SUPPLEMENTARY MATERIALS AND METHODS

Nuclear morphology assessment by Hoechst 33258 staining

CAL27 cells were plated in 24-well tissue culture plates for 24 h and incubated with different concentrations of NSC74859 (0 to 200 μM) for 24 h with detailed procedure as previous described [1]. Briefly, the CAL27 and FaDu cells were washed twice with PBS and fixed with 4% paraformaldehyde for 15 min followed by staining with 100 μg/ml of Hoechst 33258 (Beyotime, Shanghai, China) at room temperature in the dark for 30 minutes. After being washed two times with PBS, cells were examined with a fluorescence microscope (Leica) with an excitation wavelength of 340 to 360 nm [2]. The apoptotic cells containing condensed and fragmented nuclei produced bright blue fluorescence due to staining with Hoechst 33258.

Apoptosis assay

Apoptosis was quantified with an Annexin V-FITC apoptosis detection kit (BD Biosciences, San Diego, CA, USA) follow by the manufacturer's instructions as previous described [3]. Briefly, CAL27 cells were exposed to NSC74859 for 24 h, then the cells were harvested and washed with cold PBS, gently re-suspended in Annexin V binding buffer and incubated with Annexin V-FITC/PI. Finally, the cells were analyzed immediately by using a FACS Calibur flow cytometer (Becton-Dickinson, Fullerton, CA, USA).

Western blot analysis

Western blot analysis were performed as previously [1]. Briefly, CAL27 cell lines were treated with the indicated concentrations NSC74859 in DMEM for 24 h. Then the cells were lysed, after protein concentration was measured by the bicinchoninic acid (BCA) method, the total protein was separated by using 12% SEMS-polyacrylamide gelelectrophoresis and then transferred onto polyvinylidenefluoride membranes (Millipore, Billerica, MA). Membranes were blocked with 5% non-fat dry milk and powder in 0.05% Tris-buffered saline and Tween 20 (TBST) for 1 h at room temperature, then incubated overnight at 4°C with specialized antibodies. After overnight incubation, membranes were washed for three times and then incubated for 1 h at room temperature with peroxidase-conjugated secondary antibodies. Blots were then developed by West Pico enhanced chemiluminescence detection kit (West Pico, Thermo).

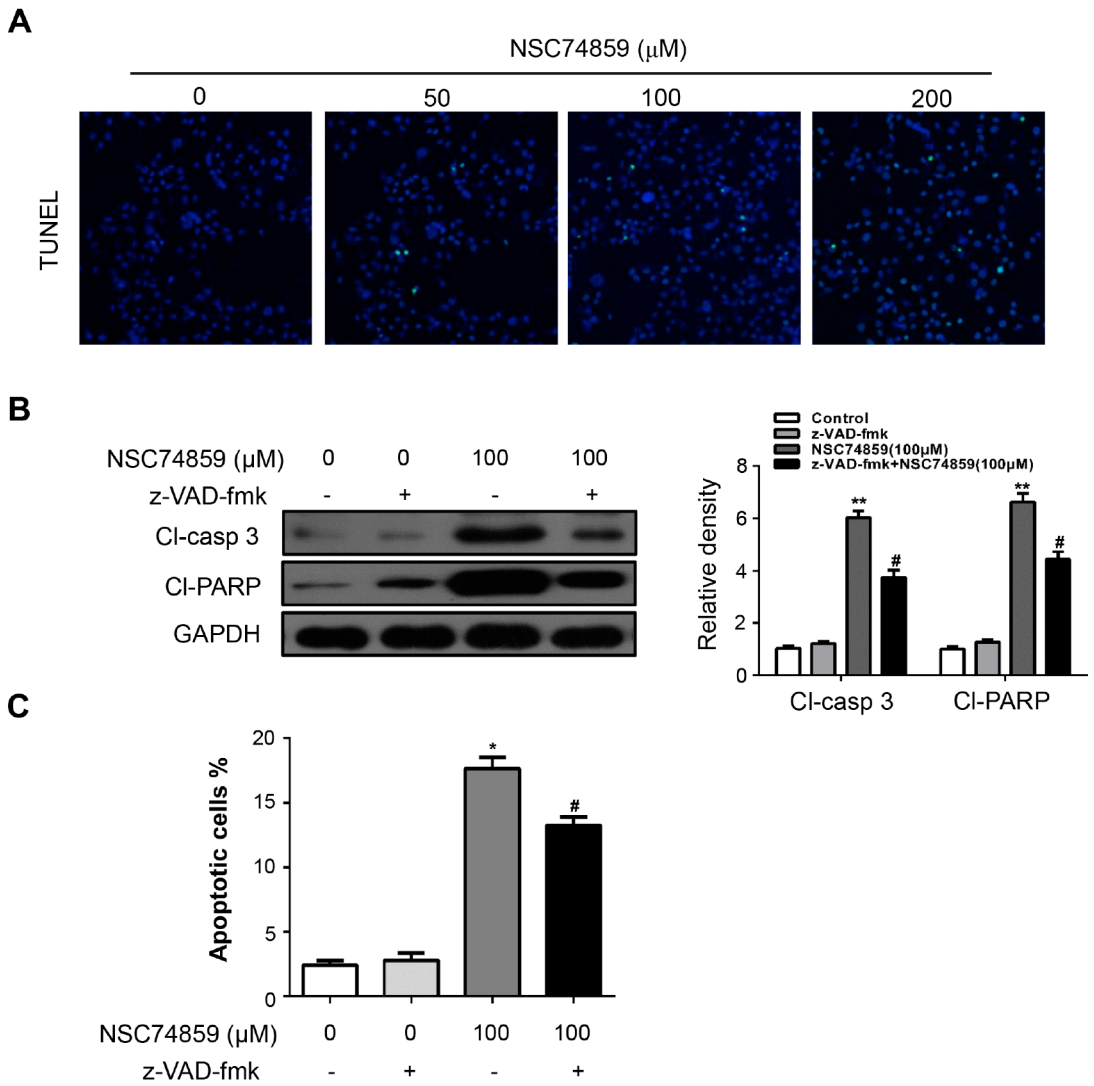
Immunohistochemistry

Immunohistochemistry (IHC) was performed as described previously [4]. Normal human oral mucosa and HNSCC samples were fixed with 4% paraformaldehyde, and then dehydrated, embedded in paraffin and sectioned at 4 μm. Sections were incubating with p-STAT3 (Cell Signaling Technology, Danvers, MA), LC3 (Cell Signaling Technology, Danvers, MA), p62 (Novus Biological, CL, USA), Bcl2 (Cell Signaling Technology, Danvers, MA), LAMP2 (Abcam, Cambridge, MA), p-Erk1/2 (Santa Cruz, CA), antibody overnight at 4°C. Horseradish peroxidase-conjugated secondary antibody was used to detect antibody binding using a diaminobenzidine substrate kit (DAKO, Carpinteria, CA, USA) according to the manufacturer's protocol.

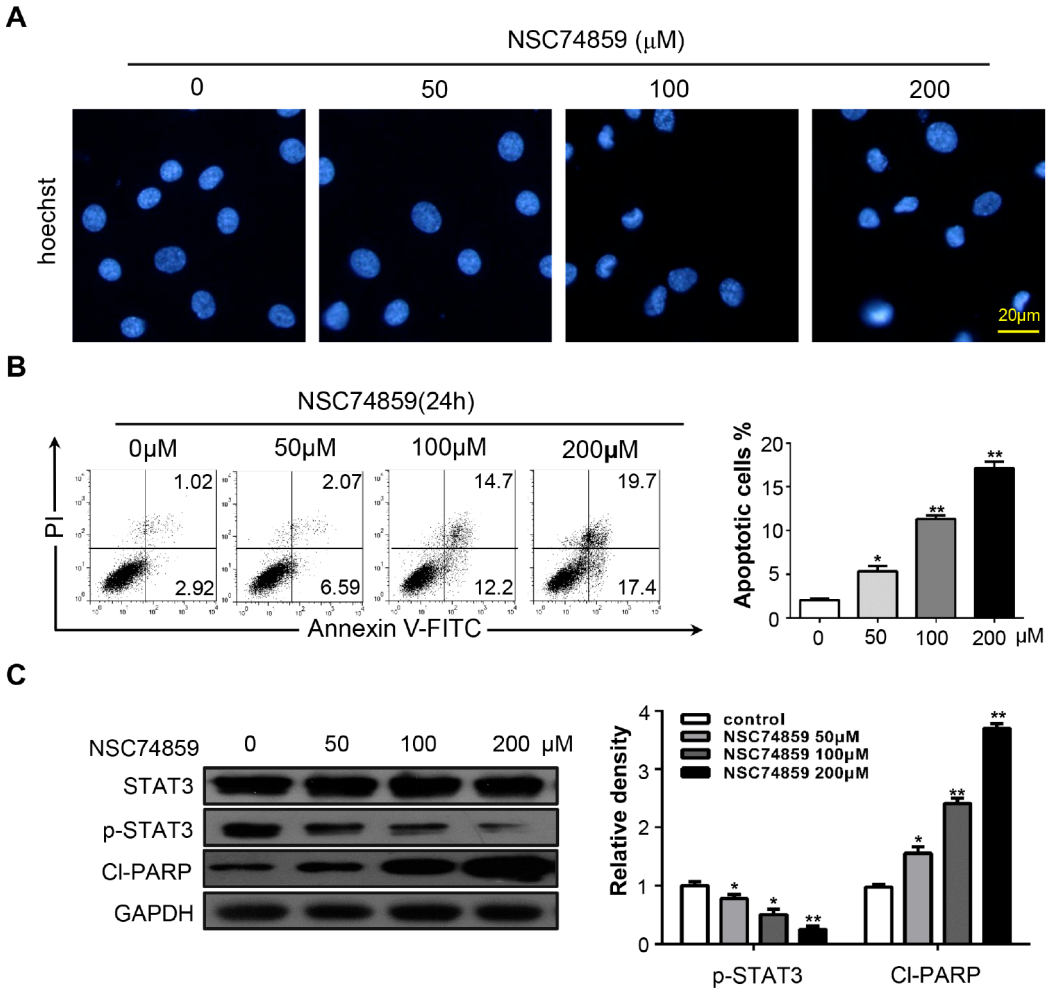
REFERENCES

1. Ma SR, Wang WM, Huang CF, Zhang WF and Sun ZJ. Anterior gradient protein 2 expression in high grade head and neck squamous cell carcinoma correlated with cancer stem cell and epithelial mesenchymal transition. Oncotarget. 2015; 6:8807–8821.
2. Wang YF, Zhang W, He KF, Liu B, Zhang L, Zhang WF, Kulkarni AB, Zhao YF and Sun ZJ. Induction of autophagy-dependent cell death by the survivin suppressant YM155 in salivary adenoid cystic carcinoma. Apoptosis. 2014; 19:748–758.
3. Sun ZJ, Chen G, Zhang W, Hu X, Liu Y, Zhou Q, Zhu LX and Zhao YF. Curcumin dually inhibits both mammalian target of rapamycin and nuclear factor-kappaB pathways through a crossed phosphatidylinositol 3-kinase/Akt/IkappaB kinase complex signaling axis in adenoid cystic carcinoma. Mol Pharmacol. 2011; 79:106–118.
4. Sun ZJ, Chen G, Zhang W, Hu X, Huang CF, Wang YF, Jia J and Zhao YF. Mammalian target of rapamycin pathway promotes tumor-induced angiogenesis in adenoid cystic carcinoma: its suppression by isoliquiritigenin through dual activation of c-Jun NH2-terminal kinase and inhibition of extracellular signal-regulated kinase. J Pharmacol Exp Ther. 2010; 334:500–512.

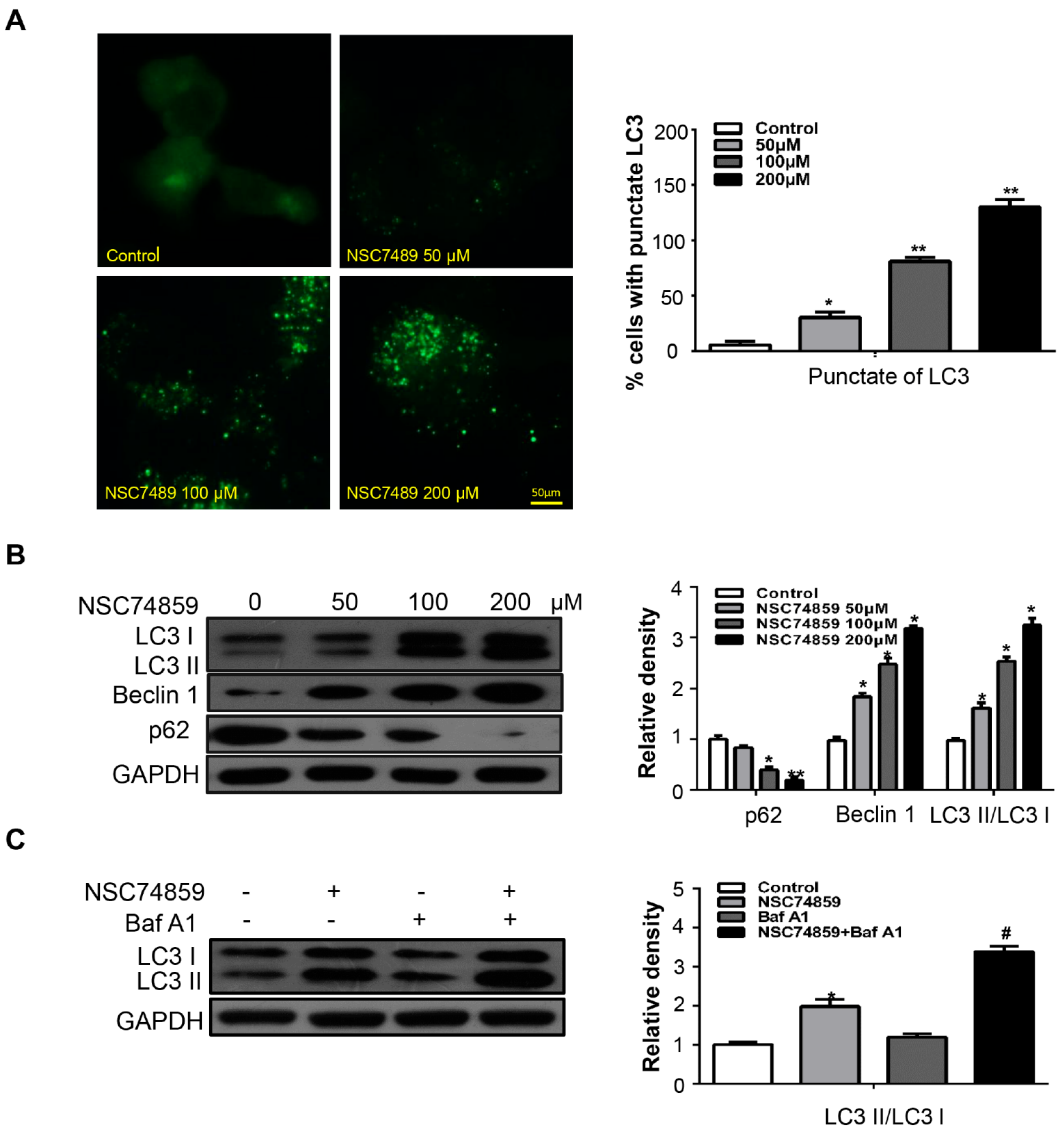
SUPPLEMENTARY FIGURES



Supplementary Figure S1: Apoptosis induced by NSC74859 is partially caspase 3-dependent in CAL27 cells. A. TUNEL staining of CAL27 cells after 24 h of NSC74859 treatment at the indicated concentrations. Positive cells were labeled with TUNEL (green). **B.** CAL27 cells were incubated with 100 μM of NSC74859, either alone or in combination with 20 μM z-VAD-fmk for 24 h, then western blot analysis was performed to assess the level of cleaved-caspase 3 and cleaved-PARP. Densitometric values were quantified using the Image J software, and the data were presented as means ± SEM of three independent experiments. ** $P < 0.01$ versus the control group, one-way ANOVA with post-Dunett analysis was used by GraphPad Prism5; #$P < 0.05$ *versus* the NCS74859 (100 μM) group, One-way ANOVA with post-Tukey analysis was used by GraphPad Prism5; **C.** CAL27 cells were treated with NSC74859 at indicated concentrations in the absence or presence of 20 μM z-VAD-fmk for 24 h, then cells were stained with Annexin V/PI and analyzed by flow cytometry after 24 h incubation. The percentages of Annexin V-positive cells were presented in bar charts. Results were represented as mean ± SEM, ** $P < 0.01$ *versus* the control group, one-way ANOVA with post-Dunett analysis was used by GraphPad Prism5; #$P < 0.05$ *versus* the NCS74859 (100 μM) group, One-way ANOVA with post-Tukey analysis was used by GraphPad Prism5.



Supplementary Figure S2: Blocking phosphorylation of STAT3 by NSC74859 induced apoptosis in FaDu cell line. A. The morphologic changes of FaDu treated with NSC74859 were captured using fluorescence microscopy with Hoechst 33258 staining. *Scale bar* 20 μm; **B.** FaDu cells were treated with 50 μM, 100 μM, 200 μM of NSC74859 for 24 h, and stained with Annexin V/PI, then analyzed by flow cytometry. The percentages of Annexin V-positive cells were presented in bar charts; the data represented mean of three independent experiments. $^{**}P < 0.01$, One-way ANOVA with post-Dunett analysis was used by GraphPad Prism5; **C.** FaDu cells were treated with different concentrations of NSC74859 for 24 h then western blot analysis was performed to assess the expression level of STAT3 and p-STAT3^{T705}, PARP and cleaved-PARP, and GAPDH served as a loading control; Relative density data were calculated by Image J, and the data represented mean of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.



Supplementary Figure S3: Blocking phosphorylation of STAT3 by NSC74859 induced autophagy in FaDu cell line. A. FaDu cells transfected with GFP-LC3 plasmid were treated with different concentrations of NSC74859 for 24 h. The formation of GFP-LC3 puncta were examined using immunofluorescence and quantified. *Scale bar* 50 μm; $^{**}P < 0.01$; **B.** FaDu cells were treated with different concentrations of NSC74859 for 24 h, then detected autophagy-associate protein LC3I/II, p62 and Beclin1 by western blot analysis; Densitometric values were quantified using the Image J software, and the data were presented as means ± SEM of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$; **C.** FaDu cells were treated with 100 μM of NSC74859 in the absence or presence of 20 nM Bafilomycin A1, then the expression of LC3II was quantified by normalization of their densitometry to GAPDH; Densitometric values were quantified using the Image J software, and the data were presented as means ± SEM of three independent experiments. $^{*}P < 0.05$ *versus* the control group, One-way ANOVA with post-Dunett analysis was used by GraphPad Prism5; $^{\#}P < 0.05$ *versus* the NCS74859 (100 μM) group, One-way ANOVA with post-Tukey analysis was used by GraphPad Prism5.