Supporting information for

Original article

Synergistic antitumor activity of artesunate and HDAC inhibitors through elevating heme synthesis *via* synergistic upregulation of ALAS1 expression

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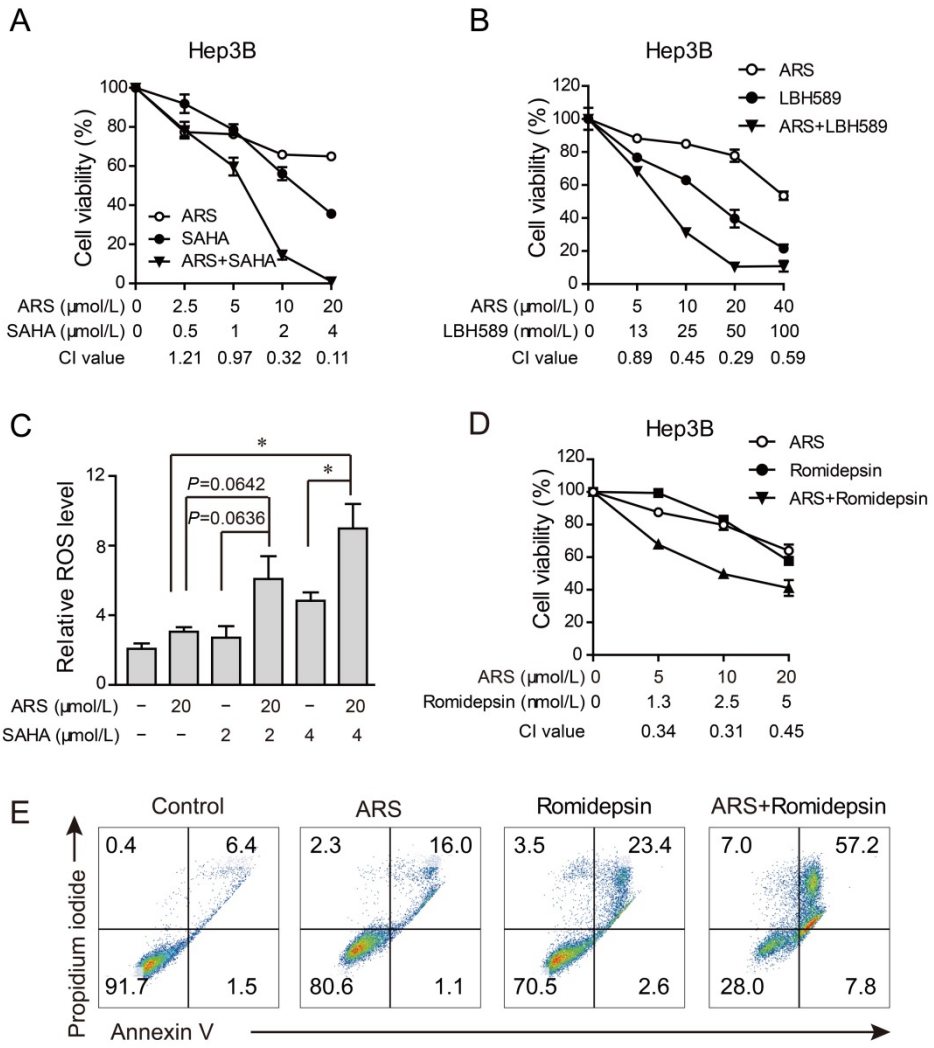

Figure S1 HDACi synergizes with ARS to inhibit Hep3B cells growth. (A)–(B) MTT assay was performed to measure the cell-proliferation inhibitory activities in Hep3B cells. Dose–response curves for ARS, HDACi [SAHA (A) and LBH589 (B)] and combinatory treatment after 72 h are shown. Error bars represent SD of triplicate experiments. CI values were calculated as indicated. (C) Intracellular ROS generation was more increased in Huh-7 cells treated with combination of ARS and SAHA for 48 h. The level of intracellular ROS was detected by a multi-detection microplate reader using the fluorescent probe DCFH-DA. $^{*}P < 0.05$, $^{**}P < 0.01$ (Student's *t*-test). (D) Cell viability of Hep3B cells treated with ARS, romidepsin and combination for 72 h. CI values were calculated as indicated. (E) Flow cytometric analysis of cell death of H1975 cells treated with vehicle control, ARS (3 μmol/L), romidepsin (1 nmol/L) or the combination for 48 h by Annexin V/PI assay.

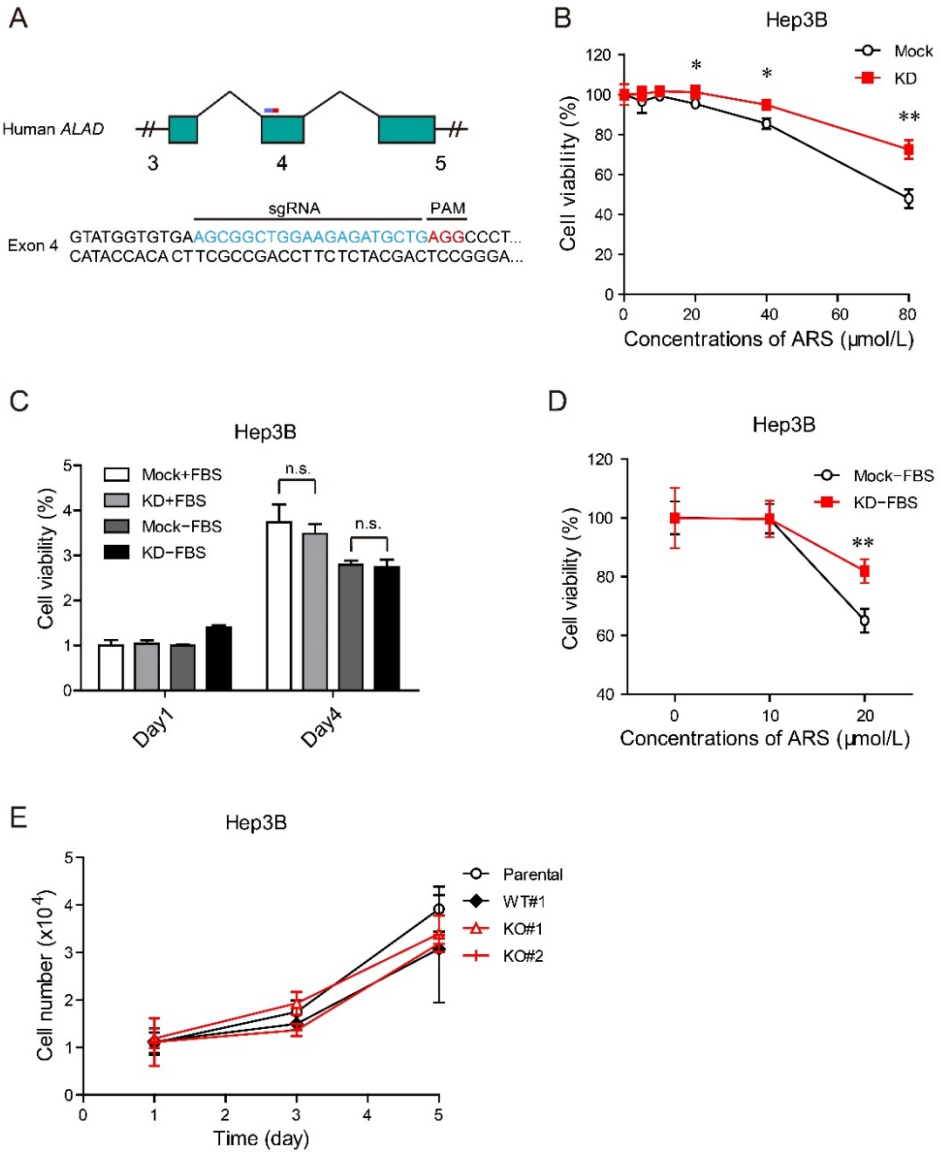

Figure S2 ALAD knockdown reduced tumor cells sensitivity to ARS. (A) Schematic of gRNA design targeting human *ALAD*. (B) ALAD KD and mock cells were treated with various concentrations of ARS for 48 h and cell viability was determined by MTT assay. (C) ALAD KD cells and mock cells were cultured in the presence (+FBS) or absence (−FBS) of 10% FBS for indicated days and cell viability were measured with CCK-8 assay. (D) ALAD KD cells and mock cells were treated with ARS in the absence of serum for 48 h. Cell viability was determined by CCK-8 assay. Error bars represent SD of triplicate experiments. Asterisks, ALAD KD *vs.* Mock; $^{*}P < 0.05$, $^{**}P < 0.01$ (Student's *t*-test). (E) Cell growth assay for Hep3B parental, WT and *ALAD* KO clones in medium containing 10% FBS. Error bars represent SD of triplicate experiments.

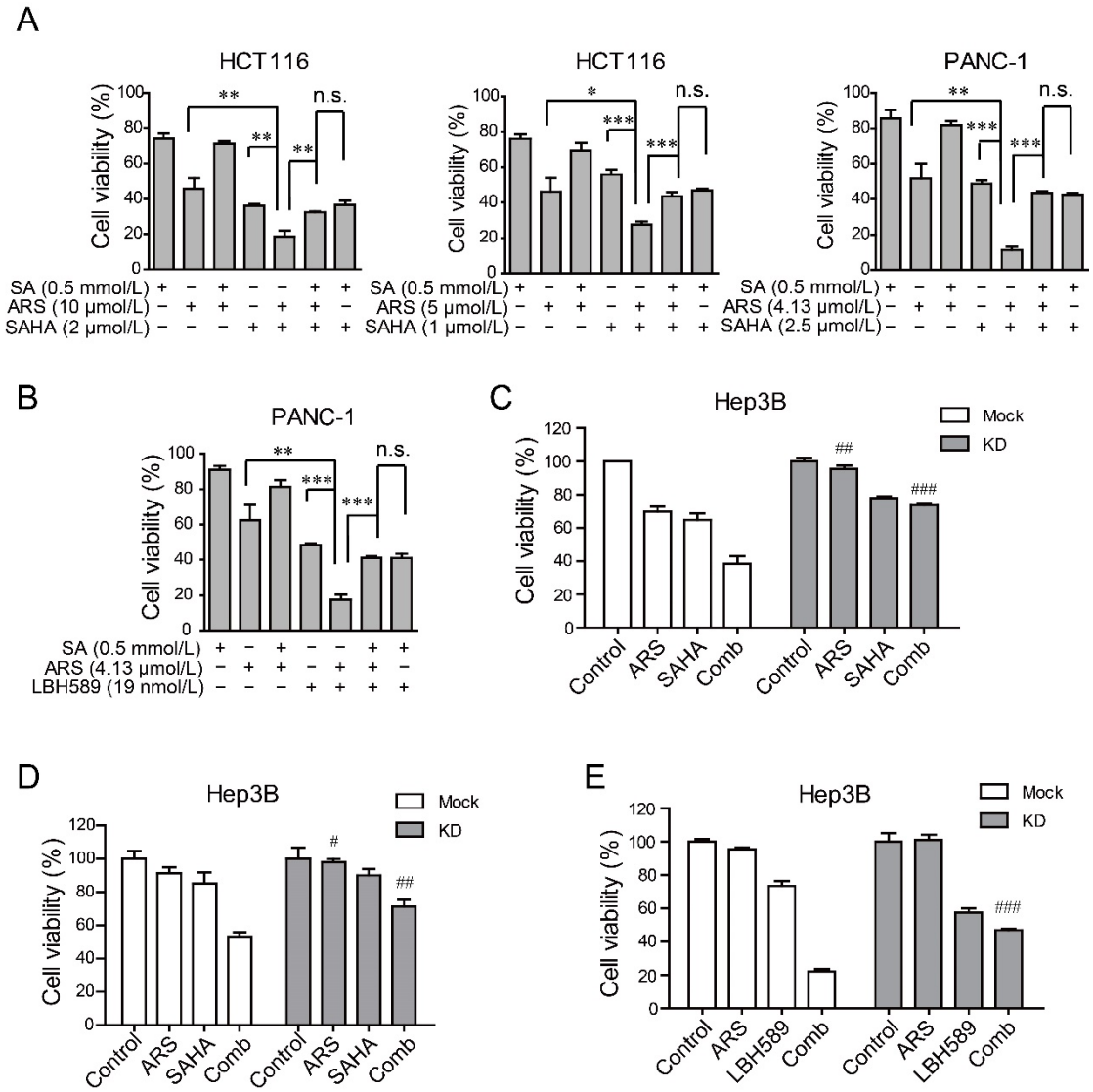

Figure S3 Blockage heme synthesis by SA or ALAD knocking down abrogates synergistic anti-tumor effect of artesunate and HDACi. (A)–(B) HCT116 cells, and PANC-1 cells were administrated with ARS, HDACi [SAHA for (A); LBH589 for (B)], SA or combination as indicated for 72 h. Cell viability was measured by MTT assay. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's *t*-test). (C)–(E) Cell viability of ALAD knockdown cells or mock cell were treated with ARS (20 μmol/L), HDACi (2 μmol/L SAHA or 50 nmol/L LBH589), or the combination for 48 h (D and E) or 72 h (C). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ (compared with mock cells with the same treatment, Student's *t*-test).

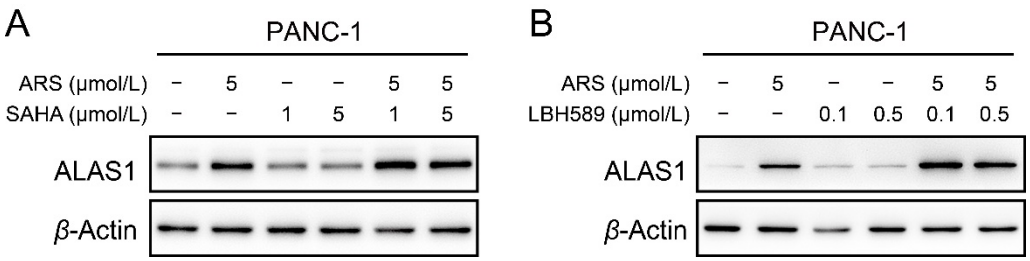

Figure S4 Alteration of ALAS1 expression and heme synthesis after treatment with ARS or HDACi or the combination. (A)–(B) Western blot analysis of ALAS1 in PANC-1 treated with ARS, HDACi [SAHA (A) or LBH-589 (B)] or the combination for 24 h.

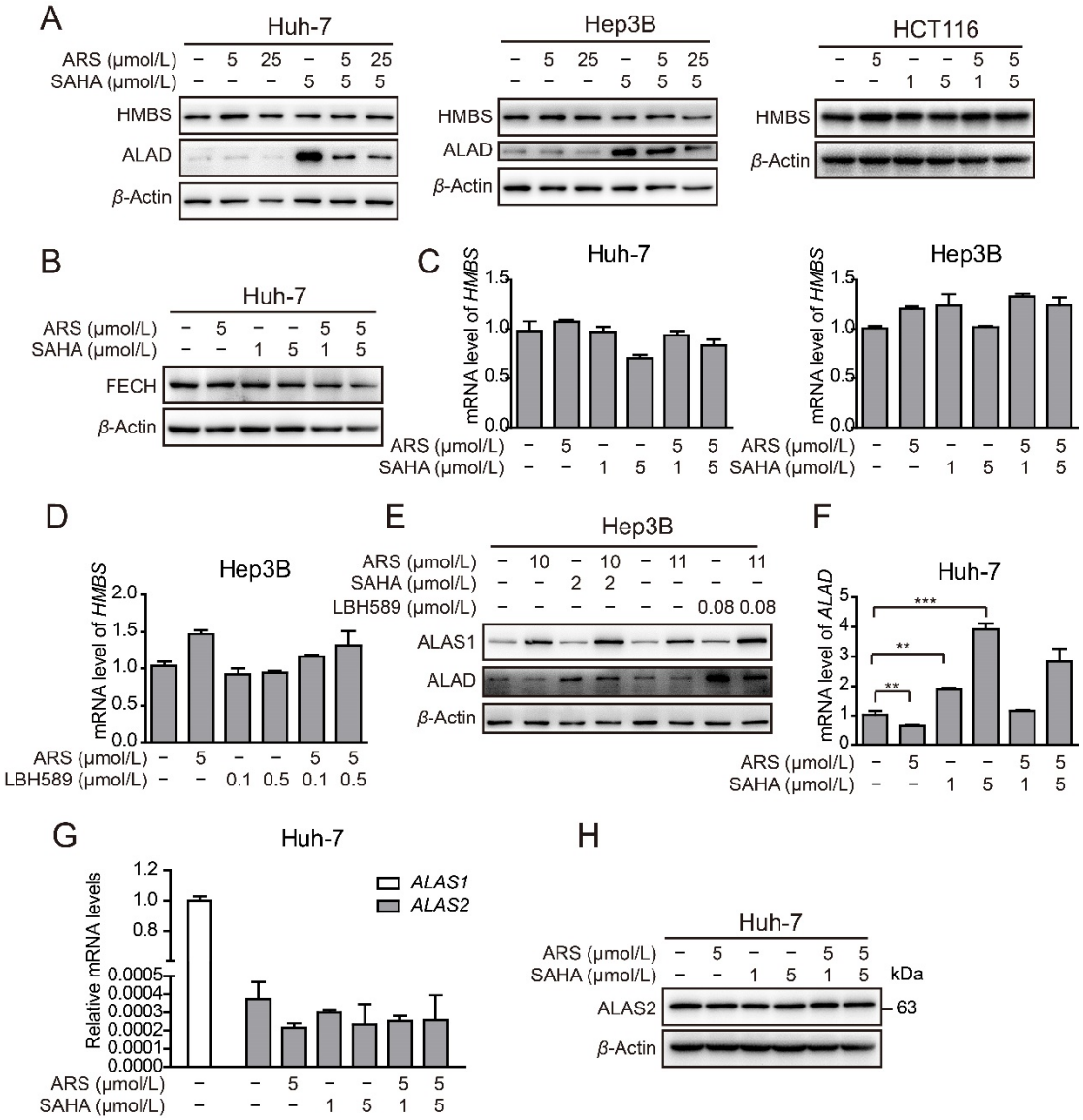

Figure S5 Expression of HMBS, FECH, ALAD and ALAS2 in combined treatment with ARS and HDACi. (A) Expression of HMBS and ALAD in tumor cells treated with ARS, SAHA or both for 24 h. (B) FECH expression in Huh-7 cells after 24 h treatment with compounds as indicated. (C)–(D) Real-time PCR analysis of *HMBS* expression in cells after administration with ARS, SAHA (C) or LBH-589 (D), or combination for 24 h. (E) Western blot validation of ALAS1 and ALAD in Hep3B cells treated with compounds as indicated. (F) Expression of *ALAD* mRNA in Huh-7 cells treated with ARS, SAHA or the combination. $^{**}P < 0.01$, $^{***}P < 0.01$ (Student's *t*-test). (G) mRNA levels of *ALAS2* in cells after administration with control, ARS, SAHA, or combination for 24 h. *ALAS1* expression in control group was normalized as 1. (E) Expression of ALAS2 in Huh-7 cells treated with compounds as indicated.

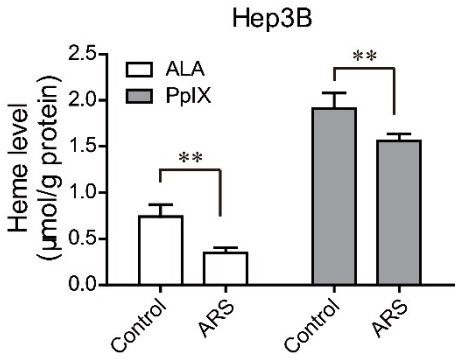

Figure S6 Cellular heme reduced after treatment with ARS. Hep3B cell were pretreated with or without 40 μmol/L of ARS for 12 h, followed with ALA (50 μmol/L) in serum free medium or PpIX (1 μmol/L) treatment for another 4 h. Heme was measured with the cell lysate. $^{**}P < 0.01$, n=3–4 (Student's t-test).