

1 **Inhibition of heat shock protein (Hsp) 27 potentiates the suppressive effect of**
2
3 **Hsp90 inhibitors in targeting breast cancer stem-like cells**

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24 **Abstract**

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26 Heat shock protein (Hsp) 90 is an ATP-dependent chaperone and its expression has
27 been reported to be associated with poor prognosis of breast cancer. Cancer stem cells
28 (CSCs) are particular subtypes of cells in cancer which have been demonstrated to be
29 important to tumor initiation, drug resistance and metastasis. In breast cancer, breast
30 CSCs (BCSCs) are identified as CD24-CD44+ cells or cells with high intracellular
31 aldehyde dehydrogenase activity (ALDH+). Although the clinical trials of Hsp90
32 inhibitors in breast cancer therapy are ongoing, the BCSC targeting effect of them
33 remains unclear. In the present study, we discovered that the expression of Hsp90 α
34 was increased in ALDH+ human breast cancer cells. Geldanamycin (GA), a Hsp90
35 inhibitor, could suppress ALDH+ breast cancer cells in a dose dependent manner. We
36 are interesting in the insufficiently inhibitory effect of low dose GA treatment. It was
37 correlated with the upregulation of Hsp27 and Hsp70. By co-treatment with HSP
38 inhibitors, quercetin or KNK437 potentiated ALDH+ cells toward GA inhibition, as
39 well as anti-proliferation and anti-migration effects of GA. With siRNA mediated
40 gene silencing, we found that knockdown of Hsp27 could mimic the effect of HSP
41 inhibitors to potentiate the BCSC targeting effect of GA. In conclusion, combination
42 of HSP inhibitors with Hsp90 inhibitors could serve as a potential solution to prevent
43 the drug resistance and avoid the toxicity of high dose of Hsp90 inhibitors in clinical
44 application. Furthermore, Hsp27 may play a role in chemoresistant character of
45 BCSCs.
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Highlight

1. Hsp90 α overexpressed in ALDH+ breast cancer stem-like cells (BCSCs).
2. Hsp90 inhibitors induced Hsp27 expression in breast cancer cells.
3. HSP inhibitors sensitized ALDH+ BCSCs toward the suppression of geldanamycin.
4. Knockdown of Hsp27 sensitized ALDH+ BCSCs toward the suppression of geldanamycin.
5. Combination of Hsp90 and HSP inhibitors is suggested as future breast cancer therapy.

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3 **Hsp90 inhibitors in targeting breast cancer stem-like cells**

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Abstract

Heat shock protein (Hsp) 90 is an ATP-dependent chaperone and its expression has been reported to be associated with poor prognosis of breast cancer. Cancer stem cells (CSCs) are particular subtypes of cells in cancer which have been demonstrated to be important to tumor initiation, drug resistance and metastasis. In breast cancer, breast CSCs (BCSCs) are identified as CD24-CD44⁺ cells or cells with high intracellular aldehyde dehydrogenase activity (ALDH⁺). Although the clinical trials of Hsp90 inhibitors in breast cancer therapy are ongoing, the BCSC targeting effect of them remains unclear. In the present study, we discovered that the expression of Hsp90 α was increased in ALDH⁺ human breast cancer cells. Geldanamycin (GA), a Hsp90 inhibitor, could suppress ALDH⁺ breast cancer cells in a dose dependent manner. We are interesting in the insufficiently inhibitory effect of low dose GA treatment. It was correlated with the upregulation of Hsp27 and Hsp70. By co-treatment with HSP inhibitors, quercetin or KNK437 potentiated ALDH⁺ cells toward GA inhibition, as well as anti-proliferation and anti-migration effects of GA. With siRNA mediated gene silencing, we found that knockdown of Hsp27 could mimic the effect of HSP inhibitors to potentiate the BCSC targeting effect of GA. In conclusion, combination of HSP inhibitors with Hsp90 inhibitors could serve as a potential solution to prevent the drug resistance and avoid the toxicity of high dose of Hsp90 inhibitors in clinical application. Furthermore, Hsp27 may play a role in chemoresistant character of BCSCs.

1. Introduction

Heat shock proteins (Hsp) are a group of proteins that were first discovered under heat shock or other chemical stimulus in a wide range of species and function as molecular chaperones that could interact with their substrates to shift the balance from denatured protein conformation toward functional conformation [1]. In addition to the molecular chaperone function, several Hsps have been reported to be overexpressed in various of cancers and display a correlation with patients' survival or response to therapy in specific cancer types and may serve as novel therapeutic targets [2]. The Hsp90 family is consisted of multiple isoforms including cytoplasmic Hsp90 α and Hsp90 β , endoplasmic reticulum Grp74 and mitochondria Trap1 [3]. Hsp90 is known to function as protein stabilizer and its client proteins includes growth receptors, Akt, hormone receptors, and hypoxia-inducible factor, etc. [4]. Many of Hsp90 client proteins are contributed to carcinogenesis and Hsp90 becomes a potential target for cancer therapy[4]. In breast cancer, the overexpression of Hsp90 α is correlated with lymph node involvement [5] and decreased overall survival [5].

17-(Allylamino)-17-demethoxygeldanamycin (17-AAG), a geldanamycin (GA)-based Hsp90 inhibitor, could eradicate ErBB2-derived xenograft [6]. 17-AAG and 17-desmethoxy-17-*N,N*-dimethylaminoethylaminogeldanamycin (17-DMAG) have also shown the clinical responses when combined with Herceptin in patients with progressive diseases after receiving Herceptin therapy [7].

Cancer stem cells (CSCs) have been discovered in varieties of malignancies, including leukemia [8] and solid tumors including breast cancer[9]. In breast cancers, the breast CSCs (BCSCs) are first defined as CD24^{-low}CD44⁺ and only these cells could form tumors when xenotransplantation into NOD/SCID mice [10]. Addition to CD24 and CD44, the activity of aldehyde dehydrogenase I (ALDH1) has also been proven to be a functional marker for both breast stem cells and BCSCs [11]. Using

1 ALDH1 specific fluorescence substrate, ALDH1 activity positive breast cancer cells
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3 could be further sorted out and be demonstrated as BCSC population by xenograft
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5 assay in NOD/SCID mouse model [11]. In addition to the tumor origin property of
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7 CSCs, BCSCs have also been suggested as metastatic origin and chemotherapy
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9 resistance of breast cancer. CD24⁻CD44⁺ BCSCs were noted to be more resistant to
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11 radiation than non-BCSCs [12]. The majority of early disseminated cancer cells in the
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13 bone marrow of breast cancer patients displayed the phenotype of CD24⁻CD44⁺ [13].
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15 Furthermore, CD24⁻CD44⁺ BCSCs isolated from primary human breast cancer
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17 specimens also expressed markers associated with epithelial-mesenchymal transition
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19 [14]. Based on their capability of growth, differentiation, and metastasis, and
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21 resistance to radiation, BCSCs have now become the hotly pursued target for therapy
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23 of breast cancer [9].
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29 Although the clinical trials of Hsp90 inhibitors as a single agent or combination
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31 with other drugs in breast cancer therapy are ongoing [15], the direct effect of Hsp90
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33 inhibitors in targeting BCSCs remains unclear. In the present study, we found that the
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35 expression of Hsp90 α was increased in ALDH⁺ BCSCs and Hsp90 inhibitors could
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37 have suppressive effect in targeting BCSCs at high dose but not sufficient at low dose.
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39 The insufficient inhibitory effect of low dose Hsp90 inhibitor treatment was possible
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41 linked with the induction of Hsp27 and could be reversed by combination with HSP
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43 inhibitors or silencing of Hsp27 by siRNA. Our data suggest that silencing of Hsp27
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45 by siRNA or HSP inhibitors may serve as a potential strategy to offer a way to
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47 increase clinical efficacy of Hsp90 inhibitors in targeting BCSCs.
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2. Materials and Methods

2.1 Cell culture and reagents

AS-B145 and AS-B244 breast cancer cells which derived from BC0145 or BC0244 xenograft human breast cancer cells [16] were provided by Dr. Alice L. Yu (Genomic Research Center, Academia Sinica, Taipei, Taiwan) and were cultured according our previous report [17]. Geldanamycin (GA) was purchase from Stressgen (Ann Arbor, MI, USA) and 17-desmethoxy-17-*N,N*-dimethylaminoethylaminogeldanamycin (17-DMAG) was purchase from LC Laboratories (Woburn, MA, USA). GA and 17-DMAG were dissolved in DMSO and stock at -20°C as 50mM.

2.2 Western blot

For western blot, cells were lysed with NP-40 lysis buffer and 25µg of total protein were separated by SDS-PAGE and transferred to PVDF membrane (Millipore, Billerica, MA, USA). Protein detection was conducted by SignalBoostTM Immunodetection Enhancer kit (Calbiochem, San Diego, CA, USA) according to manufacturer's recommendation. Briefly, primary antibody was diluted with primary antibody solution and incubated with PVDF membrane at 4°C overnight. After washing with 0.1% Tween-20/tris buffer solution, membrane was incubated with secondary antibody which diluted with secondary antibody buffer at room temperature for 1 hour. The signals were developed by ECL-plus chemoluminescence substrate (Perkin-Elmer) and captured with LAS-1000plus Luminescent Image Analyzer (GE Healthcare Biosciences, Piscataway, NJ, USA). The intensity of bands was quantified by ImageJ software (National Institute of Health, Pethesda, MA, USA). Anit-Hsp27, anti-Hsp70, anti-Hsp90α and anti-HSF-1 antibodies were purchased from Stressgen. Anti-GAPDH antibody was purchased from MDBio, Inc. (Taipei, Taiwan). Anti-β-actin antibody was purchased from Novus Biologicals (Littleton, CO,

1 USA).

2 3 4 5 **2.3 ALDEFLUOR assay**

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7 ALDEFLUOR assay kit was purchased from StemCell Technologies, Inc. (Vancouver,
8 BC, Canada) and used following manufacture's recommendation. Briefly, 1×10^5 cells
9 were suspended in 50 μ l of assay buffer and added BODIPY-aminoacetaldehyde
10 substrate to final concentration of 1 μ M. For ALDH1 inhibitor control,
11 diethylaminobenzaldehyde (DEAB) was added to final concentration of 150 μ M. Cells
12 were then incubated at 37 $^{\circ}$ C for 45min and stained with 7-AAD on ice for further
13 5min. After washed with PBS, green fluorescence positive cells in live cells (7AAD-)
14 were analyzed by flow cytometry (Epics XL, Beckman Coulter) by comparing the
15 fluorescence intensity of DEAB treated sample and these cells will be represented as
16 cells with high ALDH activity (ALDH+ cells).
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34 **2.4 Cell titer analysis**

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36 For the cell titer experiment, 1×10^4 (for AS-B145) or 2×10^4 (for AS-B244) cells/well
37 were seeded in 96-well plates and cultured for 48 h with or without compounds or
38 DMSO. Cell titers were determined by WST-1 cell viability assay reagent (BioVision,
39 Mountain View, CA, USA) using a microplate reader (Molecular Devices, Sunnyvale,
40 CA, USA) according to the manufacturer's recommendations. The absorbance values
41 at 440nm of DMSO control were set as 100% of cell titer.
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53 **2.5 Cell migration**

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55 Cell migration assay was conducted by Oris Universal Cell Migration Assembly kit
56 (Platypus Technologies, LCC, Madison, WI, USA) following manufacture's protocol.
57 Briefly, 5×10^4 cells/well/100 μ l were loaded into stopper loaded wells and incubated
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1 overnight to permit cell attachment. To start cell migration, the stoppers was removed,
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3 wash wells gently with PBS, added complete cell culture medium and incubate for
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5 16-18h. Pictures of wells were captured with inverted microscopy after fixation and
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7 stain with 0.5% crystal violet/50% EtOH. Data were analyzed with ImageJ software.
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10 11 12 **2.6 RNA interference**

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14 The Hsp27 specific siRNA oligos or negative control siRNA oligos was purchased
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16 from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA, USA). MetafecteneSI
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18 transfection reagent (Biontex, Martinsried, Germany) was used for siRNA
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20 transfection following manufacture's protocol. ALDH+ population or protein
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22 expression (Hsp27, Hsp70, HSF-1, and GADPH) of cells was determined at 48h
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24 post-transfection.
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31 32 **2.7 Statistical analysis**

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34 Statistical comparison between groups was calculated with unpaired *t*-test and p value
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36 less than 0.05 was considered to be statistically significant which was indicated in
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38 figures, separately.
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3. Results

3.1 The effect of Hsp90 inhibitors in ALDH+ BCSC population of breast cancer cells

Upregulation of Hsp90 in breast cancer tissues has been reported and we first examined the expression of Hsp90 α in ALDH- (non-BCSCs) or ALDH+ (BCSCs) cells of AS-B244 human breast cancer cells. By western blot analysis, the expression of Hsp90 α was increased to 1.43 fold in ALDH+ AS-B244 cells in comparison with ALDH- cells (Fig. 1A). We next examined the effect of Hsp90 inhibitors, GA and 17-DMAG, in targeting ALDH+ BCSCs. With ALDEFLUOR assay, the ALDH+ population of both AS-B145 and AS-B244 human breast cancer cells could be suppressed by GA treatment in a dose dependent manner (Fig. 1B and 1C). Akt is a well-known client of Hsp90 α [18] and it also plays an important role in regulation of the maintenance of BCSCs [19]. We next checked the Akt activation in AS-B145 or AS-B244 cells under GA or 17-DMAG treatment. From Figure 1D and 1E, the Akt protein and its phosphorylation was suppressed by GA or 17-DMAG treatment in a dose dependent manner. From these results, Hsp90 inhibitors could target ALDH+ BCSCs through their suppressive effect of Akt activation.

3.2 Treatment of Hsp90 inhibitors induced Hsp27 and Hsp70 expression in human breast cancer cells

It has been demonstrated that treatment of Hsp90 inhibitors in cells would cause the release of heat shock factor-1 (HSF-1) and leading to the upregulation of other HSPs, such as Hsp27 and Hsp70 [20], which may cause resistance of Hsp90 inhibitor treatment [21, 22]. We next examined if treatment of Hsp90 inhibitors also induces Hsp27 or Hsp70 expression in AS-B145 or AS-B244 breast cancer cells. Consistence with previous reports, GA or 17-DMAG could induce Hsp27 or Hsp70 expression in

1 AS-B145 breast cancer cells (Fig. 1D). GA also caused upregulation of Hsp27 or
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3 Hsp70 expression in AS-B244 cells (Fig. 1E). It suggested that the induction of Hsp27
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5 or Hsp70 may cause resistance of low dose treatment of Hsp90 inhibitors in ALDH+
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7 BCSCs. We next examined if there is a synergistic effect of HSP inhibitors in
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9 targeting ALDH+ BCSCs when combination with Hsp90 inhibitors. In addition to
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11 Hsp90, Hsp27 has also been reported to maintain the protein stability of Akt[23]. We
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13 next investigated if knockdown of Hsp27 in breast cancer cells could influence Akt
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15 activation. After transfection with Hsp27 specific siRNA, the Akt protein, as well as its
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17 phosphorylation, was not changed in comparison with negative control siRNA
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19 transfected cells (Fig. 1F). It suggests that Hsp27 is not responsible for Akt stability or
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21 activation in AS-B145 or AS-B244 cells.
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29 **3.3 HSP inhibitors sensitized ALDH+ BCSCs toward Hsp90 inhibitors**

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31 Because the Hsp27 and Hsp70 expression was induced in low dose treatment of
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33 Hsp90 inhibitors, we next investigated if the combination of HSP inhibitors could
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35 sensitize breast cancer cells toward Hsp90 inhibitors. Quercetin, which is a plant
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37 flavonoid compound found in vegetables and fruits, has been reported to inhibit the
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39 expression of Hsp27 and Hsp70 [24]. KNK437 is an inhibitor of HSF-1 and has been
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41 shown to suppress the expression of heat induced HSPs expression [25]. The ALDH+
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43 population of cells was only minor decreased when breast cancer cells were treated
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45 with low dose of GA (0.5 μ M for AS-B145 and 40nM for AS-B244) but there was a
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47 synergistic effect in combination with 20 μ M of quercetin. Similar results were also
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49 observed when combination with 20 μ M (for AS-B145) or 10 μ M (for AS-B244) of
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51 KNK437 (Fig. 2). By western blot analysis, combination with HSP inhibitors
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53 inhibited the GA-induced Hsp27 or Hsp70 expression in both AS-B145 and AS-B244
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55 breast cancer cells (Fig. 3). Furthermore, HSP inhibitors also suppressed the
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1 expression of Hsp90 α in both AS-B145 and AS-B244 breast cancer cells (Fig. 3). In
2 addition to ALDH+ population, we also examined the anti-proliferative effect of GA
3 or 17-DMAG in breast cancer cells. We first investigated the cytotoxic effects of GA
4 or 17-DMAG in AS-B244 or AS-B145 cells. GA or 17-DMAG suppressed cell
5 proliferation of AS-B244 or AS-B145 in a dose-dependent manner (Fig.4A and4B).
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7 The IC₅₀ value of GA to AS-B244 or 17-DMAG to AS-B145 was (59.4 \pm 8.4) nM or
8 (7.9 \pm 0.9) μ M, respectively (Fig. 4A and 4B). We next examined if there is a
9 synergistic effect of HSP inhibitors and Hsp90 inhibitors in cytotoxic effect to breast
10 cancer cells. Cells treated with GA only caused 11.5% reduction in cell proliferation
11 of AS-B244 cells at 40nM but caused 62.1% or 66.6% reduction when combination
12 with 25 μ M of quercetin or 20 μ M of KNK437, respectively (Fig. 4A). The synergistic
13 effect in cell proliferation was also observed in treatment of 17-DMAG. Cells treated
14 17-DMAG caused 32% reduction in cell proliferation of AS-B145 cells at 5 μ M and
15 caused 82.6% or 97.5% reduction when combination with 40 μ M of quercetin or
16 12.5 μ M of KNK437, respectively (Fig. 4B). We next tested the effect of combination
17 treatment of GA and quercetin or KNK437 in cells migration of breast cancer cells.
18 Single treatment of GA (0.5 μ M for AS-B145 or 40nM for AS-B244), quercetin
19 (20 μ M for both cell lines) or KNK437 (20 μ M for AS-B145 or 10 μ M for AS-B244)
20 did not significantly inhibit cell migration of breast cancer cells. In contrast,
21 co-treatment of GA with quercetin or with KNK437 could cause significant inhibition
22 of cell migration in both AS-B145 and AS-B244 cells (Fig. 5). These results indicate
23 that HSP inhibitors could sensitize BCSCs toward the inhibitory effects of Hsp90
24 inhibitors on ALDH+ population, cell proliferation and cell migration.
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58 **3.4 Silencing of Hsp27 potentiated the suppression effect of GA in targeting**

59 **ALDH+ BCSCs**

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1 It has been demonstrated that forced Hsp27 expression caused resistance of 17-AAG
2 treatment in PC3 prostate cancer cells [22]. We next examined if knockdown of Hsp27
3 expression could potentiate ALDH+ BCSCs to the suppressive effect of GA.
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5 Knockdown of Hsp27 expression in AS-B244 cells with specific siRNA caused 20.3%
6 reduction in ALDH+ population and 63.7% reduction when combination with 40nM
7 of GA (Fig. 6A). By western blot analysis, knockdown of Hsp27 caused
8
9 down-regulation of Hsp70 and HSF-1 protein expression, which was consistent with a
10 previous report showing that silencing of Hsp25 (Hsp27 homolog in mice) expression
11 caused reduction of Hsp70 and HSF-1 [26]. When Hsp27 knockdown AS-B244 cells
12 were treated with GA, the protein expression of Hsp70 and HSF-1 were reversed to
13 the level of negative control siRNA transfected and DMSO treated cells (Fig. 6B). It
14 also suggests that Hsp27, but not Hsp70, plays the protection role of ALDH+ BCSCs
15 in resistant to low dose of GA treatment.
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4. Discussion

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3 Ansamycin-related Hsp90 inhibitors, such as GA, 17-AAG or 17-DMAG, not only
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5 inhibit Hsp90 function through binding the ATP pocket of Hsp90 [27], but also induce
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7 heat shock response which is mediated by HSFs [28]. Under unstress condition, HSF1
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9 states as an inactive monomer within a complex which consists of Hsp90, Hsp70,
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11 FK506 binding protein 52 and p23 [29]. Cell stresses, such as heat shock or oxidative
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13 stress, or Hsp90 inhibition disrupt this complex and promote the homotrimer of HSF1
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15 which leads to upregulation of many HSPs [20, 29]. The inadvertent induction of
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17 HSPs may be responsible for resistance of Hsp90 inhibitors. Within the proteins
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19 induced by Hsp90 inhibitors, Hsp70 and Hsp27 are the most frequently observed.
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22 Combination with HSP inhibitors [21] or knockdown of Hsp70 [30] abolished
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24 17-AAG induced Hsp72 expression in malignant cells and led to additive or
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26 synergistic anti-proliferative effect. In GA or 17-AAG resistant A549 lung cancer
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28 cells, downregulation of Hsp27 by siRNA dramatically diminished the resistance [22].
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31 In our study, we also demonstrated that combination with HSP inhibitors and Hsp90
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33 inhibitors induced obvious effect on the inhibition of ALDH⁺ cells (Fig. 2), cell
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35 proliferation (Fig. 4) and cell migration (Fig. 5) of breast cancer cells. Although
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37 re-expression of Hsp70 and HSF1 was observed in Hsp27 knockdown AS-B244 cells
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39 after treated with GA, but the ALDH⁺ cells were even much more diminished than
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41 cells which treated with GA only (Fig.6). In addition, knockdown of Hsp27 in
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43 AS-B145 or AS-B244 did not change the activation status of Akt. Although Hsp27
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45 has been reported to regulate the protein stability and the activation of Akt [31, 32], its
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47 role in BCSCs seems to be Akt-independent. We have recently found that Hsp27
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49 could regulate the maintenance and tumorigenicity of BCSCs through
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51 epithelial-mesenchymal transition and NF- κ B [17]. It is interesting to search for other
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53 clients of Hsp27 in BCSCs to understand their role in maintenance of BCSCs. These
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1 results suggest that the effects of combination treatment in targeting BCSCs were due
2 to downregulation of Hsp27 expression. It also suggests that Hsp27 may play a role in
3 the drug-resistant character of BCSCs.
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7 Although ansamycin-related Hsp90 inhibitors displayed tumoricidal effects, the
8 toxicity limited the progress of their clinical evaluation in cancer therapy. GA was
9 proved to be effective against cancer in vitro but induced severe hepatotoxicity which
10 limited its clinical potential [33]. 17-AAG and 17-DMAG are two analogues of GA
11 which are designed to reduce the toxicity. Although acceptable toxicity was observed
12 in weekly injection schedule of 17-AAG, the relative short half-life and recovery of
13 client proteins were noticed [34]. The maximally tolerated dose of 17-AAG on a
14 twice-weekly schedule was 220mg/m^2 with side effects including diarrhea, fatigue,
15 nausea, etc [35]. In refractory acute myelogenous leukemia patients, combination of
16 17-DMAG and chemotherapy displayed complete responses in 3 out of 17 patients
17 but the trial was terminated because of the unfavorable toxicity [36]. Our data
18 indicated that treatment of HSP inhibitors could sensitize breast cancer cells toward
19 the inhibitory effect of low dose GA or 17-DMAG in targeting ALDH+ BCSCs.
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22 KNK437 is a weak toxic drug. At the dose of 200 mg/kg, the KNK437 concentration
23 in mice sera and in tumor was $83.26\ \mu\text{g/ml}$ ($339.5\ \mu\text{M}$) and $21.46\ \mu\text{g/g}$, respectively,
24 without obvious side effect [25]. Quercetin has been suggested to administrate as
25 $1400\ \text{mg/m}^2$ in human with limited renal toxicity [37]. When quercetin was
26 encapsulated with polyethylene glycol 4000 liposome, above $30\ \mu\text{M}$ of free quercetin
27 was maintained up to 8h in mice sera at the injection dose of 50mg/kg [38]. The
28 concentration of quercetin or KNK437 for combination treatment in targeting ALDH+
29 BCSCs in this study (up to $20\ \mu\text{M}$ for quercetin or KNK437, Fig. 2) could be reached
30 at least in mice sera under maximal injection dose in vivo. Combination therapy of
31 low dose Hsp90 inhibitors and HSP inhibitors may provide a solution in reduction of
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the toxicity of Hsp90 inhibitors when apply into clinical cancer therapy.

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5. Conclusions

In the present study, we first discovered that Hsp90 α overexpressed in ALDH+ BCSCs. The Hsp90 inhibitor, GA, could decrease ALDH+ cells in two human breast cancer cells in a dose dependent manner. In consideration of hepatic toxicity of high concentration of GA, we further tested the combination therapy of HSP inhibitors with suboptimal dosage of GA and discovered that HSP inhibitors could sensitize ALDH+ BCSCs toward GA inhibition. We also demonstrated that suppression of GA-induced Hsp27 upregulation could increase the sensitivity of ALDH+ cells toward GA treatment. Combination therapy of Hsp90 inhibitors and HSP inhibitors, especially Hsp7 inhibitors, may be considered in future breast cancer therapy.

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Disclosure Statement

The authors have no conflicts of interest.

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Figure 1. Hsp90 α expression and the effect of Hsp90 inhibitors in breast cancer cells. (A) ALDH⁻ or ALDH⁺ cells of AS-B244 breast cancer cells were sorted by cell sorter after stained with ALDEFLOUR assay. Hsp90 α expression was determined by western blot and the ratio of band intensities between ALDH⁻ and ALDH⁺ cells were calculated with after normalization with GAPDH. (B, C) AS-B145 or AS-B244 cells were treated with 0.1% DMSO or indicated concentration of GA for 48h and then harvested to analyze ALDH⁺ population of cells with ALDEFLOUR assay. The percentage of ALDH⁺ cells was calculated in comparison with DEAB-treated cells. The quantitative results were calculated from two independent experiments and displayed as bar graph. *, p<0.05; #, p<0.01. (D, E) AS-B145 (D) or AS-B244 (E) cells were treated with 0.1% DMSO or indicated concentration of GA or 17-DMAG for 48h to detect the expression of Akt, phosphor-Akt (p-Akt), Hsp27, Hsp70 and Hsp90 α by western blot. The ratio of band intensities between DMSO and drug-treated cells were calculated after normalization with the intensities of GAPDH (AS-B145) or actin (AS-B244). (F) The expression of Akt and p-Akt was determined in Hsp27-knockdown AS-B145 or AS-B244 cells at 48h post-transfection. The ratio of band intensities between DMSO and drug-treated cells were calculated after normalization with the intensities of actin.

Figure 2. HSP inhibitors sensitize breast cancer cells toward the effect of Hsp90 inhibitors in targeting ALDH⁺ BCSCs. AS-B145 cells were treated with 0.5 μ M GA alone or in combination with 20 μ M quercetin or 20 μ M KNK437 for 48h (A). AS-B244 cells were treated with 40nM GA alone or in combination with 20 μ M quercetin or 10 μ M KNK437 for 48h (B). The ALDH⁺ population of cells was determined by ALDEFLOUR assay. The quantitative results were calculated from two

1 independent experiments and shown in bar graph. * or † , p<0.05; # or ‡, p<0.01. * or
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3 # represented the statistical results after comparison with DMSO; † or ‡ represented
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5 the statistical results after comparison with GA.
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10 **Figure 3. HSP inhibitors repress GA induced Hsp27 and Hsp70 expression.** Cells
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12 were treated with GA alone (0.5µM for AS-B145 or 40nM for AS-B244) or in
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14 combination with quercetin (Q, 20µM for both cell lines) or KNK437 (K, 20µM for
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16 AS-B145 or 10µM for AS-B244) for 48h and the expression of Hsp27, Hsp70 and
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18 Hsp90α was determined by western blot. The ratio of band intensities between
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20 DMSO and drug-treated cells were calculated after normalization with actin.
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27 **Figure 4. HSP inhibitors sensitize breast cancer cells toward the**
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29 **anti-proliferative effect of Hsp90 inhibitors.** AS-B244 cells (A, C) or AS-B145
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31 cells (B, D) were treated with different concentration of GA (A), 17-DMAG (B) or
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33 indicated drugs (C, D) for 48h. Cell proliferation was determined by WST-1 substrate
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35 and the IC₅₀ value (indicated with dotted arrow) was calculated by GraFit software.
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37 The treated concentration of GA, quercetin or KNK437 for AS-B244 cells was 40nM,
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39 25µM or 20µM, respectively (C). The concentration of 17-DMAG, quercetin or
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41 KNK437 for AS-B145 cells was 5µM, 40µM or 12.5µM, respectively (D). Results
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43 were presented as 440nm absorbance relative to DMSO control. *, p<0.05 when
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45 comparison with 17-DMAG treated group; †, p<0.05 when comparison with quercetin
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47 treated group; ‡, p<0.05 when comparison with KNK437 treated group.
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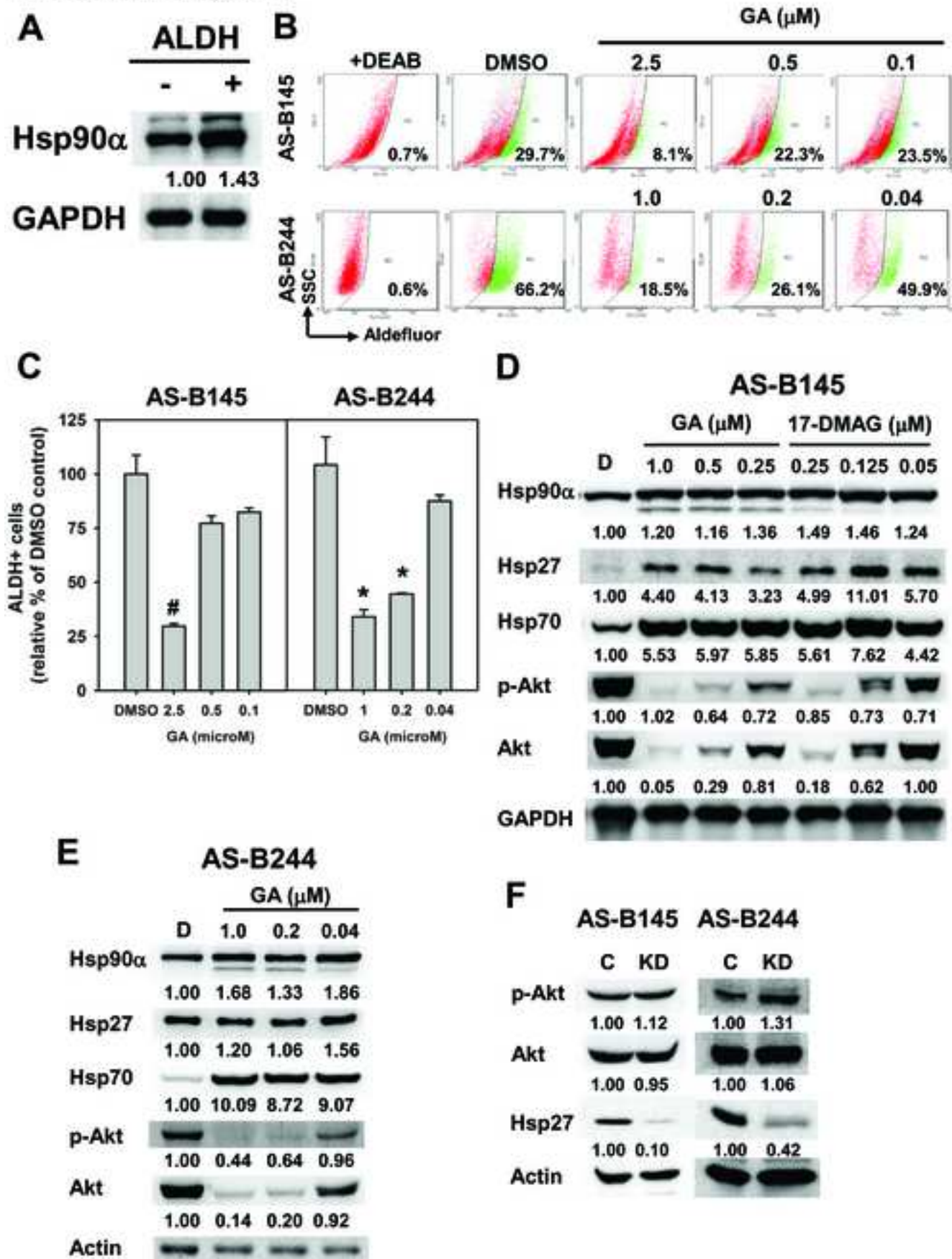
55 **Figure 5. HSP inhibitors sensitize breast cancer cells toward the anti-migration**
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57 **effect of GA.** AS-B244 (A) or AS-B145 (B) cells were seeded into wells of
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59 96-well-plate in presence of stopper for overnight and then changed drug containing
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1 medium as indicated after removal of stopper. The concentration of GA, quercetin or
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3 KNK437 for AS-B244 (A) was 40nM, 20 μ M or 10 μ M, respectively. The
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5 concentration of GA, quercetin or KNK437 for AS-B145 (B) was 0.5 μ M, 20 μ M or
6
7 20 μ M, respectively. Migrated cells were stained with 0.5% crystal violet, taken
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9 pictures and calculated with IamgeJ software. *, p<0.05; #, p<0.01 when comparison
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11 with DMSO group.
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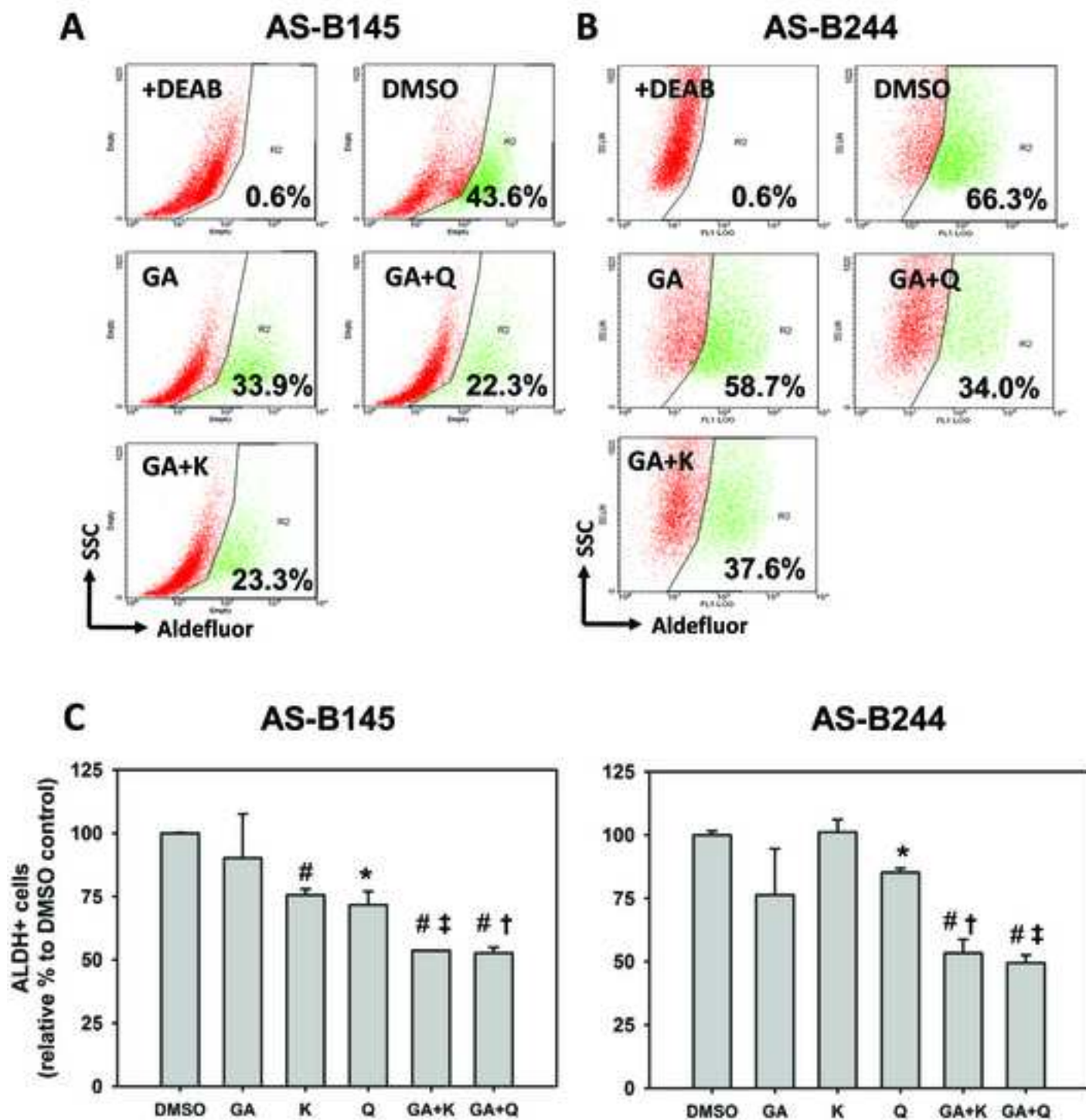
17 **Figure 6. Silencing of Hsp27 potentiates the effect of GA in targeting ALDH+**

18 **BCSCs.** (A) AS-B244 cells were transfected with 100nM negative control siRNA (ctrl
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20 siRNA) or Hsp27 siRNA (si-Hsp27) for 24h and then treated with 0.1% DMSO or
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22 40nM GA for another 48h and followed by determining the ALDH+ population by
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24 ALDEFLUOR assay. The quantitative results were calculated from two independent
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26 experiments and shown in bar graph. *, p<0.05; #, p<0.01. (B) The expression of
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28 Hsp27, Hsp70, Hsp90 α or HSF-1 (indicated with arrow) in Hsp27 knockdown cells
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30 with or without GA treatment was determined by western blot. The ratios of band
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32 intensities were calculated and presence as relative to ctrl siRNA co-treated with 0.1%
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34 DMSO group.
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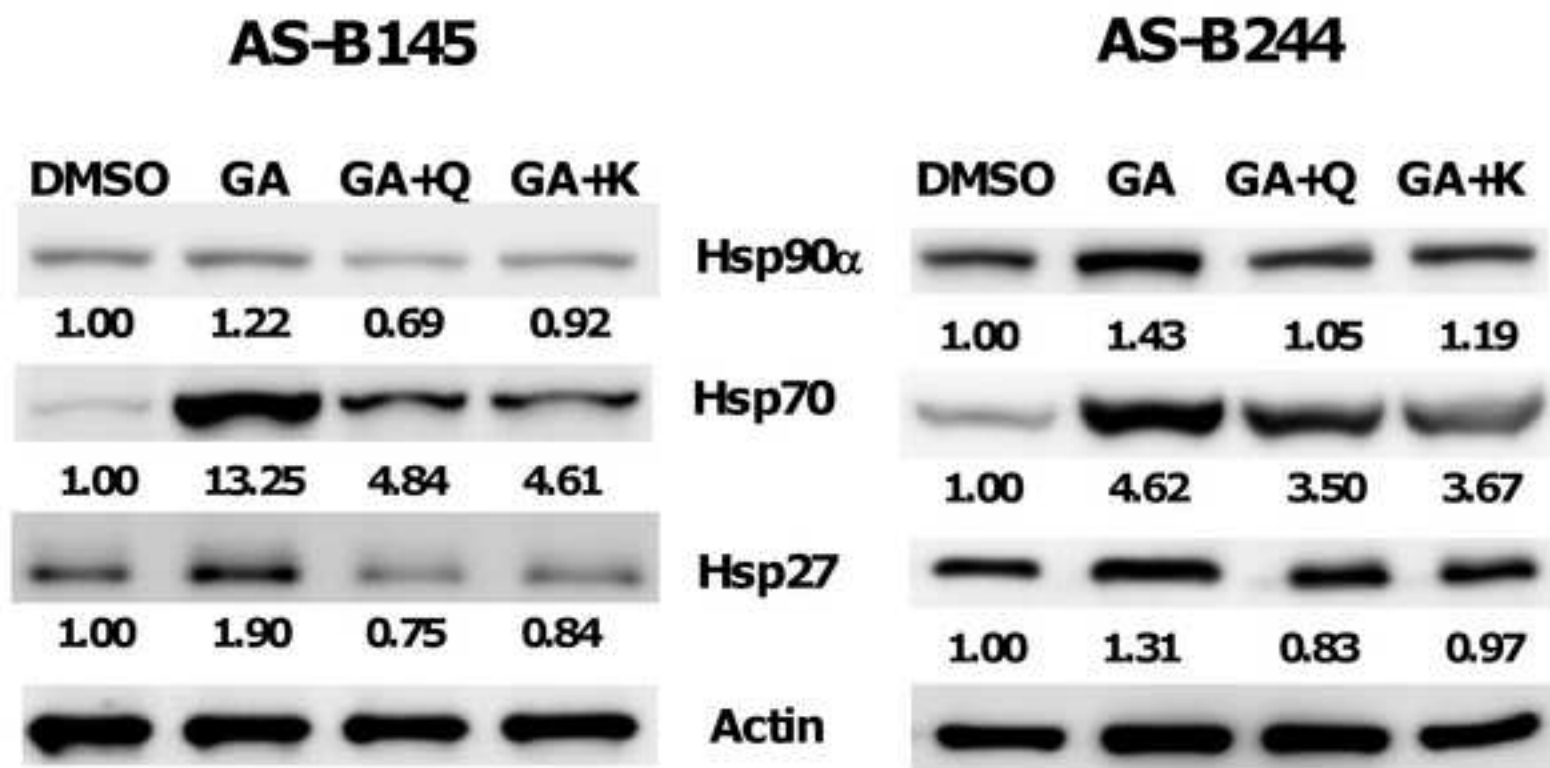
Lee CH et al. Figure 1



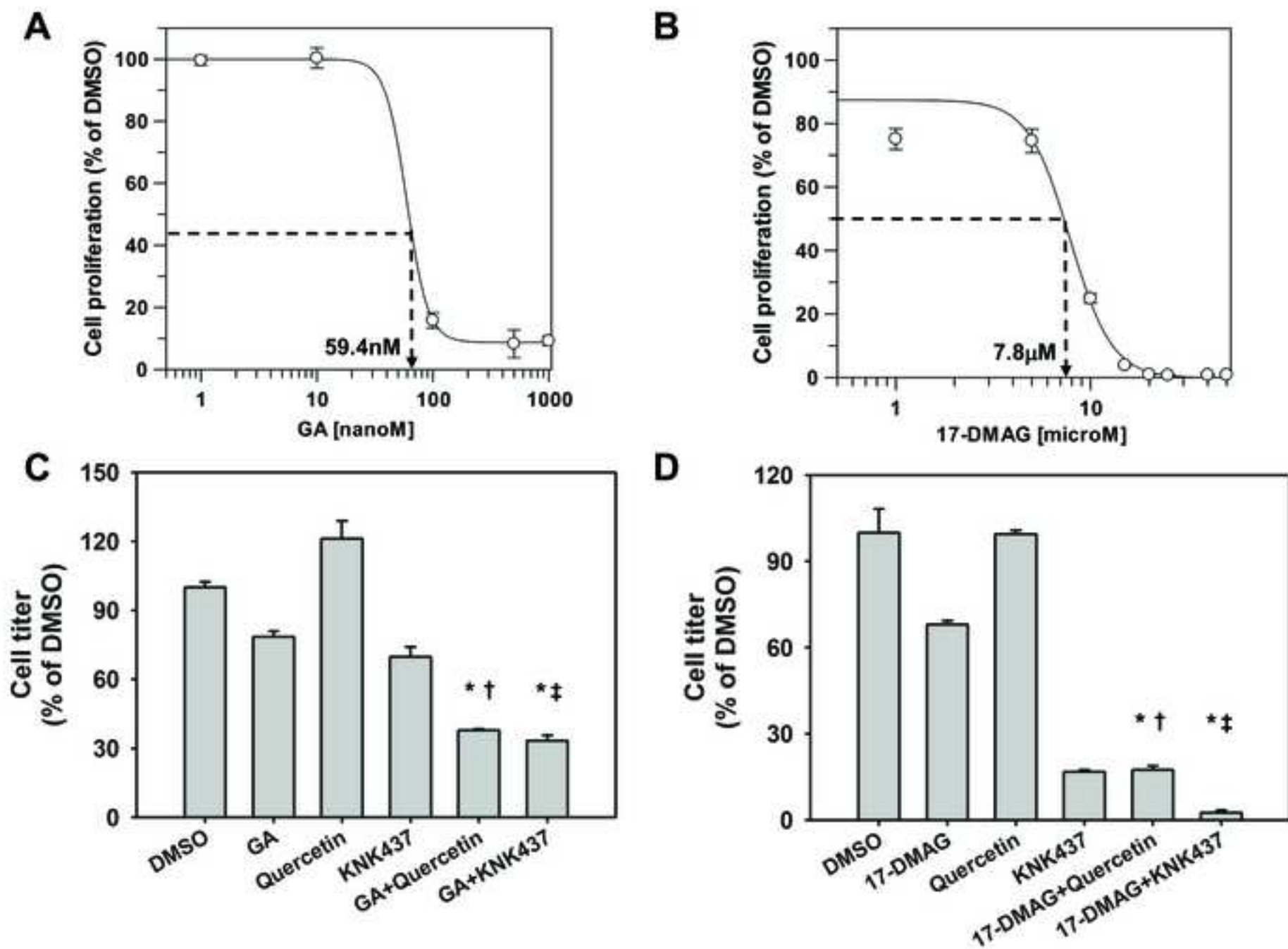
Lee CH et al. Figure 2



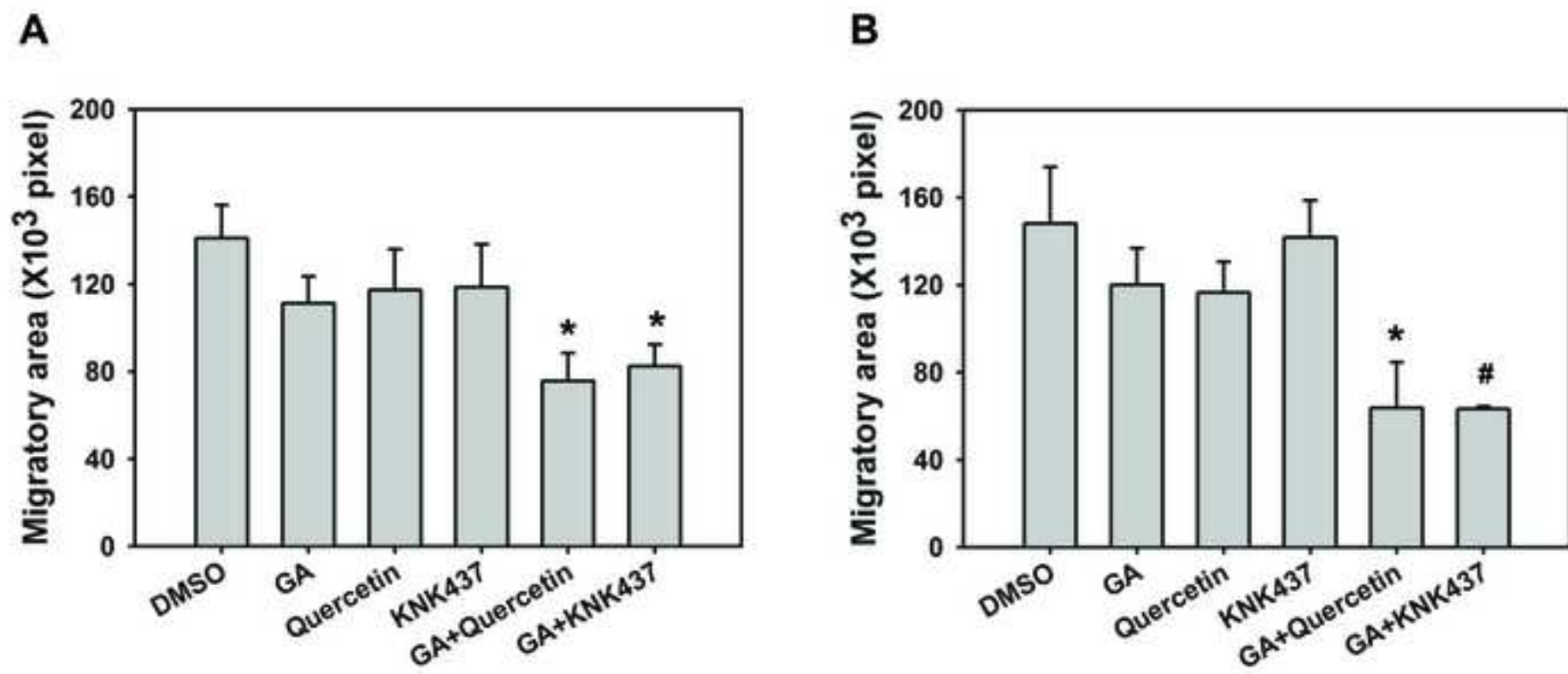
Lee CH et al. Figure 3.



Lee CH et al. Figure 4.



Lee CH et al. Figure 5.



Lee CH et al. Figure 6.

