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Entinostat is a Novel Therapeutic Agent to Treat Oral Squamous Cell Carcinoma

Running/short title: *In vitro* effects of Entinostat on OSCC

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ABSTRACT

Introduction: Alterations of the epigenome may influence cancer initiation and progression. At the cellular level, histones are key regulators of chromatin accessibility and gene transcription; thus, inhibition of histone deacetylase enzymes (HDACs) constitutes an attractive target for therapy. In this study, we investigated the effects of the HDAC inhibitor Entinostat on oral squamous cell carcinoma (OSCC).

Materials and Methods: We tested the effects of Entinostat on OSCC cell lines. Cell viability and growth were analyzed using MTT assay. Cell cycle analysis, cell apoptosis, cancer stem cells (CSCs) content, and the concentration of reactive oxygen species (ROS) in OSCC tumor cells were assessed using flow cytometry. The expression of histones and cell cycle regulatory proteins were examined by western blot.

Results: Administration of Entinostat resulted in reduced proliferation of OSCC cells, followed by cell cycle arrest at the G0/G1 phase, as well as substantial tumor apoptosis. We also found an increase in ROS production and significant reductions in CSCs. We also found that Entinostat caused increased acetylation histones H3 or H4, and changes in the expression of cell cycle-associated proteins such as p21.

Conclusion: This study indicates that Entinostat is a potential novel therapeutic agent for OSCC by halting tumor proliferation, inducing cytotoxicity and intracellular ROS, and attacking the CSCs.

Keywords: Oral Cancer; Epigenetics; HDAC inhibitor; Apoptosis; Cancer Stem Cells.

INTRODUCTION

Oral cancer is one of the leading cancers with a nearly 50% mortality rate, and the overall 5-year survival rate is approximately 60% . Tobacco, alcohol, and HPV infection are the most important risk factors for oral squamous cell carcinoma (OSCC) (1). Conventional therapy for OSCC includes multimodal approaches consisting of surgery, radiation, chemotherapy, and combinations based on the stage at the time of diagnosis (2, 3). Recent advances in cell and molecular biology have made personalized medicine viable.

Epigenetic modifications and posttranslational protein alterations have become novel targets for anticancer therapies. The acetylation of the ε-amino group present at lysine residues of histone proteins plays a central role in gene transcription and cell survival . Histone acetyltransferases (HATs) and histone deacetylases (HDACs) control the acetylation levels of histones and chromatin accessibility (4, 5).

Entinostat (SNDX-275 and MS-275) is the first amino-benzamide-based HDAC inhibitors (HDACi) currently being tested in several clinical trials (6-8). Entinostat is a bioavailable class I HDACi with a long half-life. In vitro and in vivo studies have shown that administration of Entinostat as a single or combined therapy represents a promising drug for managing breast and esophageal cancers due to its antiproliferative and pro-apoptotic effects (9, 10). Nevertheless, there is a gap in the current knowledge of the potential efficacy of Entinostat in managing OSCC. Here we investigate the biological effects of Entinostat on the behavior of OSCC.

MATERIAL AND METHODS

Cell lines and culture conditions

Oral squamous cell carcinoma cell lines WSU-HN6 and WSU-HN12 (primary and metastatic OSCC from the tongue respectively) and an immortalized keratinocyte (HaCat) cell line were kept in a 5% CO_2 humidified incubator at 37°C, and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% antibiotic.

Cell Proliferation, Viability, and IC50

Cell viability was assessed by the MTT assay (MTT Cell Proliferation Assay Kit, Trevigen, Gaithersburg, MD, USA) following the manufacturer's protocol. The experiments were done in sextuplicate. Dose-response curves (IC50) for Entinostat were

performed using a drug concentration range of 0.0001μM to 1000μM for 24 hours. Control cells received vehicle alone.

Cell cycle assay

Cells were seeded and treated with Entinostat and vehicle for 24h. Cells were fixed with 100% cold ethanol, following the addition of propidium iodide solution (PI-RNAse) and incubation at room temperature in the dark for 20 min. Samples were analyzed by flow cytometry using Accuri C6 Plus Flow Cytometer (BD Biosciences, San Jose, CA, USA) and Accuri CFlow Plus software. The assays were run in triplicates.

Cell apoptosis assay

Cell apoptosis was assessed using CellEvent Caspase-3/7 Green Flow Cytometry Assay (Life Technologies, Carlsbad, California, USA) according to the manufacturer instructions. Briefly, 1 μL of CellEvent Caspase-3/7 Green Detection Reagent was added to samples containing 1 mL of cell suspension at $1x10^6$ cells/mL and incubated for 25 minutes at 37 °C. Then, 1 μL of 1 mM SYTOX AADvanced dead cell stain solution was added to samples and incubated for 5 minutes at 37 °C. A minimal of triplicates assays were performed. The flow cytometry was used to analyze the samples.

ROS detection assay

Cells were trypsinized and centrifuged at 400xg for 5 minutes. Next, cells were resuspended in fresh medium at the concentration of 1×10^5 cells/mL. Cells were incubated with ROS/Superoxide solution (Enzo Life Sciences, New York, USA) along with the administration of Entinostat or vehicle as control for 1 hour at 37°C in the dark. Unstained cells were used to determine the negative population. Quality controls were performed using the ROS inducer Pyocyanin as positive control, and ROS inhibitor N-acetyl-L-cysteine as negative control. ROS levels were identified by flow cytometry.

Cancer Stem Cell assay

Tumor cells receiving Entinostat or vehicle control were trypsinized, counted, and resuspended in AldeFluor assay buffer ($1x10^6$ cells/mL) containing ALDH substrate (Aldefluor kit - StemCell Technologies, Durham, NC). The assay was carried out following the manufacture's protocol. Cells were stained with anti-CD44 antibody

conjugated with APC (clone G44-26, BD Biosciences, San Jose, CA, USA). Negative controls received the ALDH inhibitor diethylaminobenzaldehyde (DEAB). Additional control samples stained for CD44 and unstained cells were also used in the assay. All experiments were performed in sextuplicate. All samples were analyzed using flow cytometry.

Western blot analysis

Entinostat and vehicle-treated cells were washed with cold PBS, lysed, and proteins were denatured (boiled) following run on SDS-Page electrophoresis gel. Western blotting was performed using standard techniques. Primary anti-human antibodies (suppl. table 1) were diluted in 5% bovine serum albumin (BSA) or 5% milk/TBS-T) and added to the WB membrane (4^{o}C/overnight). The secondary antibodies (horseradish peroxidase-HRP substrate) were diluted in 5% milk/TBS-T (1:3000 dilution). The signal was captured using the enhanced chemiluminescence (ECL) system. GAPDH protein was used as a loading control.

Statistical analysis

Statistical analyses for cell viability assays and flow cytometry experiments were carried out by Student`s t-test, Kruskal Wallis, and Welch's t-test using GraphPad software by PRISM (GraphPad Software, San Diego, CA, USA). Dose-response curves were created using GraphPad version 7.0. All flow cytometry experiments were repeated in sextuplicate. The p values $\leqq 0.05$ reflects statistically significant. Asterisks indicate statistical significance (*$p \leqq 0.05$; **$p \leqq 0.01$; ***$p \leqq 0.001$; ****$p \leqq 0.0001$).

RESULTS

Entinostat reduces OSCC cell viability and SIRT1 levels

The mammalian genome is packed into the chromatin, which consists of DNA together with histone and nonhistone proteins. The epigenome constitutes a group of molecules and chemical compounds capable of modifying gene transcription. Changes to the epigenome can contribute to the dysregulation of key cellular processes, including cellular proliferation (11). Here, we investigated whether Entinostat-induced inhibition

of HDAC 1 and 3 would affect the proliferation of OSCC tumor cells. We found that the administration of Entinostat resulted in a dose-dependent reduction in the proliferation of cancer cells (Fig. 1A-C). Next, we performed the cell viability assay and determined the IC50 values for all cell lines, which were 18.74 μM for keratinocyte, 0.54 μM for WSU-HN6, and 23.31 μM for WSU-HN12 (Fig. 1D-F). We also analyzed the ability of Entinostat in modifying the epigenome of tumor cells through the identification of the acetylation levels of histones H3 and H4. We observed increased acetylation of histone H3 and H4 in control, WSU-HN6, and WSU-HN12 cells, compared to the vehicle-treated cells. Notably, there was a decrease of the enzyme deacetylase SIRT1 in all cells, confirming the efficacy of Entinostat as a potent inhibitor of HDAC (Fig. 1G).

Entinostat activates p21 and induces cell cycle arrest

Next, we investigated the impact of Entinostat on the tumor cell cycle using flow cytometry. Overall, we found that Entinostat stimulates cells to undergo G0/G1 cell cycle arrest in all analyzed cell lines (Fig. 2A-F). While OSCC tumor cells did not present an increase in the number of cells undergoing DNA synthesis (S phase) upon administration of Entinostat (ns - $p>0.05$), we observed that normal epithelial cells progressed from G0/G1 to S phase during Entinostat treatment compared with the vehicle-treated group ($p \leqq 0.001$) (Fig. G-I). Our data support the fact that the administration of Entinostat is efficient in enhancing the number of cells to undergo G0/G1 cell cycle arrest, while only normal keratinocyte cells responded to the treatment by increasing DNA synthesis (Fig. 2G).

Non-histone targets of HDAC may include transcription factors and other proteins involved in cancer development and progression (12). Interestingly, we observed a reduced expression of the p53 protein in keratinocyte cells after Entinostat treatment; and the absence of this protein was found in the WSU-HN12 cells treated with vehicle or Entinostat (Fig. 2J). p53 is considered to be one of the first non-histone targets for acetylation and deacetylation. In fact, HDACs can deacetylate p53 affecting its transcriptional activity (13). Furthermore, we observed that p21 was upregulated in all cells receiving Entinostat (Fig. 2J).

Entinostat triggers the production of ROS and induces cellular apoptosis

Previous studies have reported that keratinocytes have a lower reactive oxygen species (ROS) levels compared to cancer cells (14). Here, we hypothesize that cancer cells are more vulnerable to HDAC inhibitors due to its sensitivity to the accumulation of ROS intracellular levels. Indeed, we found that ROS levels are higher in OSCC cells treated with Entinostat. Nonetheless, all cell lines responded to the administration of Entinostat by significantly accumulating ROS (keratinocytes and WSU-HN6 - $p \leqq 0.05$; WSU-HN12 - $p \leqq 0.01$). Similar to ROS, we also observed the accumulation of Superoxide Dismutase (SOD) ($p \leqq 0.05$) (Fig. 3A-C). Following, we also explored the levels of cells undergoing apoptosis in the treated group compared with vehicle controls. We observed that Entinostat significantly induced programmed cell death in all cell lines (Fig. 4A-F).

Administration of Entinostat decreases the population of CSC and BMI1 levels

CSCs correspond to a subpopulation of cancer cells presenting self-renewal properties and enhanced engraftment abilities capable of generating new tumors (15). These cells are also involved in the development of tumor resistance to chemotherapy and further tumor recurrences. Here investigated whether administration of Entinostat would impact the population of CSC from OSCC, and with that, paving the way for epigenetic modifications as novel therapeutic strategies to manage head and neck cancers. We evaluated the effect of Entinostat on CSCs using well-characterized OSCC stem cell markers CD44 and ALDH (16). We observed a reduction of more than 50% of CSC after the administration of Entinostat for 24h (Fig. 5 A-D). These results suggest that the HDAC inhibitor Entinostat is effective in reducing the population of CSC from OSCC tumors. We also analyzed the expression of BMI1, a Polycomb group repressor, involved in governing the self-renewal capacity of CSCs (17). Our experiments demonstrated that the administration of Entinostat reduced BMI1 expression (Fig. 5E). We observed no change in the protein levels of PTEN (phosphatase and tensin homolog deleted on chromosome 10) upon administration of Entinostat (Fig. 5E). PTEN is a tumor suppressor gene frequently found mutated or down-regulated in cancer. Meng and collaborators showed that PTEN activation by blocking HDAC6 contributed to cancer inhibition (18). Therefore, non-selective HDAC inhibitors do not interfere with the endogenous levels of the tumor suppressor PTEN that corroborated to the potential use of Entinostat as a therapy for OSCC.

In summary, the administration of Entinostat to OSCC leads to cell cycle arrest, apoptosis, and accumulation of ROS and superoxide dismutase, along with the depletion of CSCs (Fig. 6).

DISCUSSION

Changes to the epigenome can contribute to cancer formation and progression. Epigenetic modifications are associated with the deregulation of important biological processes, such as cell cycle progression and DNA repair, which can be controlled by environmental stressors. Enzymes that participate in epigenetic events are often found dysregulated in human cancers. More specifically, alterations of two families of chromatin enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs), are reported in many tumors (19). The identification and targeting of epigenetic-associated dysfunctional enzymes have spawned the development of new inhibitors capable of targeting the epigenome (5). Fine-tuning the balance between HDACs and HATs are proved to be an effective anti-tumor therapy, which led to the development of HDAC inhibitors (5). HDACi interferes with the cancer cell cycle, triggering cellular differentiation and death, decrease angiogenesis, as well as modify the immune response. HDACi is currently approved for the treatment of T-cell lymphoma (e.g., Belinostat, Romidepsin, and Vorinostat), and multiple myeloma (Panobinostat), while additional HDACi are under clinical investigation for hematopoietic cancers .

Entinostat is a synthetic small-molecule benzamide derivative that inhibits HDAC activity. The efficiency of Entinostat was demonstrated in animal models leading to the hyperacetylation of histones and consequent increased gene expression. The present study has demonstrated that even at low concentrations, Entinostat is able to reduce the cellular viability of OSCC cells in a dose-dependent fashion. These effects were observed in the WSU-HN6 cell line (IC 50: 0.54 μM). Nevertheless, the WSU-HN12 cell line required a higher inhibitory drug concentration (IC 50 - 23.31 μM), similar to the inhibitory concentration affecting the normal keratinocytes. Although Entinostat was effective on OSCC cells, it was not selective to cancer cells. Other studies have also shown that histone deacetylase inhibitors, such as Vorinostat and Resminostat, cause cytotoxicity in normal keratinocytes (20, 21).

Mutations and inactivation of the p53 have been reported in more than 50% of all carcinomas (22). It is well known cancer cells response to chemotherapeutic agents

may depend on the p53 pathway. WSU-HN12 cells have p53 mutations resulting in loss of p53 protein. This may explain why WSU-HN12 (p53 null cells) required higher IC50 doses of Entinostat compared to the WSU-HN6.

We also evaluated the effects of the Entinostat on the cell cycle progression. We found that Entinostat is effective in inducing cell cycle arrest in G0/G1 in all cell lines we have tested, including normal keratinocyte cell line. We have, however, observed that Entinostat could selectively inhibit tumor cells entry on S phase when compared to normal keratinocyte cells. Parallel to these observations, an increase in p21 tumor suppressor protein (Cdkn1a) levels was also observed in all cells independent from the p53 status. Aligned with our results, the knockdown of p53 in colon carcinoma cell lines interfered with cytotoxicity of the HDAC inhibitors, which also induced the expression of Acetyl-H4 and p21 (20, 23). The process of the cell cycle is regulated by cyclin-dependent kinase (Cdk)-cyclin complexes in eukaryotic cells. During carcinogenesis, the cell cycle is frequently found deregulated, and Cdks are often involved in the maintenance of tumor proliferation. In cancer cells, p21 binds and inactivates cyclin/Cdk complexes resulting in G1 cell cycle arrest (24). Here we found that Entinostat mediates the accumulation of p21 followed by increased cell cycle arrest in G0/G1.

Histone modifications, including the deacetylation of histones H3 and H4, can be induced by HDACs. These modifications result in the decondensation of the chromatin and subsequent gene repression (25). Thus, as an HDAC inhibitor, Entinostat is effective in enhancing histone acetylation in OSCC cells. In this study, we observed increased expression of acetylated histone H3 and H4 after treatment with HDACi. Entinostat decreased the expression of the SIRT1, a nicotinamide adenine dinucleotide (NAD+)-dependent deacetylase that applies its biological action by deacetylating histones and non-histone protein. Interestingly, recent investigations showed that Entinostat is a potent inhibitor of cellular nicotinamide adenine dinucleotides (NAD^+), which in turn affects SIRT1 (26, 27). Indeed, inhibition of NAD^+ decreases sirtuins, such as SIRT1, SIRT3, and SIRT6 (27). SIRT1 is upregulated in many cancers, including breast, ovarian, pancreatic, colorectal, prostate, gastric, and hepatocellular carcinoma . SIRT1 enhances cancer cell survival by improving resistance to apoptosis, inducing angiogenesis, and resistance to chemotherapy (28, 29). These findings are related to unsatisfactory clinical outcomes in cancer patients, like higher tumor stage,

lymph node metastasis, chemoresistance, and elevated cell proliferation (29). Thus, SIRT1 has been an attractive target for cancer therapy.

Epigenetic modifications can affect HNSCC stem cells, which are an important factor for tumor development and progression (4, 30). CSCs also contribute to radio-chemotherapy resistance, which can result in tumor relapse. Thereby, targeting CSCs remains an attractive therapeutic strategy. Current methods used to recognize and isolate CSCs from the head and neck cancers involve the identification of the membrane marker CD44 combined with high enzymatic activity of aldehyde dehydrogenase (ALDH) Here we showed that CSCs are sensitive to Entinostat. Also, the expression of BMI1, a protein related to CSC proliferation and growth, was decreased in the OSCC cells. These results indicate that chemical inhibition of HDAC reduces the number of CSCs.

Entinostat and other HDAC inhibitors are entering into clinical trials. A multicenter phase II study testing Entinostat and 5-azacitidine (5-AZA) in women with advanced hormone-resistant or triple-negative breast cancer was initiated in 2017 (7). Initially, recruited patients underwent a regiment of 5-AZA and Entinostat for 28 days, and the epigenetic therapy was further prolongated with the addition of endocrine therapy. Results from the optional prolonged therapy suggested that some women benefit from epigenetic therapy, along with the reintroduction of endocrine therapy beyond progression. Overall, the combination therapy of 5-AZA and Entinostat was well tolerated by the patients. Additionally, the PEMDAC study was the first trial to assess the addition of the HDAC inhibitor Entinostat in the management of melanoma of the eye treated with Pembrolizumab, an anti-PD1 drug (8).

All things considered, our findings demonstrated that patients with OSCC might benefit from therapies using HDAC inhibitors such as Entinostat. Entinostat promoted a cytotoxic effect on OSCC as well as cell cycle disruption and increased production and accumulation of intracellular levels of ROS. Combined, Entinostat increased the number of tumor cells undergoing apoptosis and reduced the CSCs. These findings suggest that Entinostat is a promising therapeutic agent in the management of oral cancer.

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Conflict of Interest Statement

The authors declare that they do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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Figures Legend

Figure 1: Effect of Entinostat on oral cancer growth and expression of histones H3 and H4. A-F: Graphic representation of Entinostat concentrations used in the MTT assay to determine the 50% inhibitory concentration of cell viability (IC50) of each cell

line in 24 h of treatment and their respective dose-response curves (percentage mean ± SEM). Note that Entinostat decreased the cell proliferation of OSCC lines and keratinocytes. IC50 for keratinocyte is 18.74 μM, WSU-HN6 is 0.54 μM, and WSU-HN12 is 23.31 μM. **G:** Entinostat increased the expression of acetylated histones H3 and H4 and reduced the protein levels of SIRT1.

Figure 2: Effect of Entinostat on cell cycle and expression of regulatory proteins. A-I: Representation of the cell distribution in the G0/G1, synthesis (S) and G2/M phases of the cell cycle after 24 h of treatment with Entinostat using the IC50 values previously defined for each cell line. There were statistically significant increases of G0/G1 and S phases in keratinocytes (**** = $p \leqq 0.0001$), WSU-HN6 (* = $p \leqq 0.05$), and WSU-HN12 (**** = $p \leqq 0.01$) (NS ≥ 0.05. Not significant; percentage mean ± SEM). **J:** Entinostat reduced p53 expression in keratinocyte. The absence of p53 was observed in WSU-HN12. Entinostat induced expression of p21 in all cell lines.

Figure 3: Effect of Entinostat on the production of reactive oxygen species (ROS) and superoxide. A-C: After 24-hour treatment with Entinostat, using the IC50 values defined for each cell line, there was an increase in the generation of both ROS and superoxide in all cell lines (fold increase mean ± SEM; * = $p \leqq 0.05$. ** = $p \leqq 0.01$).

Figure 4: Effect of Entinostat on cell death in all cell lines. A-F: Flow cytometry was used to analyze cell death events. Results are shown in dot plot diagrams. The apoptotic cells are shown in red. The bar graph displays the results of the apoptosis analyses. Each cell line was treated with Entinostat or vehicle for 24 h. Note that Entinostat resulted in apoptotic cells, especially in the WSU-HN12 cells. (percentage mean ± SEM; ** = $p \leqq 0.01$, **** = $p \leqq$ 000.1).

Figure 5: Flow cytometric analysis of OSCC cells treated with Entinostat for the expression of ALDH and CD44, and effect of Entinostat on BMI1 expression. A-D: After 24-hour treatment using the IC50 values previously defined for each cell line, there was a statistically significant reduction of stem cells (percentage mean ± SEM; * = $p \leqq 0.05$; ** = $p \leqq 0.01$). Cells exposed to Entinostat for 24 h were collected and assessed for ALDH activity and CD44 positivity using fluorescence activated cell

sorting (FACS). Dot plots display the cancer stem cells (CSC or $CD44^+$ and $ALDH^{bright}$ cells showed as green dots, and tumor cells (TC) are displayed as black dots. The results for the analyses of cancer stem cells are represented in the bar graphs. **E:** Entinostat led to the reduction of BMI1 protein levels after treatment. Entinostat does not affect PTEN protein levels.

Figure 6: Schematic representation shows the main effects of Entinostat treatment on oral squamous cell carcinoma. Entinostat treatment of oral squamous cell carcinoma (OSCC) cells resulted in cell cycle arrest, tumor cell apoptosis, accumulation of ROS and superoxide, as well as depletion of cancer stem cells (CSC).

Figure 1

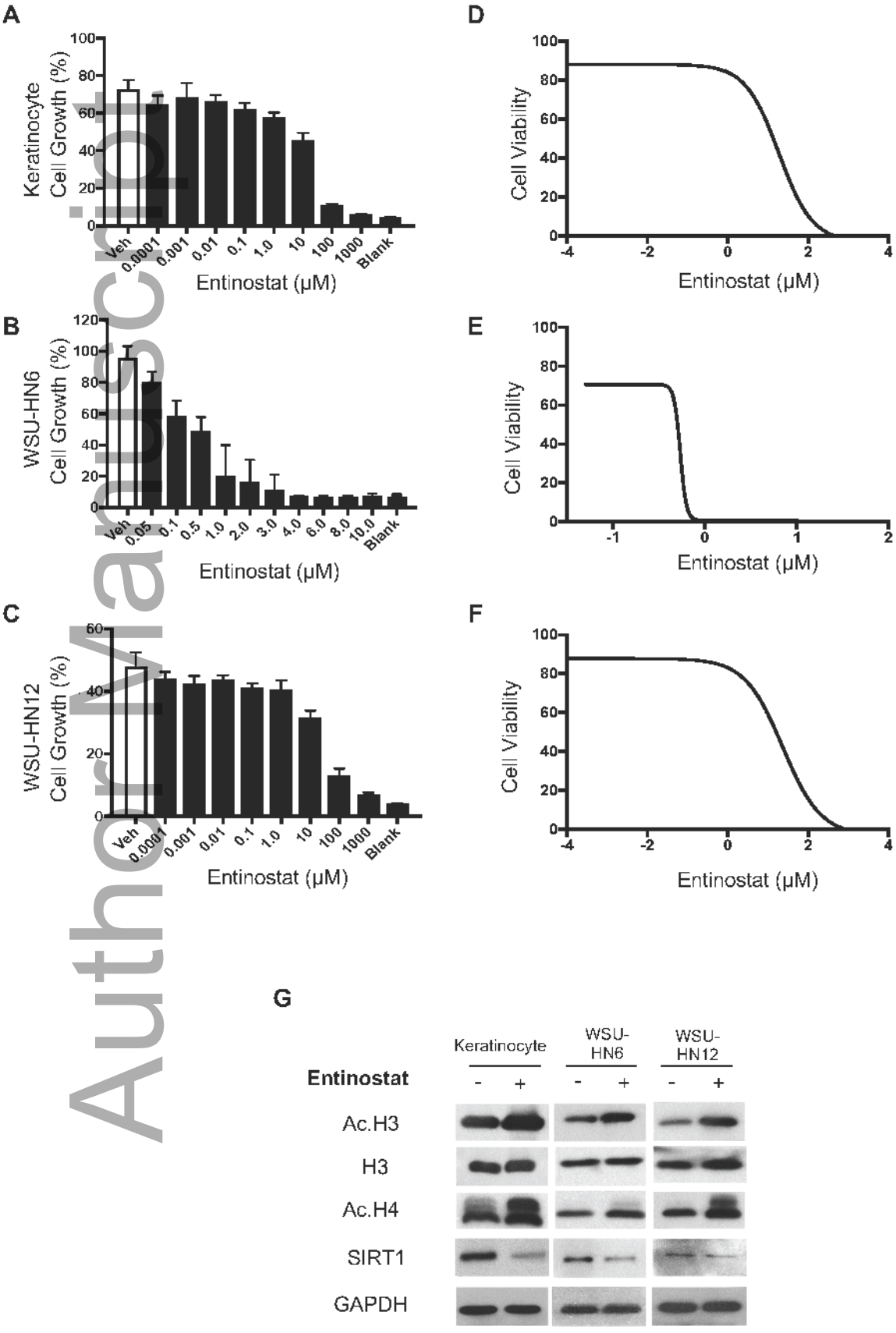

Figure 2

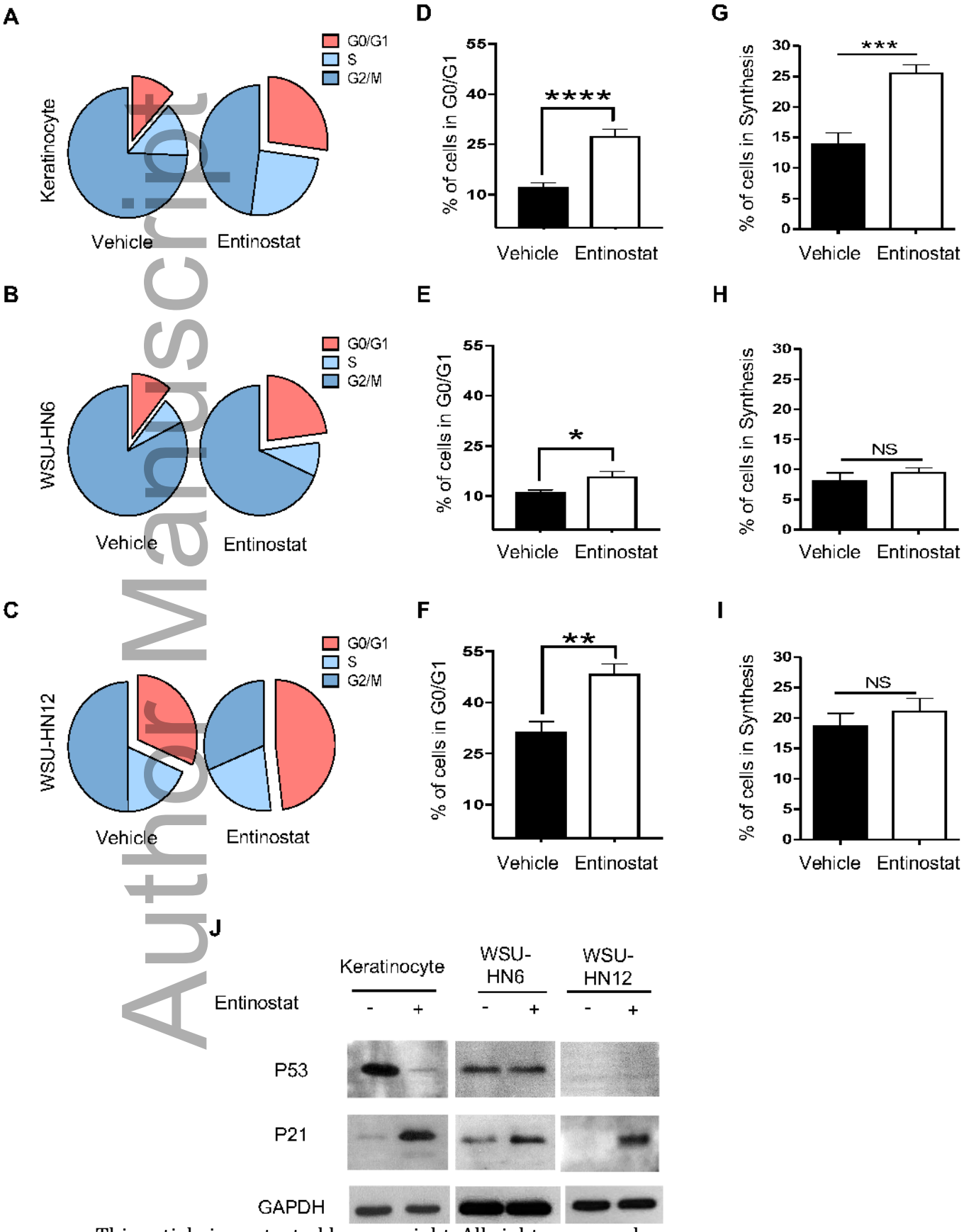

Figure 3

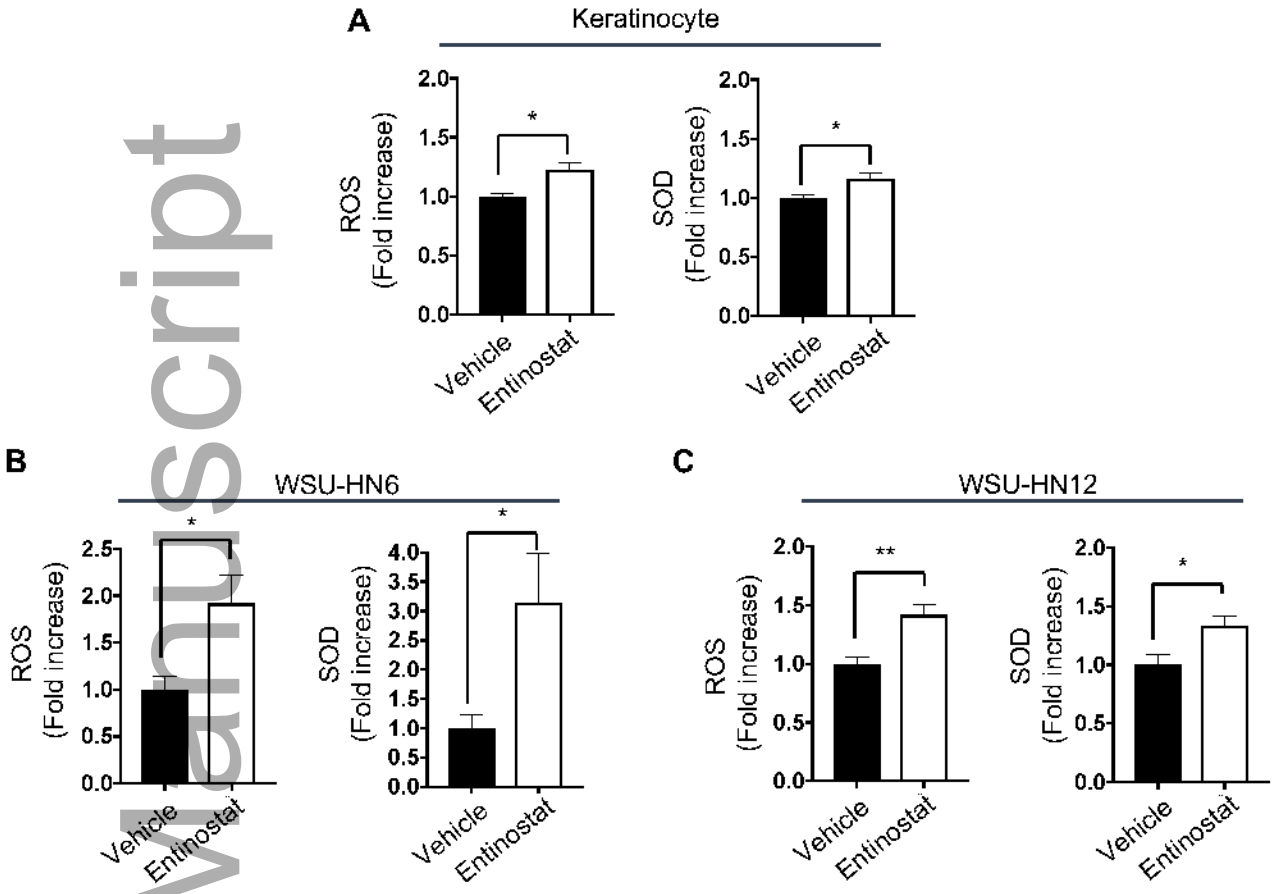

Figure 4

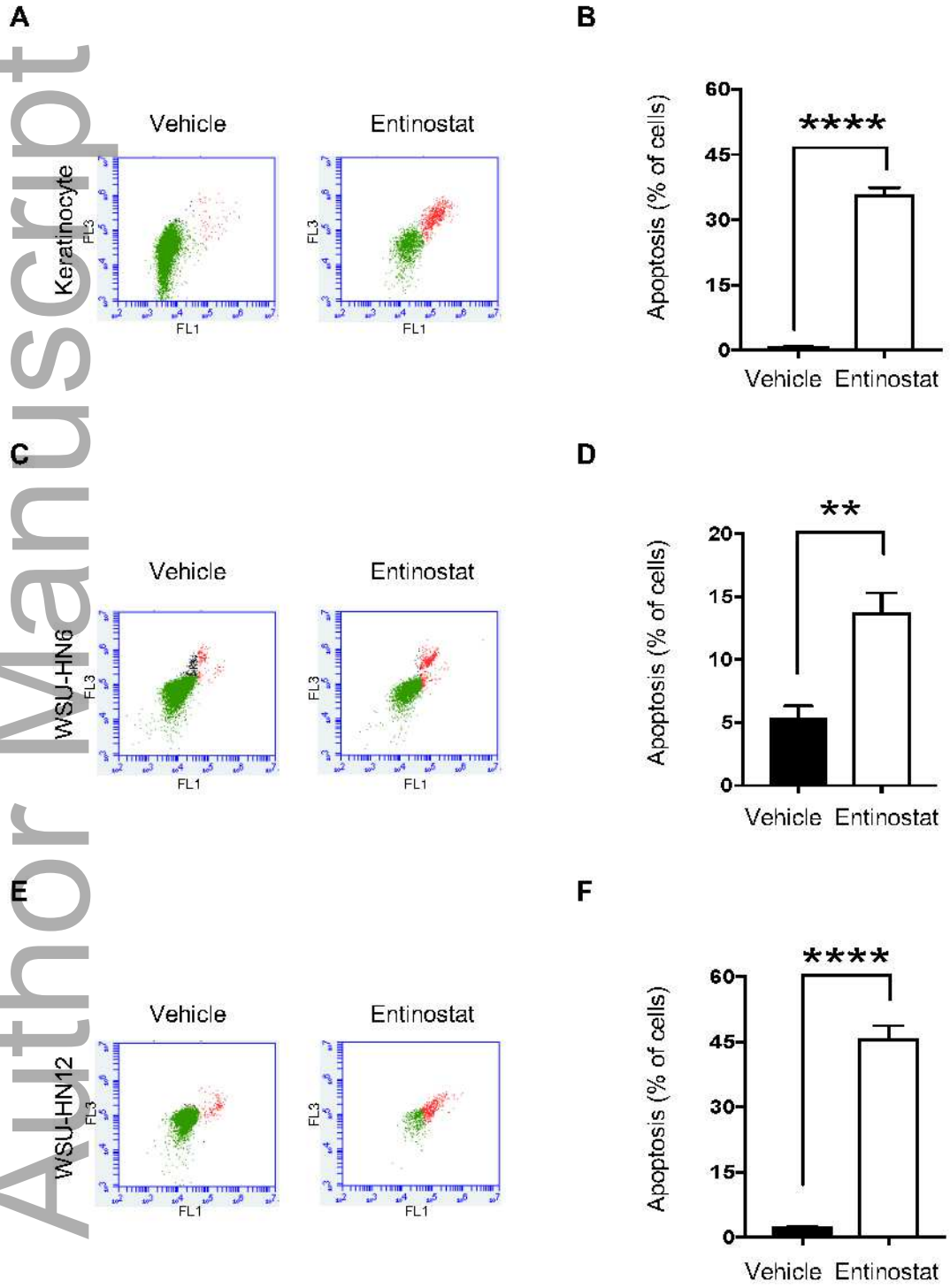

Figure 5

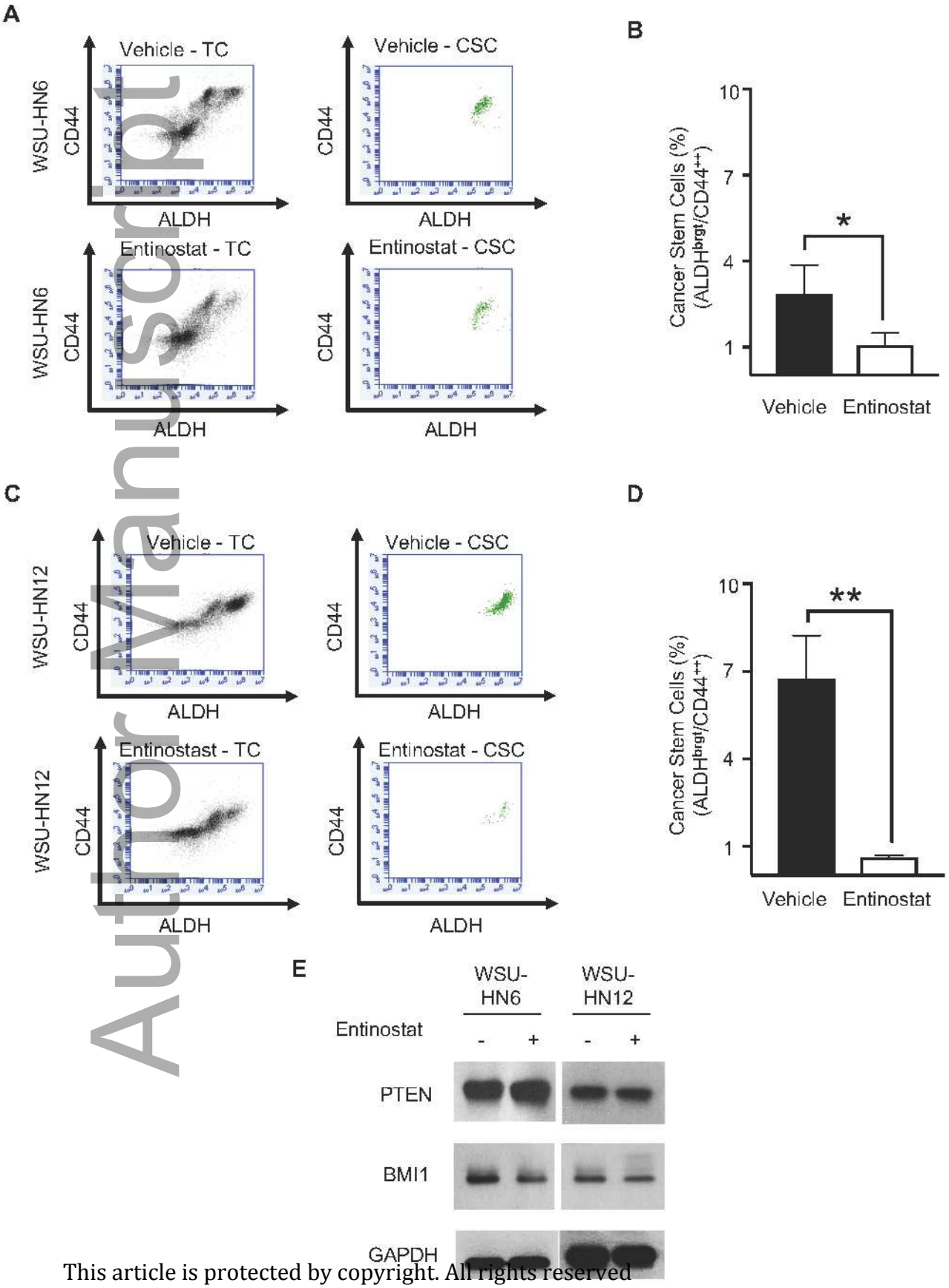

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Figure 6