

Figure S1 Screening drug from FDA-approved drug library

Figure S2 CDK4/6 and RB1 or p-RB1 S807/S811 has positive correlation

Figure S3 Dronedarone could not bind with c-MYC and inhibit the activation of p-RB1 Figure

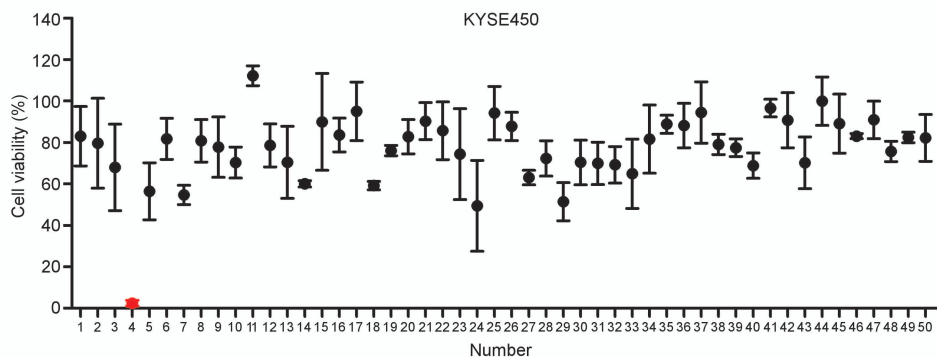
S4 Depletion of CDK4/CDK6 blocked ESCC at G0/G1 phase

Figure S5 Dronedarone could not arrest the ESCC cell cycle after knocking out CDK4 or CDK6

Figure S6 Knocking out CDK4 or CDK6 could not arrest the ESCC cell cycle after
rescuing the expression of RB1 protein

Figure S7 Dronedarone inhibited ESCC growth *in vivo*

A



B

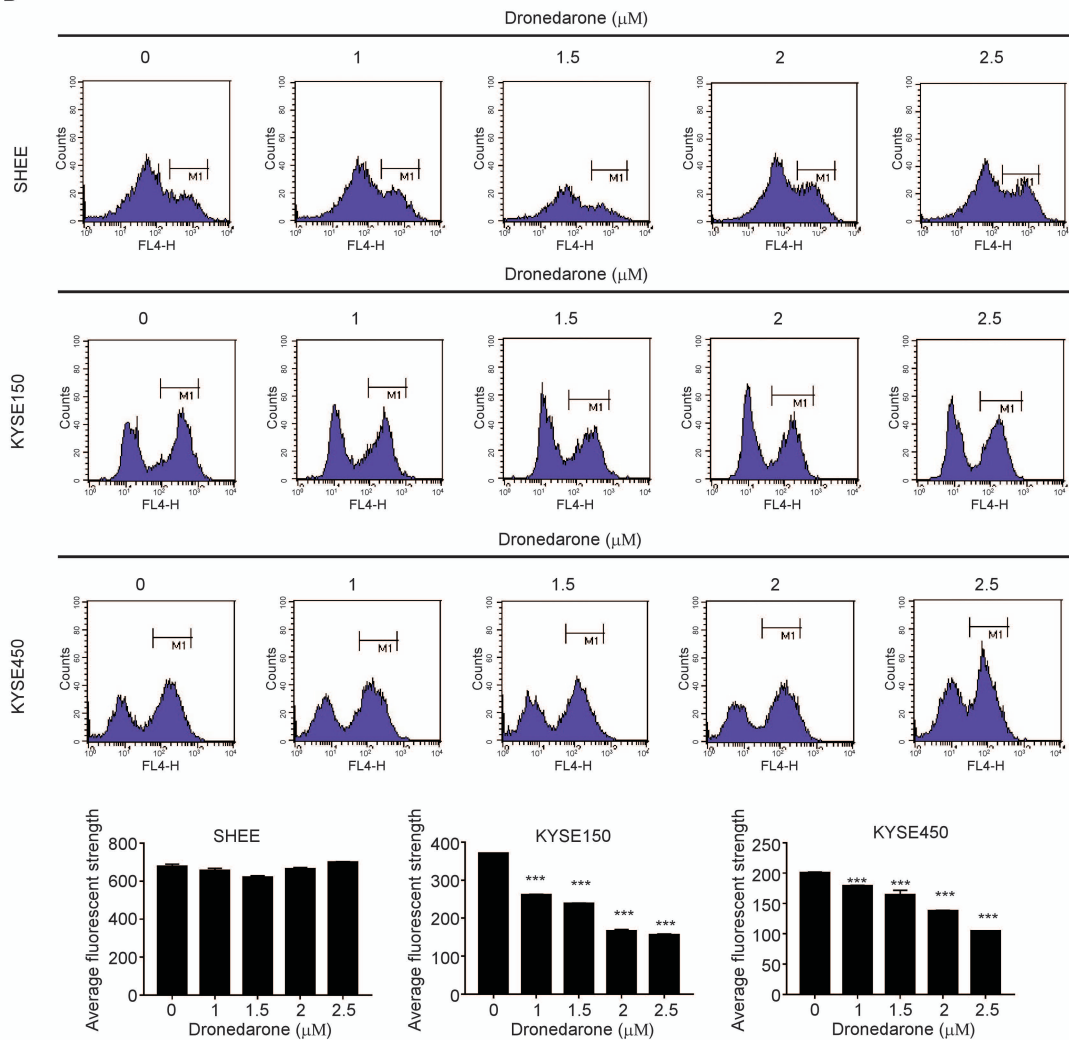
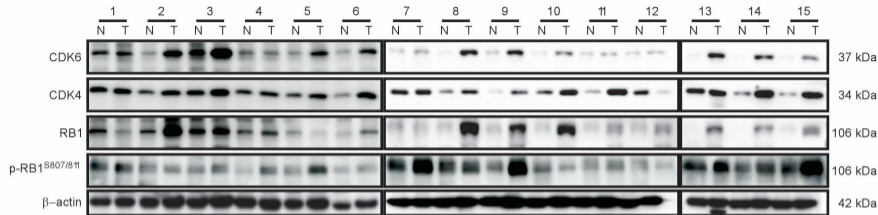


Figure S1 Screening drug from FDA-approved drug library.

(A) Cytotoxicity of FDA-approved drugs on KYSE450 cells was measured by treating cells with 50 μM of the drugs and measuring cell viability at 48 h.

(B) Effect of dronedarone on SHEE, KYSE150, and KYSE450 cells was evaluated using the EdU incorporation assay. Cells were treated with dronedarone (0, 1, 1.5, 2 and 2.5 μM), and the average fluorescent strength was measured after 48 h. The data were analyzed by homogeneity of variance and ANOVA. ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$)

A



B

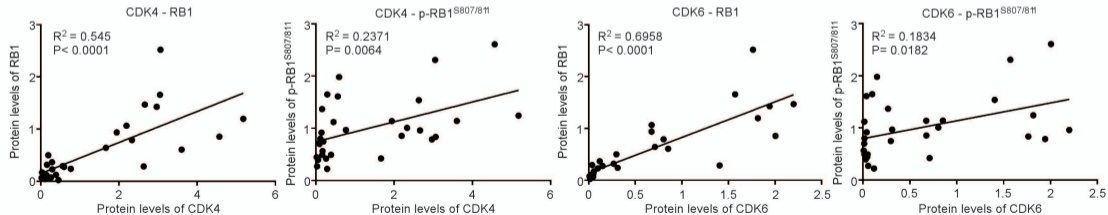


Figure S2 CDK4/6 and RB1 or p-RB1 S807/S811 has positive correlation.

(A) The CDK4, CDK6, RB1, and p-RB1 S807/811 protein levels were measured in 15 pairs of normal and tumor tissues from ESCC patients by Western blotting.

(B) The correlation between CDK4, RB1, and p-RB1 S807/811 was analyzed; the correlation between CDK6, RB1, and p-RB1 S807/811 was analyzed.

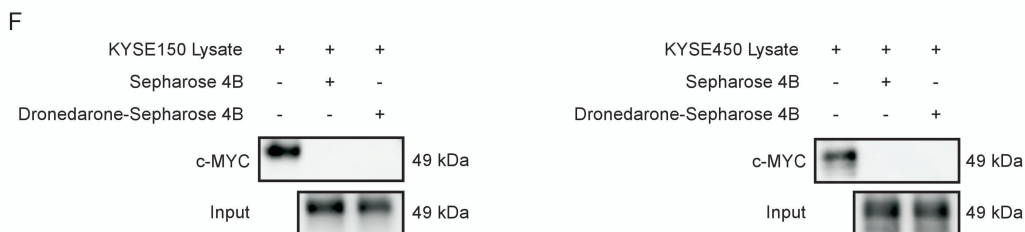
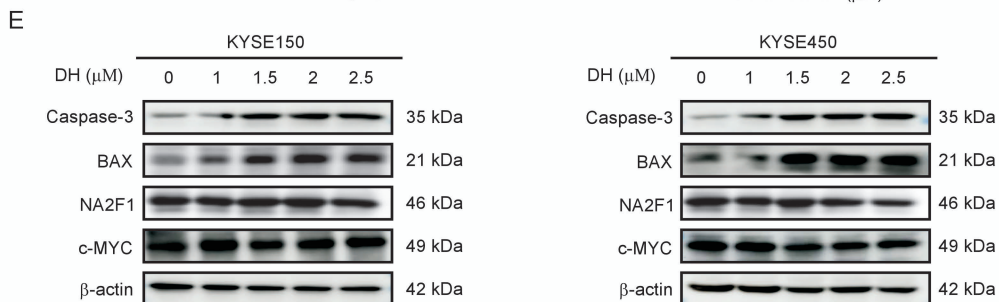
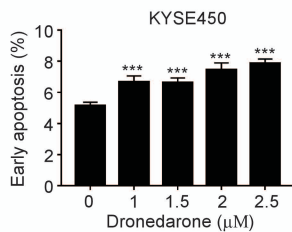
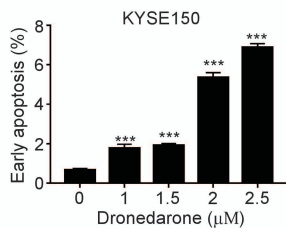
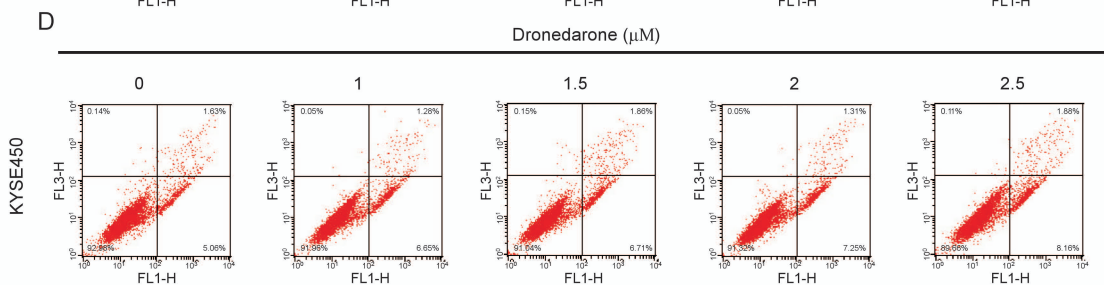
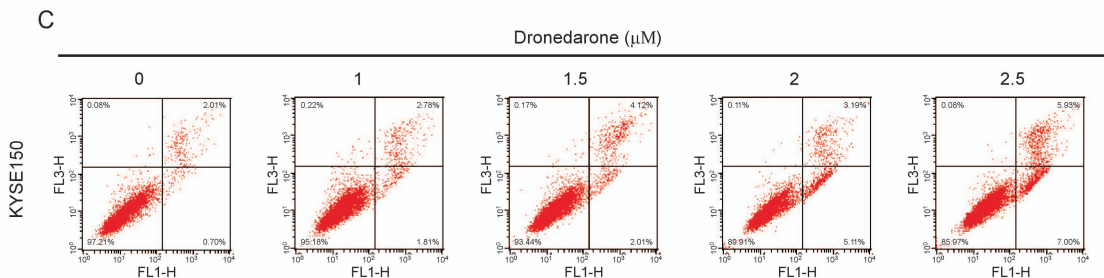
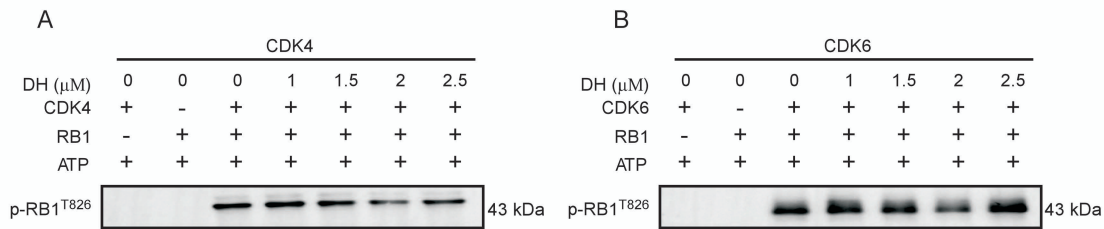


Figure S3 Dronedarone could not bind with c-MYC and inhibit the activation of p-RB1 T826 by CDK4 and CDK6.

(A and B) *In vitro* kinase experiment assay showed dronedarone (0, 1, 1.5, 2 and 2.5 μ M) could not inhibit the activation of p-RB1 T826 by CDK4 (A) and CDK6 (B).

(C and D) After being treated with different concentrations of dronedarone 0, 1, 1.5, 2 and 2.5 μ M) for 24 h, the cell cycle of both KYSE150 (C) and KYSE450 (D) cells was analyzed. Data were analyzed by homogeneity of variance and ANOVA. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

(E) Protein levels of Caspase-3, BAX, NA2F1, and c-MYC were measured in KYSE150 and KYSE450 cells treated with various concentrations of dronedarone 0, 1, 1.5, 2 and 2.5 μ M) by Western blotting.

(F) The pull-down assay was conducted to examine the interactions between dronedarone and c-MYC.

Figure S4 Depletion of CDK4/CDK6 blocked ESCC at G0/G1 phase.

(A) The CDK4 and CDK6 mRNA expressions in normal tissues, adenocarcinoma, and squamous cell carcinoma were analyzed using the TCGA database.

(B) The EdU incorporation assay was used to analyze the cell growth of sgCDK4 and sgCDK6 in KYSE150 and KYSE450 cells by measuring the average fluorescent strength at 48 h. The data were analyzed using homogeneity of variance and ANOVA.

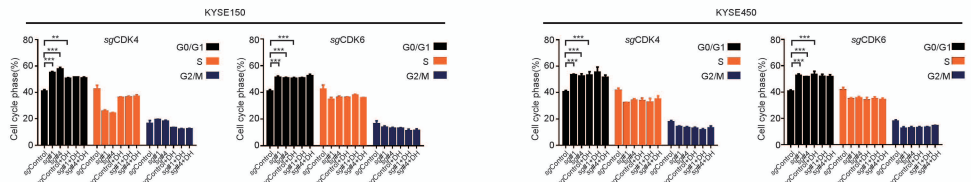
(* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

(C) The images were taken from the cell cycle of KYSE150 and KYSE450 sgCDK4 and sgCDK6 cells for 24 h. Data were analyzed by homogeneity of variance and ANOVA. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

A



B



C

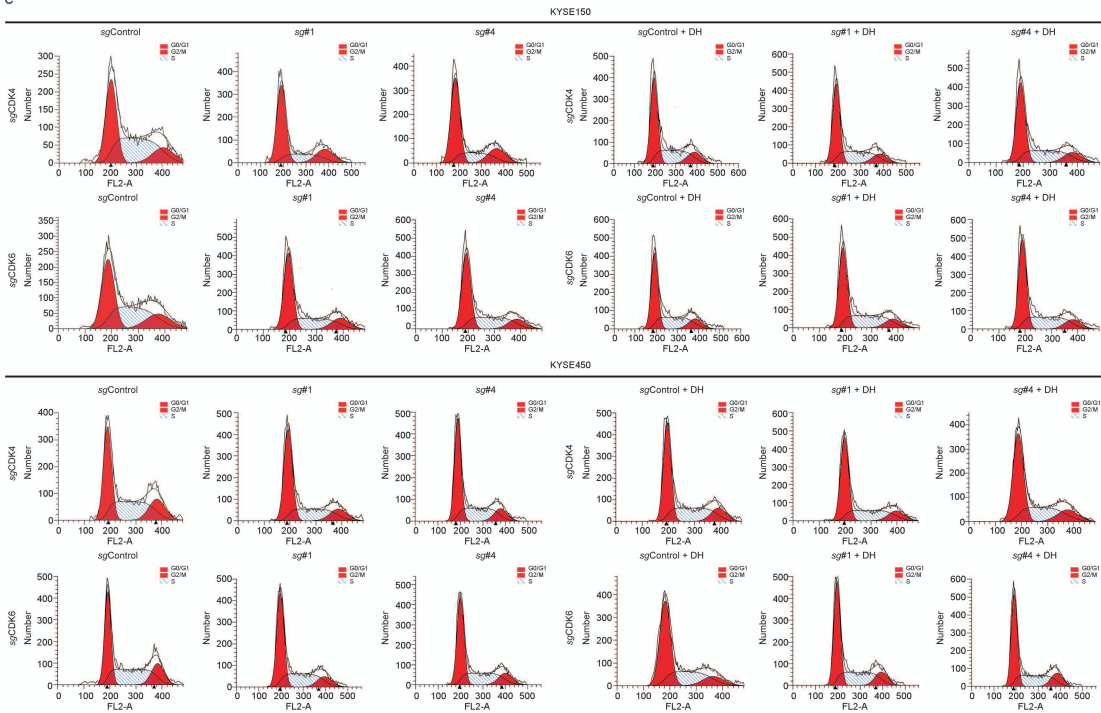
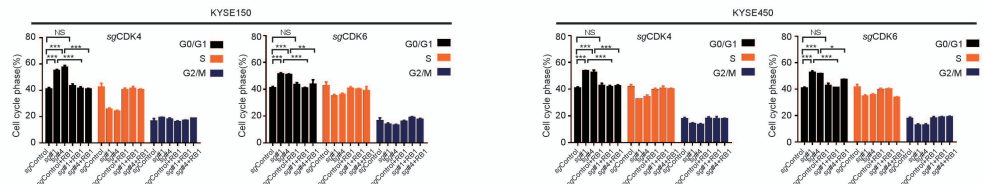


Figure S5 Dronedarone could not arrest the ESCC cell cycle after knocking out CDK4 or CDK6

(A) Protein levels of RB1 were measured in sgCDK4/sgCDK6 cells after overexpressing RB1 in KYSE150 and KYSE450 cells.

(B and C) The cell cycle of sgCDK4 and sgCDK6 ESCC cells was analyzed after treatment with dronedarone (0, 2.5 μ M) for 24 h. Data were analyzed by homogeneity of variance and ANOVA. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

A



B

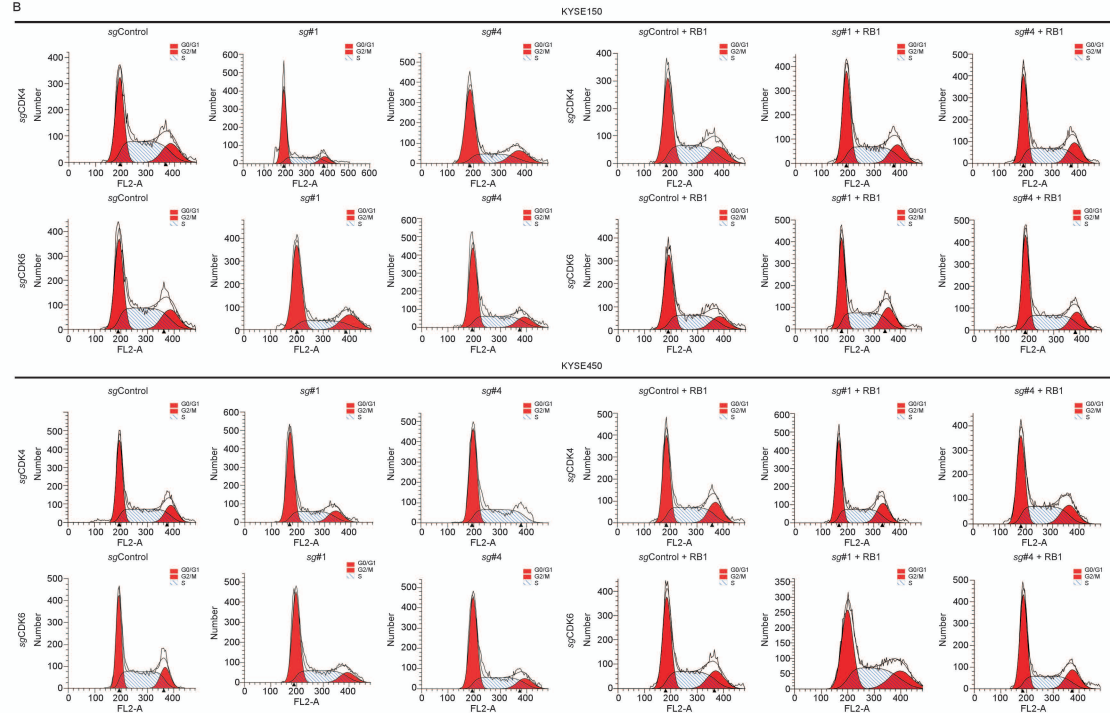


Figure S6 Knocking out CDK4 or CDK6 could not arrest the ESCC cell cycle after rescuing the expression of RB1 protein.

(A and B) The cell cycle of sgCDK4 and sgCDK6 ESCC cells was analyzed after overexpressing RB1 for 24 h. Data were analyzed by homogeneity of variance and ANOVA. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

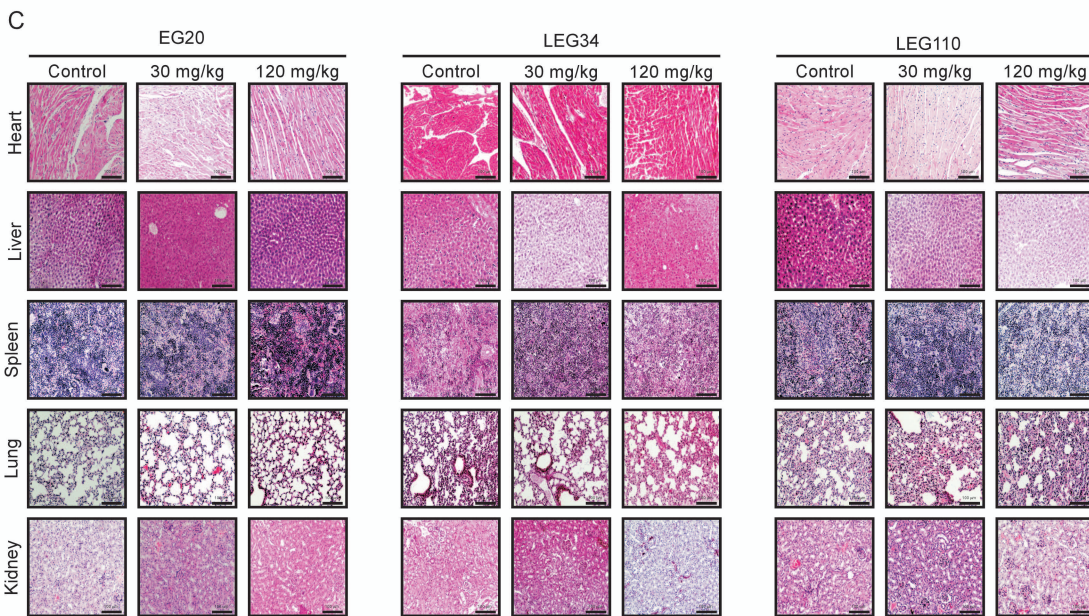
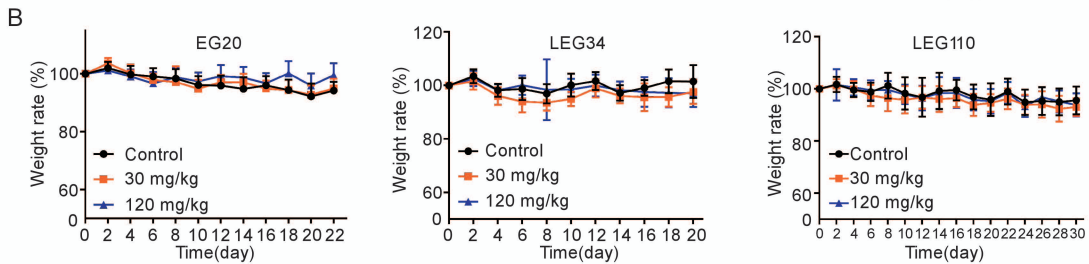
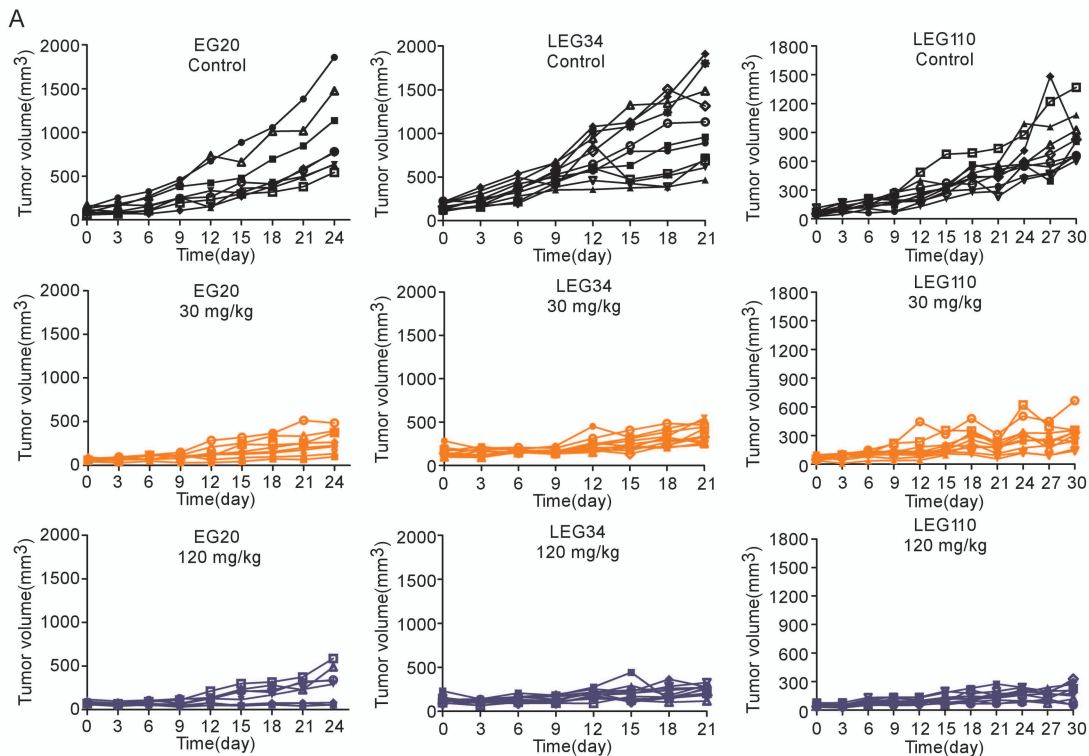


Figure S7 Dronedarone inhibited ESCC growth *in vivo*.

(A) The tumor volume per mouse in cases EG20, LEG34, and LEG110 was displayed.

(B) The body weight of the groups treated with different doses of dronedarone was shown for cases EG20, LEG34, and LEG110.

(C) The typical images showed HE staining of mice's heart, liver, spleen, lungs, and kidneys in cases EG20, LEG34, and LEG110. Scale bar: 100 μm .