitors have been underway (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 demonstrates it alone has potential to prevent relapse, or in combination with conventional therapy, treat relapsed or refractory hematological cancers. Using a low dose of 17-AAG to target CSCs may also lessen toxicity arising from off target effects, as many of the signaling pathways utilized by CSCs are shared with their normal counterparts (47). Lastly 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 demonstrate that low doses of 17-AAG selectively target lymphoma CSCs through HSP90α-mediated HIF1α 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 two subsets, providing important considerations for future drug development in patients with malignant lymphoma/leukemia. The regulatory mechanism between HSF1, HSP90α and HIF1α that is required for the maintenance of both 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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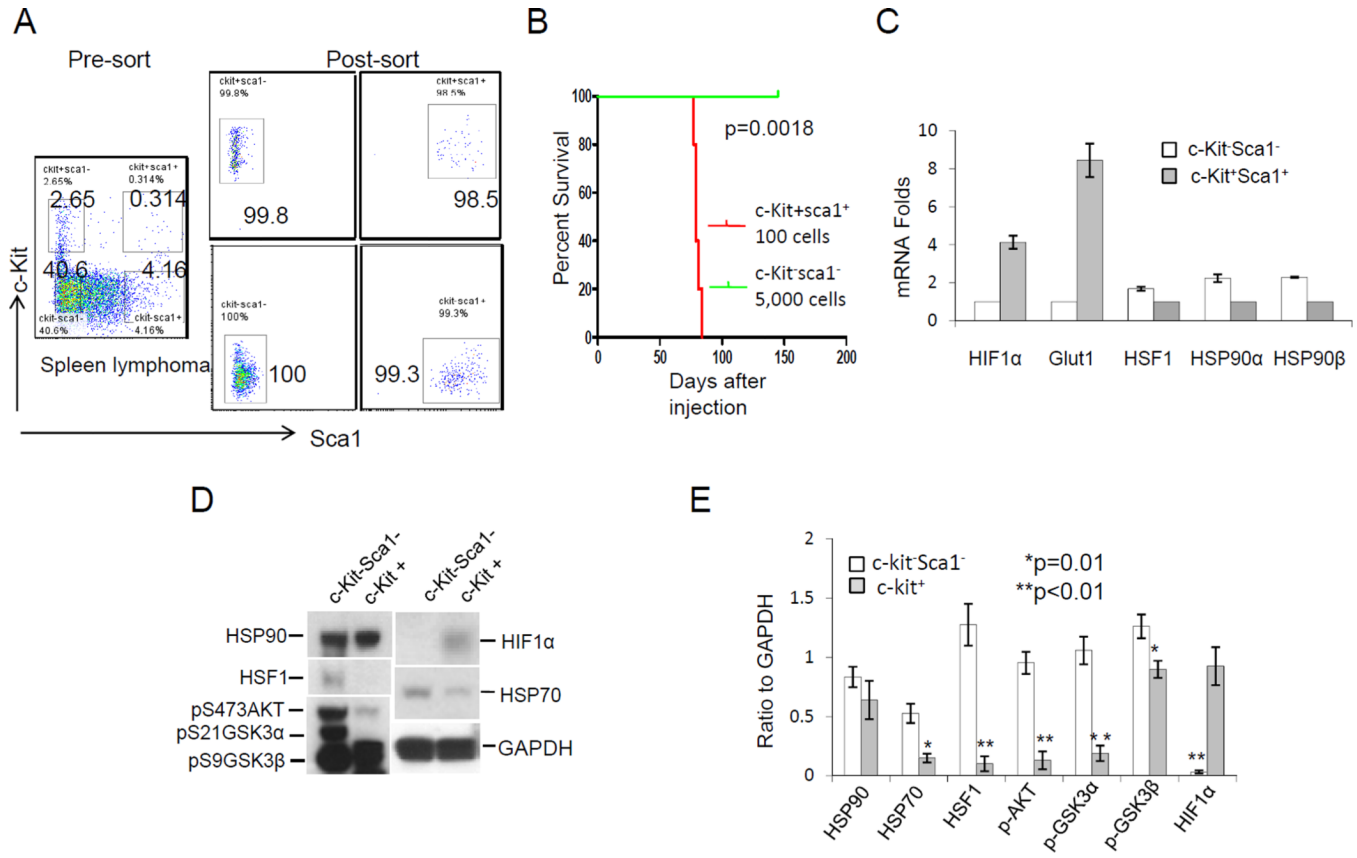

Figure 1.

The distinct features of lymphoma CSCs (c-Kit$^+$Sca1$^+$) and differentiated cells (c-Kit$^-$Sca1$^-$). A. Lymphoma cells were sorted by BD FACSAria sorting system. Lymphoma cells freshly isolated from spleen tissues of TGB lymphoma mice were stained with c-Kit and Sca1 and sorted into four subsets. B. Sorted subsets of c-Kit$^+$Sca1$^+$ and c-Kit$^-$Sca1$^-$ were injected into B10BR mice and lymphoma-initiating capacity of the two subsets is expressed based on the mouse survival rate. C. Quantitative RT-PCR showing the relative fold increase in mRNAs of indicated genes in the c-Kit$^-$Sca1$^-$ and c-Kit$^+$Sca1$^+$ subsets; the subset with lower mRNA levels is arbitrarily set to 1. D, E. The levels of indicated proteins in the subsets of c-Kit$^-$Sca1$^-$ and c-Kit$^+$ (c-Kit$^+$Sca1$^+$ combined with c-Kit$^+$Sca1$^-$). The relative ratios of bands to GAPDH in E were calculated by densitometry (E).

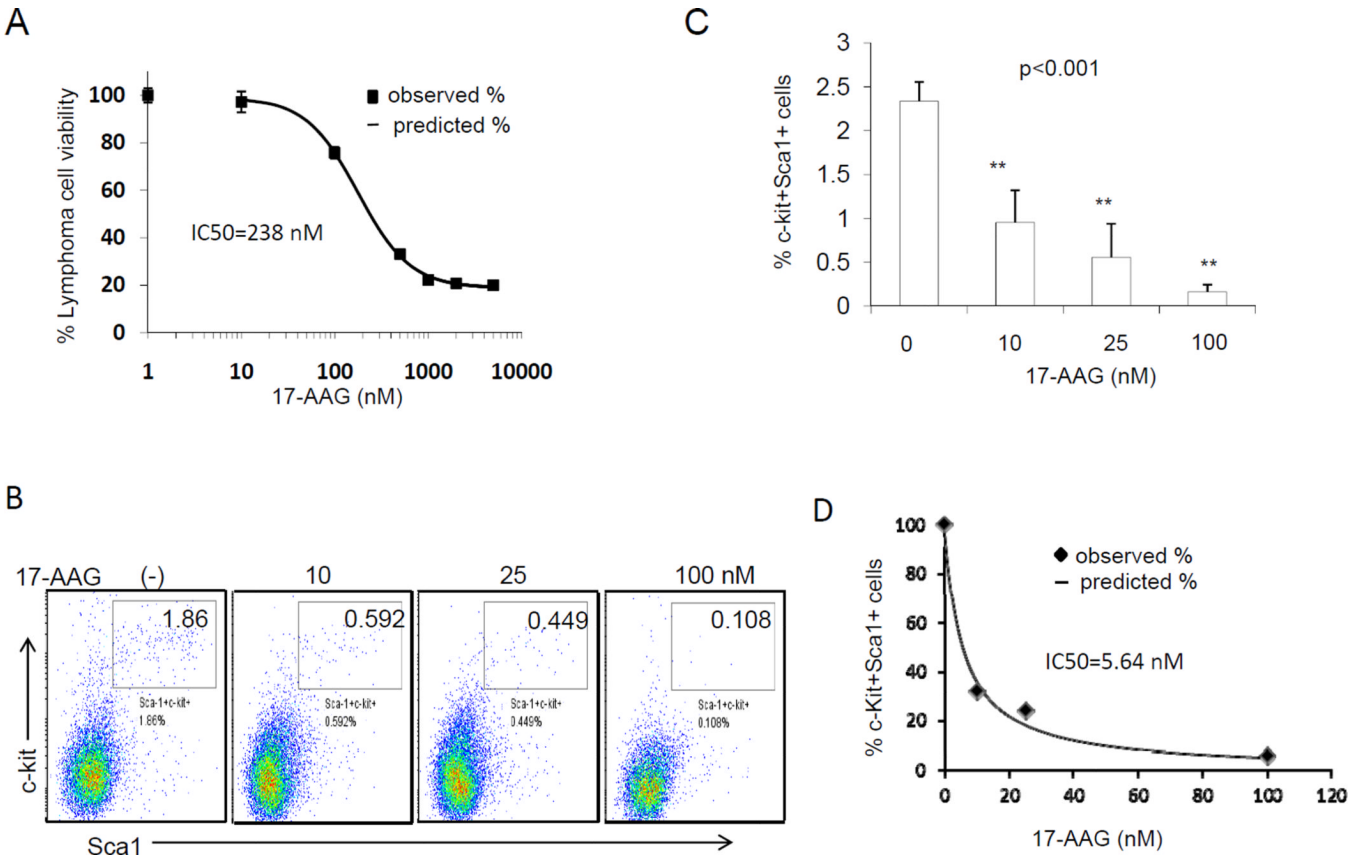

Figure 2.

17-AAG eliminates lymphoma CSCs. A. MTT assay determined the IC50 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 performed. B–D. 17-AAG selectively eliminated the c-Kit$^+$Sca1$^+$ 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$^+$Sca1$^+$ cells from triplicate experiments was quantified by flow cytometry (C). The predicated IC50 of 17-AAG to c-Kit$^+$Sca1$^+$ cells is shown in D.

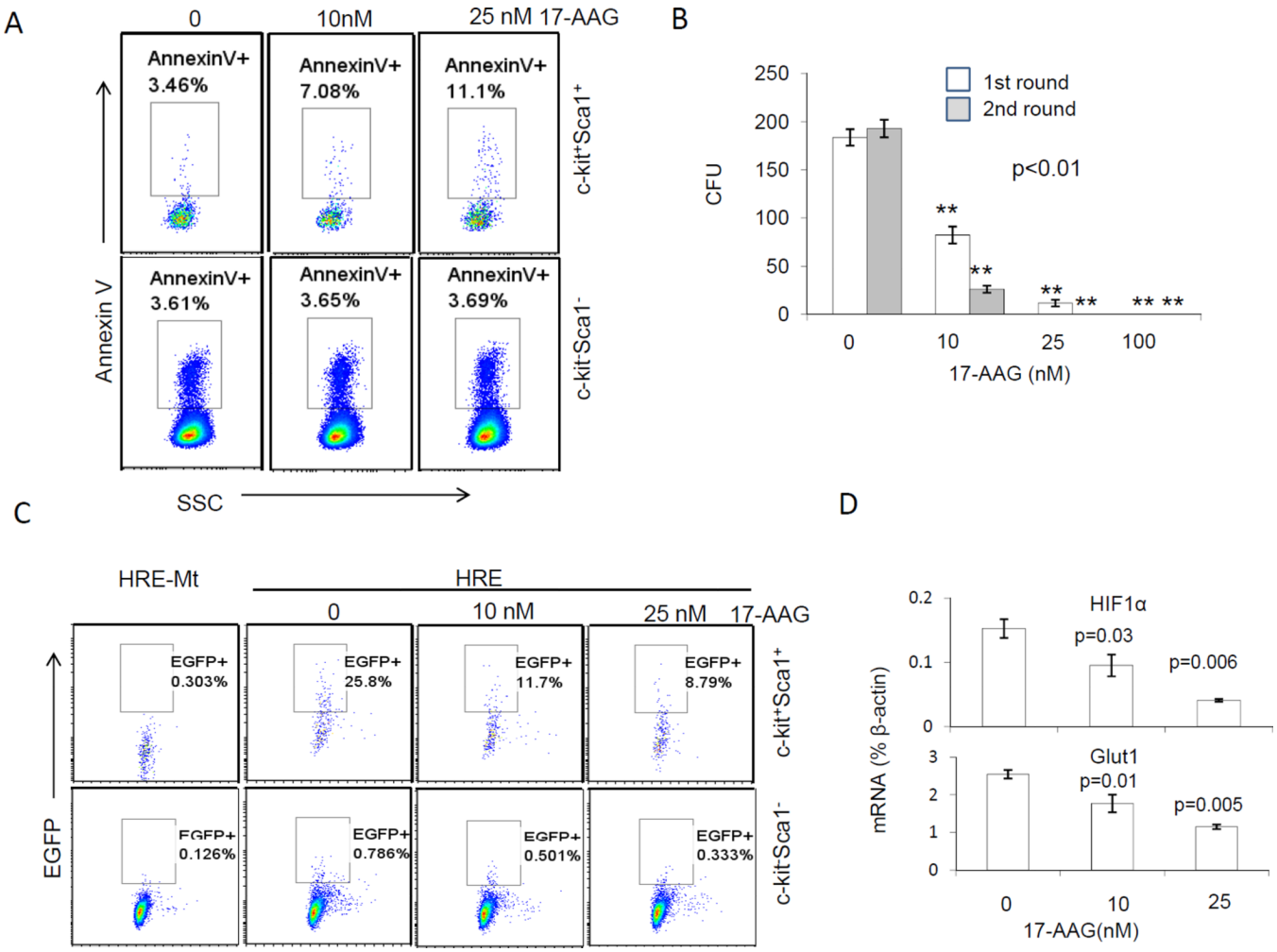

Figure 3.

17-AAG selectively induces CSC apoptosis through inhibition of HIF1α transcriptional activity. A. 17-AAG preferentially induced the apoptosis of c-Kit$^+$Sca1$^+$ but not c-Kit$^-$Sca1$^-$ cells. Twenty four hours after 17-AAG treatment, cultured lymphoma cells were stained with an antibody cocktail of c-Kit, Sca1, and Annexin V. Annexin V$^+$ cells on gated c-Kit$^+$Sca1$^+$ or c-Kit$^-$Sca1$^-$ subsets were analyzed by flow cytometry. B. 17-AAG dramatically reduced the colony formation units (CFUs) of lymphoma CSCs. After treatment with increasing concentrations of 17-AAG, the lymphoma cells were seeded in 1% methylcellulose culture medium, and CFUs were counted, and the colony replating assay was performed after a 7 day culture. Data shown are means ± SD of colony numbers in triplicates and are representative of three independent experiments. C–D. 17-AAG suppressed HIF1α 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 two 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 HIF1α and Glut-1 (D). The percentage of mRNA relative to β-actin is shown. All data presented in this figure have been repeated at least twice.

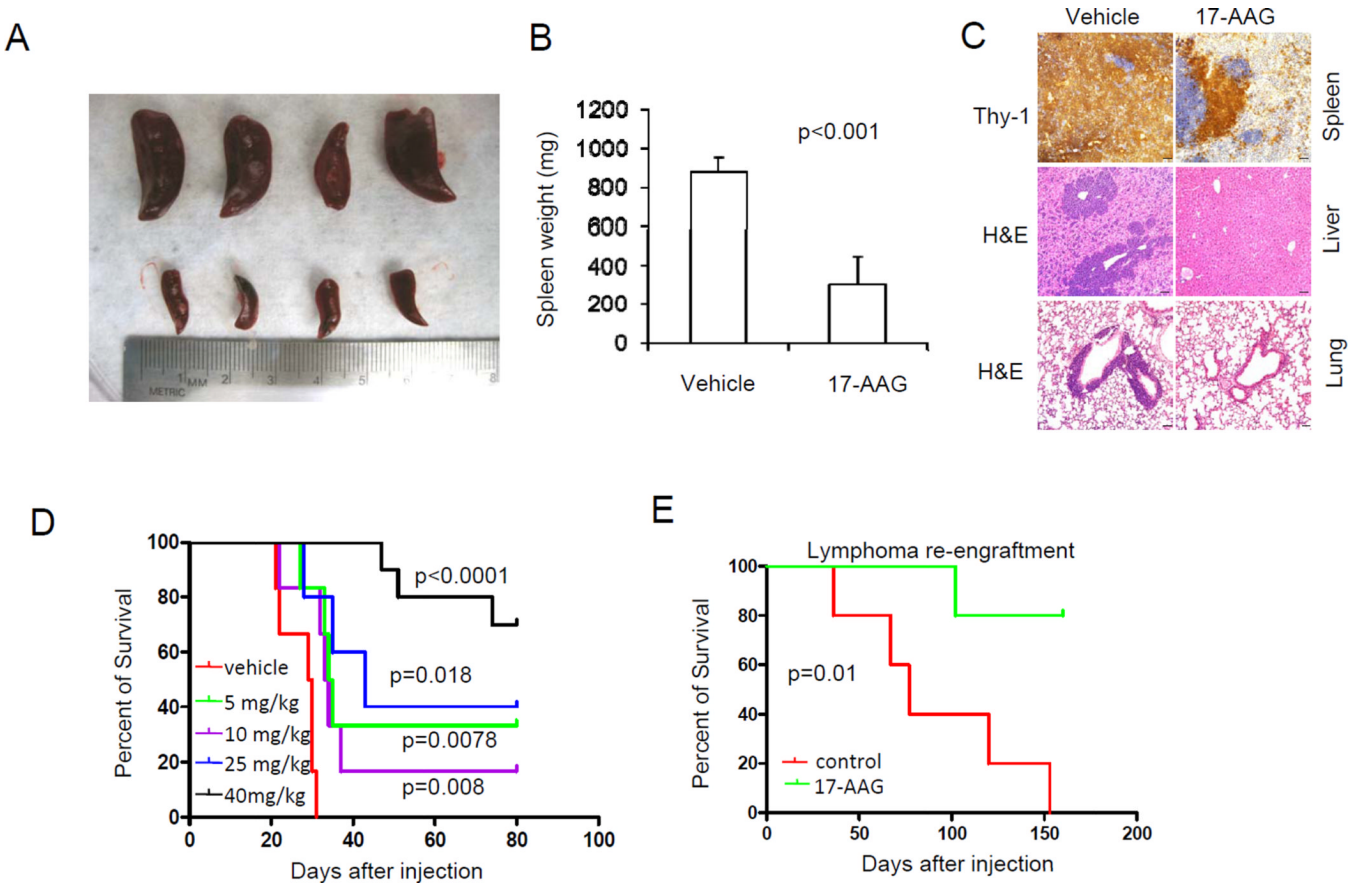

Figure 4.

17-AAG suppresses lymphoma growth in lymphoma transplanted congenic recipient mice. A–D. B10.BR mice were inoculated with 5×10^5 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 three weeks. After the mice were monitored up to 80 days post tumor cell injection, four of the surviving mice from 40 mg/kg or vehicle control group were sacrificed to measure the spleen sizes (A) and weights (B); two mice from 25mg/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. The survival rate was evaluated and data shown are representative of two independent experiments (D). E. 17-AAG reduced the self-renewal ability of lymphoma CSCs. B10.BR mice were implanted by intraperitoneal injection with 1×10^5 spleen 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.

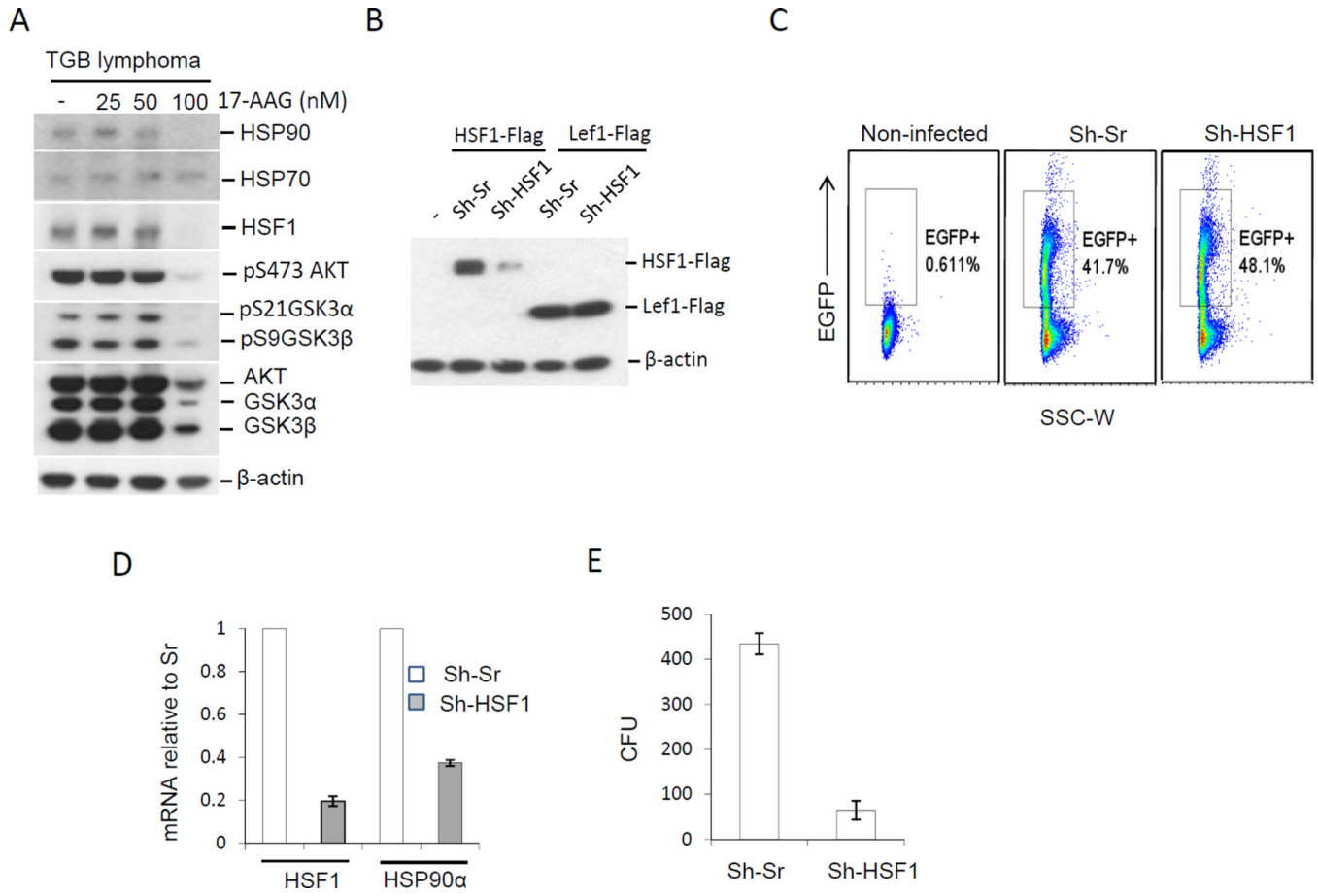

Figure 5.

HSF1 is required for the maintenance of lymphoma CSCs. A. Lymphoma non-CSC cells resisted low doses of 17-AAG treatment. After treatment with 25 to 100nM of 17-AAG for 24 hrs, the cultured lymphoma cells were lysed for Western-blot to detect the expression of chaperones, HSF1, and the AKT-GSK3 pathway. B. Knockdown efficiency of HSF1 by Sh-RNA. Twenty four hours after cotransfection with the plasmids for Sh-HSF1, Flag-tagged HSF1, or with other combined plasmids as indicated, the HEK293 cells were lysed for Western-blot to detect the HSF1-silencing efficiency using anti-Flag antibody. Lef1 (lymphocyte enhancer factor 1). C–E. Knockdown of HSF1 abrogated the colony formation ability of lymphoma CSCs. After infection with the lentivirus for Sh-HSF1 or scrambled control, one portion of infected cells was cultured in expanded medium for 48 hours to check the infection efficiency (C). Relative mRNA levels of HSF1 and HSP90α to scrambled control (Sr), which is arbitrarily set as 1, were determined in sorted EGFP-positive cells expressing Sh-HSF1 or Sh-Sr (D). The other portion of infected cells after infection were directly seeded in triplicate in 1% methylcellulose medium for CFUs assays (E). EGFP-positive colonies were counted under fluorescence microscope after 7 days of culture. All data presented in this figure have been repeated at least twice.

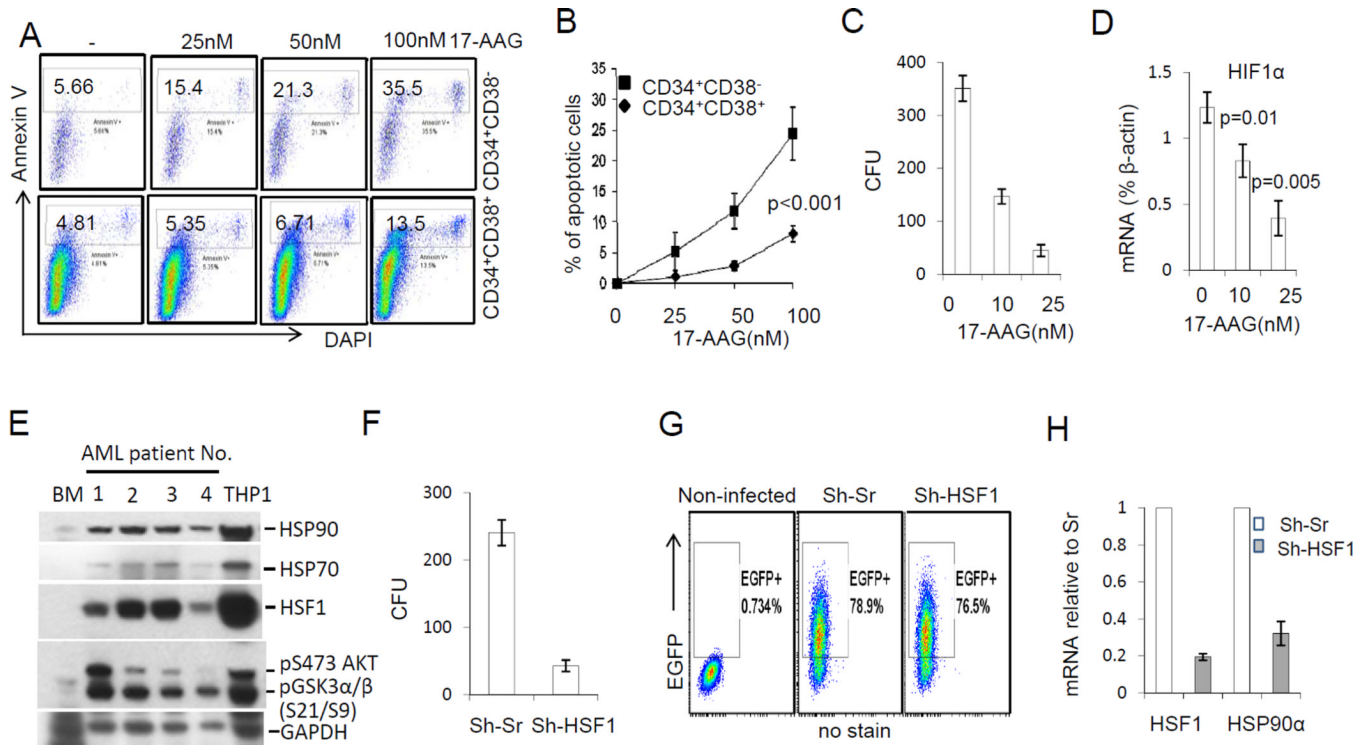

Figure 6.

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 % Annexin V^+ 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 ± SD of the colony numbers in triplicate plates and are representative of those from at least three independent experiments. The percentage of mRNA levels of HIF1α relative to β-actin is shown in D. E. The expression patterns of human AML cells. Compared to normal mouse bone marrow (BM) cells, the four 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 HSP90α to scrambled control (Sr), which is arbitrarily set as 1, in sorted EGFP-positive cells expressing Sh-HSF1 or Sh-SrI (H). All data presented in this figure have been repeated at least twice.

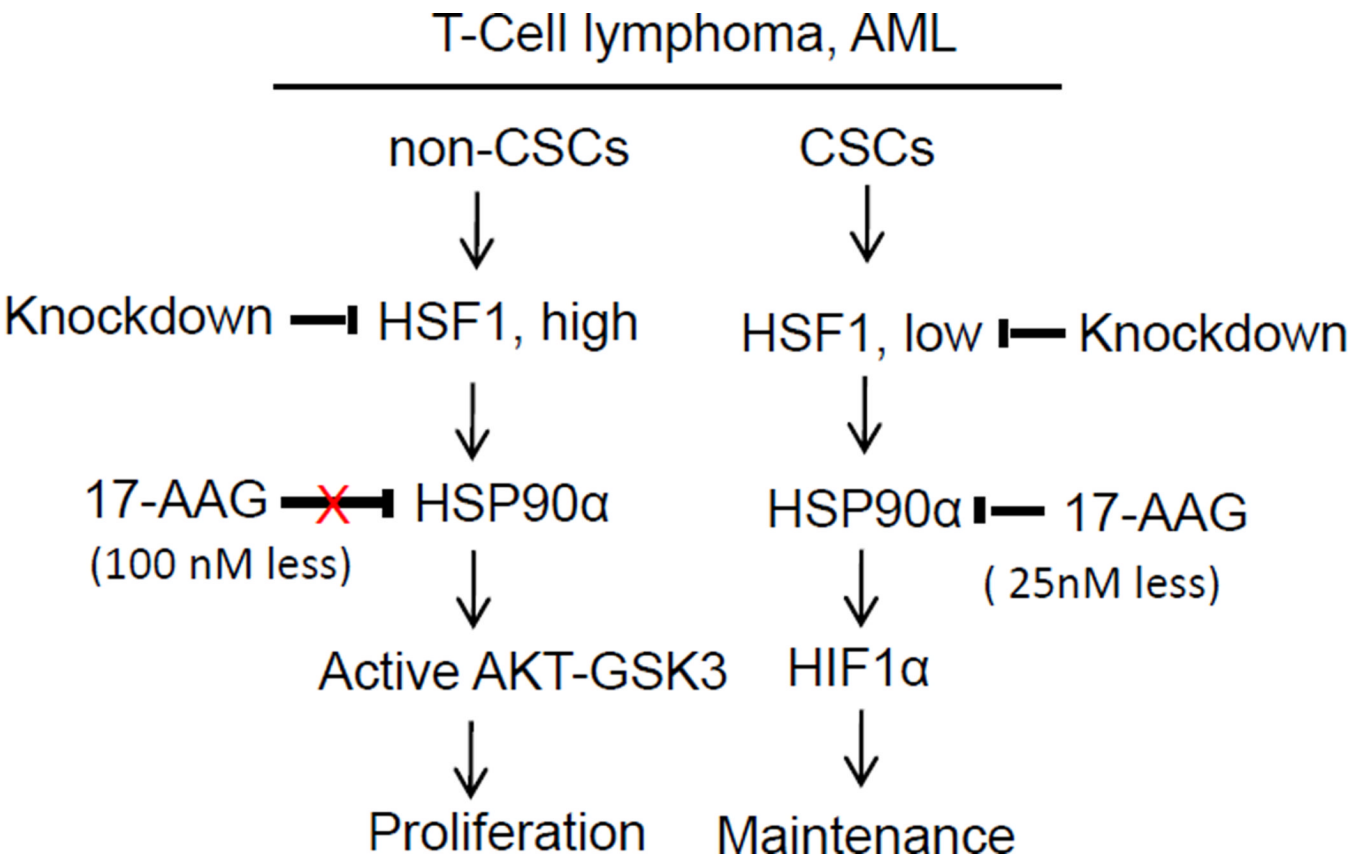

Figure 7.

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 through ensuring HSP90α expression and chaperone-mediated activation of the AKT-GSK3 pathway, that 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 HSP90α inhibition by 17-AAG, thus leading to the loss of HSP90α-mediated HIF1α maintenance of CSCs. Knockdown of HSF1 disrupts both CSC's and Non-CSC's activities without a need of HSP90 inhibition by 17-AAG.