Pharmacological Inactivation of Skp2 SCF Ubiquitin Ligase Restricts Cancer Stem Cell Traits and Cancer Progression

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Introduction:

1. chemotherapy and radiotherapy represent two major options for cancer treatment through inducing p53-dependent cellular senescence and apoptosis;

2. developing cancer treatment strategies via boosting p53-independent senescence and/or apoptosis responses is a key to the success of advanced cancer treatments;

3. Skp2 is an F box protein, constituting one of the four subunits of the Skp1-Cullin-1-F-box (SCF) ubiquitin E3 ligase complex. Skp2 regulates apoptosis, cell-cycle progression, and proliferation by promoting the ubiquitination and degradation of p27, and Skp2 SCF complex can also trigger nonproteolytic K63-linked ubiquitination of Akt;

4. targeting aerobic glycolysis has recently emerged as a promising strategy for cancer therapies, as cancer cells display elevated glycolysis irrespective of the presence or absence of oxygen, which warrants cancer cell proliferation and survival. Aerobic glycolysis is orchestrated by Akt, whose activation is achieved by its membrane translocation and subsequent phosphorylation. SKP2-E3 ligases are utilized to trigger nonproteolytic K63-linked ubiquitination of Akt, targeting E3 ligase of Akt may serve as a promising strategy to tame cancer glycolysis.
Ubiquitin mediated protein degradation:
Figure 1. Identification of the Skp2 Inhibitor, which Impairs Skp2 SCF E3 Ligase Activity by Preventing Skp2-Skp1 Binding

(A) The identified potential binding pockets on the interface of Skp2-Skp1 complex.

(B) In vitro Skp2-Skp1 binding assay with or without compound #25.

(C) In vivo Skp2-Skp1 binding assay with or without compound #25 in PC3 cells.

(D) In vivo p27 ubiquitination assay in 293T cells transfected with p27, His-Ub, along with Xp-Skp2 in the presence of DMSO or compound #25. WCE, whole cell extracts.

(E) In vitro Skp2-mediated p27 ubiquitination assay was performed with or without Flag-Skp2-SCF or p27 in the presence of DMSO or compound #25.

(F) PC3 cells were treated with DMSO or compound #25 at different doses for 24 hr and harvested for immunoblotting (IB) assay.

(G) In vivo Akt ubiquitination assay in 293T cells transfected with various constructs in the presence of DMSO or compound #25.

(H) In vitro Skp2-mediated Akt ubiquitination assay was performed with or without Flag-Skp2-SCF or GST-Akt in the presence of DMSO or compound #25.

(I) LNCaP cells were serum starved in the absence or presence of compound #25 for 24 hr, stimulated with or without EGF, and harvested for IB assay.
The Physical Binding of Skp2 Inhibitor to Skp2 Prevents Skp2-Skp1 Complex Formation

Figure 2. Skp2 Inhibitor Directly Interacts with Skp2 at Trp97 and Asp 98 Residues

(A) Chemical structure of compound #25. 3-(1,3-benzothiazol-2-yl)-6-ethyl-7-hydroxy-8-(1-piperidinylmethyl)-4H-chromen-4-one (top). The docking between compound #25 and predicted pocket 1 of Skp2. The residues in green lines form hydrogen bonding/hydrophobic/aromatic stacking interactions with compound #25. The yellow dashed lines represent hydrogen bonds between compound #25 and Skp2 (bottom).

(B) In vivo Skp2-Skp1 binding assay in 293T cells transfected with Skp2 or its various mutants.

(C) In vivo p27 ubiquitination assay in 293T cells transfected with various constructs in the presence of DMSO or compound #25.

(D) In vitro binding of Skp1 with Skp2 WT or various mutants in the presence of DMSO or compound #25.

(E) The extracted ion chromatography spectra demonstrating the quantity of compound #25 bound to GST alone, GST-Skp2 WT, W97A mutant, and D98A mutant (retention time 24 min.).
Figure 3. The Skp2 Inhibitor Specifically Diminishes E3-Ligase Activity of Skp2-SCF Complex, but Not Other F box SCF Complexes

(A–D) 293T cells transfected with various constructs in the presence of DMSO or compound #25 was treated with MG132 for 6 hr followed by in vivo ubiquitination assay.

(E) LNCaP cells were treated with DMSO, Skp2 inhibitors, or MLN4924 for 24 hr and harvested for IB assay.
>Skp2 Inhibitor Restricts Cancer Cell Survival through Triggering p53-Independent Cellular Senescence and Inhibiting Aerobic Glycolysis

Figure 4. Inhibition of E3-Ligase Activity of Skp2-SCF Complex Results in Cancer Cell Death, Glycolysis Defects, and Cellular Senescence

(A) Prostate cancer cells and normal epithelial cells (PNT1A) were treated with various doses of compound #25, followed by cell survival assay.

(B) Lung cancer cells and normal fibroblasts (IMR90) were treated with various doses of compound #25, followed by cell survival assay.

(C) PC3 cells were treated with or without compound #25 for 4 days and harvested for senescence assay.

(D and E) Lactate production was measured in PC3 (D) or LNCaP (E) cells treated with DMSO, LY294002, or compound #25.

(F) Apoptosis (programed cell death) rate was determined in PC3 cells treated with DMSO or compound #25.

(G and H) PC3 cells with or without Skp2 knockdown (G) or PC3 cells stably expressed with Skp2 WT, W97A, or D98A mutants (H) were treated with various doses of compound #25, followed by cell survival assay. Cell survival percentage of each stable cell lines treated with various doses of compound #25 was normalized to that treated with DMSO. Results are presented as mean values ± SD. *p < 0.05; **p < 0.01.
Structure-Activity Relationships of Skp2 Inhibitor

Figure 5. The Structure-Activity Relationship of Compound #25 Derivatives

(A and B) In vitro Skp2-Skp1 binding assay in the presence of DMSO, compound #25, or its derivatives.

(C) Structure-activity relationship (SAR) of compound #25 and its derivatives. #25-5 is illustrated separately due to its unique core structure.

(D) In vivo p27 ubiquitination assay in 293T cells transfected with various constructs in the presence of DMSO, compound #25, or its derivatives with MG132 treatment.

(E) PC3 cells were treated with various doses of compound #25 or its derivatives, followed by cell survival assay.
Figure 6. Skp2 Inactivation Diminishes Cancer Stem Cell Properties

(A) Populations of ALDH+ cells were determined by FACS analysis in PC3 cells treated with vehicle or compound #25.

(B) Populations of ALDH+ cells were determined by FACS analysis in PC3 cells with control or Skp2 knockdown.

(C) Populations of ALDH+ cells were determined by FACS analysis in PC3 cells with control or Skp2 knockdown treated with vehicle or compound #25.

(D) Populations of ALDH+ cells were determined by FACS analysis in PC3 cells with DMSO or LY294002.

(E) Populations of ALDH+ cells were determined by FACS analysis in LNCaP cells treated with DMSO, LY294002, or compound #25.

(F and G) Prostate sphere self-renewal ability-formation assay in PC3 (F) and LNCaP (G) cells treated with DMSO, LY294002, or compound #25. Results are presented as mean values ±SD; **p < 0.01.
Pharmacological Skp2 Inactivation Exhibits Strong Antitumor Activities In Vivo

Figure 7. Skp2 Inhibitor Heightens Cancer Cell Sensitivity to Chemotherapy and Suppresses Tumor Growth in Human Tumor Xenografts (A and B) PC3 cells in the absence or presence of compound #25 were treated with Dox (doxorubicin) (A) or CPA (cyclophosphamide) (B), followed by cell survival assay. (C and D) Nude mice bearing A549 (C) or PC3 (D) tumor xenografts were administration with or without compound #25 via i.p. injection. Mean tumor volumes ±SD are shown; n = 6 mice per group. (E and F) The quantification results (E) and representative images (F) of histological analysis of p27, p21, pAkt, and Glut1 in PC3-induced tumor xenografts. “Low” indicates 40 mg/kg and “high” indicates 80 mg/kg of compound #25 was injected into mice. Scale bar indicates 100 mm.
The working model depicts how Skp2 inhibitor prevents Skp2-SCF complex formation and results in tumor suppression.
Summary:

1. Skp2 E3 ligase is overexpressed in numerous human cancers and plays a critical role in cell-cycle progression, senescence, metabolism, cancer progression, and metastasis.
2. In the present study, the authors identified a specific Skp2 inhibitor using high-throughput in silico screening of large and diverse chemical libraries.
3. This Skp2 inhibitor selectively suppresses Skp2 E3 ligase activity, but not activity of other SCF complexes.
4. It also phenocopies the effects observed upon genetic Skp2 deficiency, such as suppressing survival and Akt-mediated glycolysis and triggering p53-independent cellular senescence.
5. Strikingly, we discovered a critical function of Skp2 in positively regulating cancer stem cell populations and self-renewal ability through genetic and pharmacological approaches. Notably, Skp2 inhibitor exhibits potent antitumor activities in multiple animal models and cooperates with chemotherapeutic agents to reduce cancer cell survival.
6. Our study thus provides pharmacological evidence that Skp2 is a promising target for restricting cancer stem cell and cancer progression.