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Research paper

Identification of Novel Cdc7 Kinase Inhibitors as Anti-Cancer Agents that Target the Interaction with Dbf4 by the Fragment Complementation and Drug Repositioning Approach

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ABSTRACT

Background: Cdc7-Dbf4 is a conserved serine/threonine kinase that plays an important role in initiation of DNA replication and DNA damage tolerance in eukaryotic cells. Cdc7 has been found overexpressed in human cancer cell lines and tumor tissues, and the knockdown of Cdc7 expression causes an p53-independent apoptosis, suggesting that Cdc7 is a target for cancer therapy. Only a handful Cdc7 kinase inhibitors have been reported. All Cdc7 kinase inhibitors, including PHA-767491, were identified and characterized as ATP-competitive inhibitors. Unfortunately, these ATP-competitive Cdc7 inhibitors have no good effect on clinical trial.

Methods: Here, we have developed a novel drug-screening platform to interrupt the interaction between Cdc7 and Dbf4 based on *Renilla reniformis* luciferase (Rluc)-linked protein-fragment complementation assay (Rluc-PCA). Using drug repositioning approach, we found several promising Cdc7 inhibitors for cancer therapy from a FDA-approved drug library.

Findings: Our data showed that dequalinium chloride and clofoctol we screened inhibit S phase progression, accumulation in G2/M phase, and Cdc7 kinase activity. In addition, *in vivo* mice animal study suggests that dequalinium chloride has a promising anti-tumor activity in oral cancer. Interestingly, we also found that dequalinium chloride and clofoctol sensitize the effect of platinum compounds and radiation due to synergistic effect. In conclusion, we identified non-ATP-competitive Cdc7 kinase inhibitors that not only blocks DNA synthesis at the beginning but also sensitizes cancer cells to DNA damage agents.

Interpretation: The inhibitors will be a promising anti-cancer agent and enhance the therapeutic effect of chemotherapy and radiation for current cancer therapy.

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1. Introduction

Cdc7 is a highly conserved serine/threonine kinase from yeast to human and also known as Dbf4/Drf1-Dependent Kinase (DDK). Cdc7 forms a complex with Dbf4, an activation subunit, to generate an activate kinase complex [1]. Cdc7/Dbf4 kinase phosphorylates and activates the putative MCM helicase complex and Cdc45 to facilitate the initiation of DNA replication, which is the first step required to establish a competent replication fork for semiconservative DNA synthesis [2].

Cdc7 and Dbf4 are overexpressed in many cancer cell lines and in certain primary tumors [3,4]. Aberrations in DNA replication are a major cause to tumorigenesis and genome instability, a hallmark of cancer cells [5]. Indeed, overexpression of Cdc7 is associated with tumor advanced clinical stage, cell cycle deregulation, and genomic instability in ovarian [6], breast cancer [7], lung adenocarcinoma [8], and oral cancer [9]. Additionally, Dbf4 overexpression is associated with lower relapse-

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Research in context

Evidence before this study

So far Cdc7 kinase inhibitors for cancer therapy available are ATP-competitive compounds, which are suffered from the problem of specificity due to sequence and structural similarity.

Added value of this study

Here we developed a platform based on luciferase-linked complementation assay, a *Renilla* Luciferase Protein Fragment Complementation assay (*R*Luc-PCA), to interrupt the Cdc7 interaction with Dbf4. We identified the drugs that selectively target Cdc7-Dbf4 *via* interrupting their interaction after we screened a library collection of the US FDA-approved drug compounds according to their effects on the *R*luc-PCA Cdc7-Dbf4 reporter. Our results indicate that Dequalinium chloride, a *bona fide* Cdc7-Dbf4 kinase inhibitor, has antitumor activity *in vivo* in preclinical cancer models. In addition, we found that dequalinium chloride and clofoctol sensitize the effect of platinum compounds and radiation due to synergistic effect.

Implications of all the available evidence

This approach will open an avenue to the identification of new aspects of specific Cdc7 inhibitors that have a synergistic effect with platinum compounds and radiation.

free survival in cutaneous melanoma [10]. Similar to its substrate, MCM2–7, increased Cdc7 level is thought to link to the proliferation of tumor cells [11]. Upregulation of Cdc7 and Dbf4 in numerous tumor cells makes Cdc7 an attractive target for cancer therapy [4,12]. Moreover, knockdown of Cdc7 was shown to cause cell death in cancer cells, but not in normal cells, in which p53-dependent pathways arrest the cell cycle in G1 phase. The apoptotic response induced in cancer cells by Cdc7 depletion is not mediated by p53 [13], but is activated by the stress-activated protein p38 MAPK in an ATR-dependent manner [14]. Thus, the fact that differential killing activity of Cdc7 inhibition has allowed for the development of small molecules targeting Cdc7 kinase for cancer therapy [4,15–21]. However, all Cdc7 inhibitors available so far target ATP binding region of the kinase, which will influence other kinase function due to sequence and structural similarity.

In the present study, we have developed a *Renilla reniformis* luciferase-based protein-fragment complementation assay (*R*luc-PCA) platform to identify chemicals with specificity to interrupt the protein-protein interaction between Cdc7 and Dbf4. The PCA strategy allows the detection of protein complex formation by fusing each of the proteins of interest to two fragments of a "reporter" protein [22,23]. Binding of the two proteins of interest brings the unfolded fragments into proximity, allowing for folding and reconstitution of measurable activity of the *R*luc [23–26]. We identified the drugs that selectively target Cdc7-Dbf4 *via* interrupting their interaction after we screened a library of the US Food and Drug Administration (FDA)-approved drug compounds according to their effects on the Rluc-PCA Cdc7-Dbf4 reporter. Our results indicate that Dequalinium chloride, a *bona fide* Cdc7-Dbf4 kinase inhibitor, has antitumor activity *in vivo* in preclinical cancer models. Overall, *R*luc-PCA assay system will be a powerful screening method for identifying specific Cdc7 inhibitors to develop novel anticancer drugs.

2. Materials and methods

2.1. Construction of plasmids

Fragments (Luc1:1-110aa; Luc2:111-310aa) of the *R*luc gene were PCR-amplified (template, pRL-TK; Promega). Cdc7 and Dbf4 were subcloned into the 5′ end of the 10-aa linker $(GGGGS)_2$ and the *R*luc-PCA fragments (Luc1 or Luc2; pcDNA3β).

2.2. Cell culture, reagents, and immunoblot analysis

293 T cell lines were plated into 6-wellplate and grown in DMEM (Gibco) supplemented with 10% FBS (Invitrogen), 1% penicillin/streptomycin(Gibco) at 37 °C in 5% CO2 Transient transfections with PolyJet (SignaGen) according to the manufacturer's instructions. The reactions were terminated and immunoblotted with anti-Cdc7 (ThermoFisher) and Anti-Dbf4 (LTK BioLaboratories [27],). Libraries used are: 1175 FDA-approved drug library (SelleckChem, L1300-01). FDA approved drugs supplied as pre-dissolved DMSO solutions.

2.3. Peptide synthesis and peptide visualization

Peptide (HPFFKDM) label with Nuclear Localization Signal (PKKKRKV) and FITC was synthesized (Yao-Hong Biotechnology Inc.). Peptide (CLHPHQPSHPRAASPR) was synthesized (Kelowna International Scientific Inc.) for control. The cells were transfected with the ProteoJuice Protein Transfection Reagent (Merck) according to the manufacturer's instructions.

Peptide were transfect with 5 μM to HeLa cell in 6 well culture dish, incubate 24 h at 37 °C in 5% CO2. Label with ProLong® Gold Antifade Reagent with DAPI (Invitrogen), and examined under a fluorescence microscope BX51 (Olympus).

2.4. Bioluminescence assay

293T cells were co-transfected with plasmid DNA (cdc7-luc1: dbf4-luc2=1:1) using Maestrofectin transfection reagent (Omics Bio). Co-transfected cells (≈5×104 cells) were transferred to 96-well white-walled plates (Costar). For drug screening, 10 μM of compounds of FDA-approved drug library were added for 24 hrs and then the cells were subjected to bioluminescence analysis. *R*luc activities were monitored for the first 2 seconds after addition of the ViviRen™Live Cell Substrate (Promega).

2.5. Cell viability assay

Cell viability assay was examined using the CellTiter 96 AQueous One Solution Cell Proliferation Assay Kit (Promega, USA) according to the manufacturer's recommendations. Percentage of cell viability was analyzed and normalized against the untreated controls.

2.6. Irradiation treatment

OEC-M1 cells were irradiated at different doses (0, 2, and 5 Gy). X-irradiation was performed with a 160 kV RS 2000 X-ray biological Irradiator (Rad Source Technologies, USA) at a dose rate of 16.55 mGy/s at 25 mA.

2.7. In vitro kinase assay

In vitro kinase assay was performed using 293 T cells that is transiently transfected with FLAG-tagged Dbf4 and Cdc7 plasmid. Anti-FLAG immunoprecipitates of Cdc7-Dbf4 kinase were incubated with the purified GST-MCM2 (aa1-169) as a positive control in Cdc7 kinase

buffer (25 mM HEPES pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 10 μM ATP) and in the presence of 10 μCi [γ-^{32}P]ATP and phosphatase inhibitors (10 mM NaF, 50 mM β-glycerophosphate) at 30 °C for 40 mins, followed by the addition of SDS-PAGE sample buffer to stop reaction. Phosphorylated radioactive proteins were separated by SDS-PAGE and detected by autoradiography of the dried gels.

2.8. Co-immunoprecipitation assay

The total cell lysate from 293 T cells was incubated with control or indicated antibodies and rotated for 2 h at 4 °C. The immunocomplex was captured by incubating with protein A/G-agarose beads (Merck, USA) for 3 h at 4 °C with constant rotation. Then the beads were washed three times with ice-cold NETN buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris-Cl pH 8.0, 0.5% NP-40 (*v*/v)) and ready for Western blot analysis.

2.9. Animal studies

Male BALB/c nude mice of 5–6 weeks of age were obtained from The National Laboratory Animal Center (Taipei, Taiwan). The use and care of the animals were approved by the Institutional Animal Care and Use Committee of NHRI. Mice were subcutaneously inoculated with OEC-M1 oral carcinoma cells (1×10^6); bearing a palpable tumor (100–150 mm^3) were selected and randomized into control and treated groups. Tumor-bearing mice of 19.1 ± 1.1 g body weight were given twice a week with either compound 7 or compound 9 by oral gavage at 15 mg/kg [28] and 250 mg/kg [29] in two different vehicle formulations: pure water and olive oil (Fragata Extra Virgin, Spain), respectively. Tumor dimension was measured by calipers and tumor mass was calculated using the formula: Mass (mg) = Tumor volume (mm^3) = D^2 x L/2, where D and L are the shortest and longest diameter in mm, respectively.

2.10. Statistical analysis

Multivariate Cox proportional hazard model was used to estimate hazard ratios with adjustments for age and gender. We considered a statistical significance if a *P*-value was <0.05. All data was analyzed using the R statistical software (version 3.1.1). Parametric Student's *t*-test was used to judge the significance of difference between conditions of interest. The association between Cdc7 protein level (scoring of the IHC staining) and the quantified clinicopathological features of tumors were examined by χ2 test. Cox proportional hazard regression model and log-rank test was applied for analysis of survival data. In all analysis, a *P* value of <0.05 was considered as statistically significant (Student's *t*-test, *p < 0.05, **p < 0.01, and ***p < 0.001).

3. Results

3.1. Design of the Rluc-PCA

The purpose of our study was to create a *R*luc-PCA for screen chemicals to interrupt the interaction between Cdc7 and Dbf4. We chose to generate a *R*luc-PCA, a widely used bioluminescence reporter, because of its simplicity and sensitivity [30]. Spilt points of a reporter protein that dissects into two PCA fragments are generally chosen based on the following criteria: (i) the cut sites without interference from the catalytic activity, (ii) the fragments fold recognizable three-dimensional (3D) subdomains, and (iii) the cut sites are in nonstructured regions [23,31]. The structure of *R*luc, isolated from the marine "sea pansy" *R. reniformis*, has been solved (PDB ID code 2PSD). In addition, Rluc-PCA has been successfully applied to identify novel direct modulators of protein kinase A and G protein-coupled receptors signaling [31]. Based on these previous studies, spilt point between amino acids 110 and 111 was chose to dissect the luciferase into two fragments, which provide reconstitution of the bioluminescence activity in the PCA experiment (Fig. 1A).

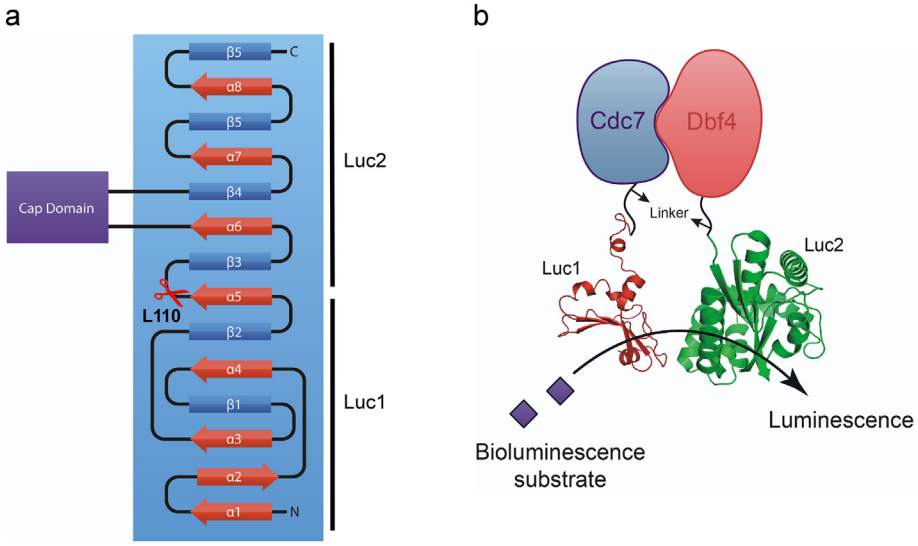

Fig. 1. *Renilla* luciferase-based protein fragment complementation assay (*R*luc-PCA) for the study of Cdc7-Dbf4 interaction. (A) *R*luc is a 310 a.a. protein and contains two major domains, Cap domain and α/β hydrolase domain. *R*luc was split into two fragments between L110 and P111, which is located between α5 and β3 motif in α/β hydrolase domain. Fragment a.a.1–110 named Luc1; a.a 111–310 named Luc2. (B) Schematic representation of the PCA strategy using *R*luc fragments to study the interaction between Cdc7 and Dbf4. The association of Cdc7 and Dbf4 induces the assembly of two *R*luc fragments, leading to increasing *R*luc-PCA activity. A linker peptide was inserted to connect Luc1 and Cdc7 as well as Luc2 and Dbf4.

3.2. Characterization of the interaction in Cdc7-Dbf4 complex in vivo using the Rluc-PCA reporter

The general scheme for construction and detection of the *R*luc-PCA Cdc7-Dbf4 sensor fusing complementary fragments of *R*luc to the C-termini of Cdc7 and Dbf4 was shown (Figs. 1B and 2A). The expression efficiency of the Rluc-PCA Cdc7-Dbf4 sensor, Cdc7-Luc1 and Dbf4-Luc2, was examined by Western blotting analysis (Fig. 2A, bottom). We then measured the first 3 s of bioluminescence by monitoring the luciferase activities after addition of the *R*luc *in vivo* substrate (ViviRen™). Expression of individual PCA fusion protein in 293 T cells was unable to detect bioluminescence signal, confirming the specificity of the assay. Only coexpression of Cdc7-Luc1 and Dbf4-Luc2 gave significant bioluminescence signals (Fig. 2B). These results indicated that a direct protein-protein interaction between Cdc7 and Dbf4 is necessary to support reconstitution of *R*luc enzyme activity. Intriguingly, neither excess Cdc7 nor excess Dbf4 gave good bioluminescence signals, suggesting a stoichiometric relationship between Cdc7 and Dbf4 interaction.

3.3. Specificity of the interaction in Cdc7-Dbf4 complex using the Rluc-PCA reporter

To confirm specificity of the interaction in Cdc7-Dbf4 complex using the *R*luc-PCA reporter, we first designed a peptide named Cdc7-Dbf4

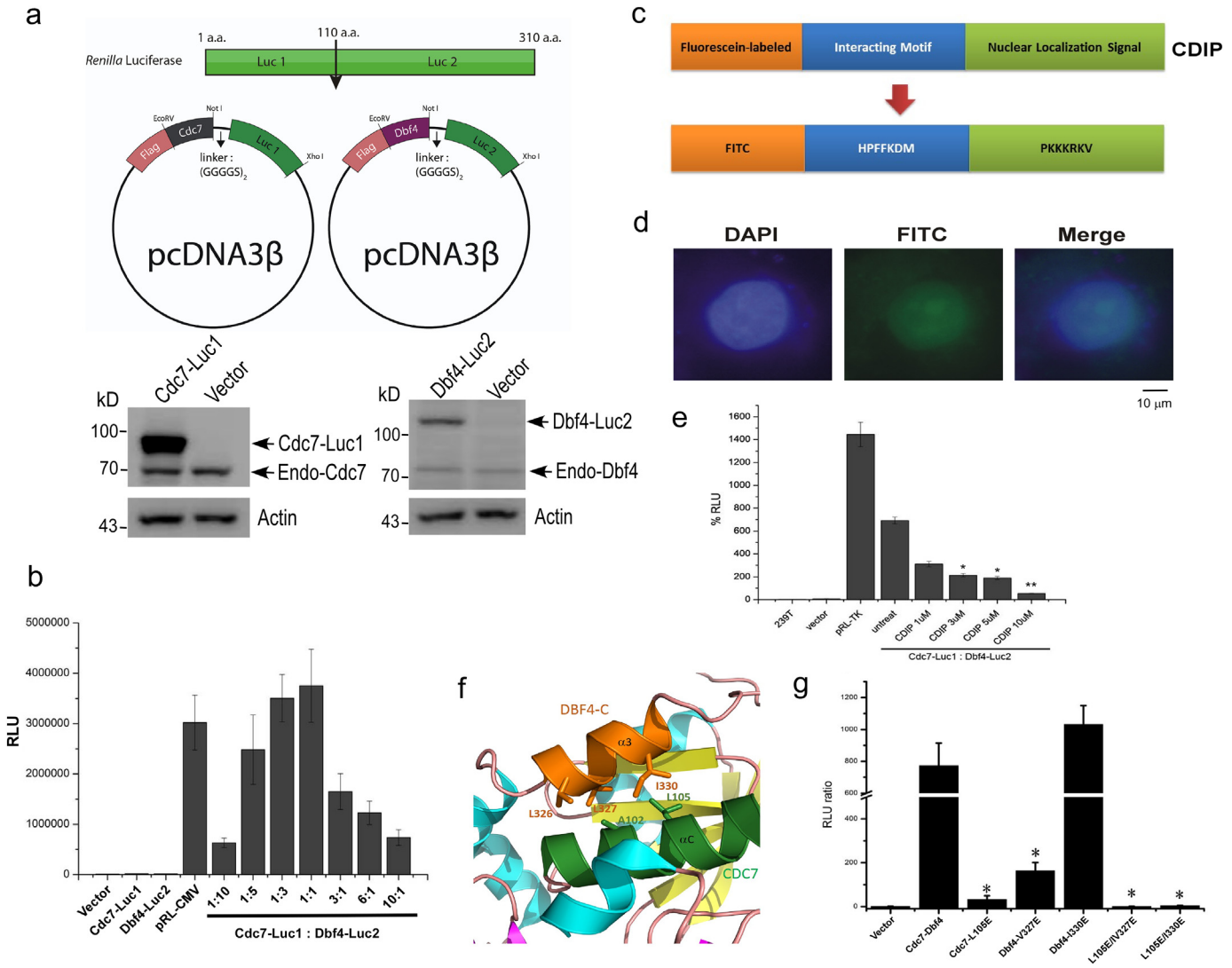

Fig. 2. A reporter of Cdc7-Dbf4 interaction based on the *R*luc-PCA strategy. (A) Schematic representation for the design of an *R*luc-PCA-based reporter of Cdc7-Dbf4 interaction. Cdc7 and Dbf4 were fused to Luc1 or Luc2 of *Renilla* luciferase with flag tag in a pcDNA3β vector. The expression of Flag-Cdc7-Luc1-pcDNA3β (Cdc7-Luc1) and Flag-Dbf4-Luc2-pcDNA3β (Dbf4-Luc2) was examined by Western blotting analysis after transiently transfection into 293 T cells (bottom). Cdc7-Luc1 is recognized by anti-Cdc7 antibody shown in left panel and Dbf4-Luc2 by anti-Dbf4 in right panel. Endo Cdc7 and Endo Dbf4 stands for endogenous Cdc7 and Dbf4, respectively. (B) Characterization of Cdc7-Dbf4 *R*luc-PCA platform using measurement of *Renilla* luciferase activity. *R*Luc signals were detected after transfection of different ratio of Cdc7-Luc1:Dbf4-Luc2. Transfection of pcDNA3β vector only and individual Cdc7-Luc1 and Dbf4 Luc2 was used as negative controls. RLU, relative luminescence units. (C) Schematic representation for the design of Cdc7-Dbf4 interrupter peptide (CDIP). CDIP includes FITC, interacting motif, and nuclear localization signal segment. (D) Confirmation of the nuclear location of CDIP in cultured cells. The co-localization between FITC and DAPI was examined under fluorescence microscope after 24 h peptide transfection. (E) Specificity of the Cdc7-Dbf4 Rluc-PCA platform was examined using Cdc7-Dbf4 interrupter peptide (CDIP). The cells transfected Cdc7-Luc1/Dbf4-Luc2 (1:1) or not were transfected with none, CDIP (1 μM and 10 μM) or 10 μM Control peptide after 36 h. After another 12 h, luciferase signals were detected by a luminometer. (F) Structural view on the interface between Cdc7 and Dbf4. Protein chains are shown with selected residues as sticks and indicated. The colors of the protein structure are shown as cartoons, with Cdc7 (the αC helix) in dark green and Dbf4 (motif C) in orange. Selected amino acids are shown in sticks. Each amino acid is referred to using its one-letter abbreviation. The structure around the interface between Dbf4 motif C and Cdc7 αC helix was built based on the crystal structure of human Cdc7-Dbf4 complex (PDB code: 4F99) [33]. (G) Specificity of the Cdc7-Dbf4 Rluc-PCA platform was examined by mutating the amino acid residues in the interface of Cdc7 and Dbf4. The cells were transfected with Cdc7-Luc1/Dbf4-Luc2 (1:1), Cdc7-Luc1/Dbf4-Luc2 with mutation, or with none. After 48 h, luciferase signals were detected by a luminometer.

interrupter peptide (CDIP) to inhibit the interaction between Cdc7 and Dbf4. We chose a Dbf4/ASK interacting motif-2 (DAM-2) on the C terminus of Cdc7 as a base of CDIP, which is identified as a Dbf4-binding motif and is essential for Cdc7 kinase activation by Dbf4 [32]. We thus designed a fluorescent dye-labeled peptide (FITC-HPFFKDM) and fused a SV40 nuclear-localizing signal (PKKKRKV) to the C terminus of DAM-2, allowing the peptide to enter nucleus (Fig. 2C). Therefore, we first ensured that CDIP is able to translocate to nucleus properly. The result showed that FITC signal is colocalized with DAPI by using a fluorescence microscopy, suggesting that CDIP indeed is transported into nucleus (Fig. 2D). Next CDIP was applied to confirm specificity of the interaction between Cdc7-Dbf4 in the *R*luc-PCA reporter platform. The result demonstrated that the *Renilla* luciferase signal is slightly decreased after addition of 1 μM CDIP and significantly decreased at 10 μM CDIP, suggesting that bioluminescence signals in the *R*luc-PCA reporter specifically represent the interaction between Cdc7 and Dbf4 (Fig. 2E).

To further confirm the specificity of Cdc7-Dbf4 Rluc-PCA reporter, we tried to interrupt the interaction by mutating the amino acid residues in the interface between Cdc7 and Dbf4 according to the crystal structure of human Cdc7-Dbf4 complex [33]. The effector domains of Dbf4, containing conserved motif M and C, are major contact sites with Cdc7 kinase. Dbf4 motif C is essential and sufficient to activate Cdc7 kinase activity by binding to and stabilizing the canonical αC helix of N-terminal lobe of Cdc7 kinase through C298 and V327 [33]. We rebulit the structure of the interface between Dbf4 motif C and Cdc7 αC helix (Fig. 2F). The potential amino acid residues involving the interface between Dbf4 motif C and Cdc7 αC helix include L326, V327, I330 of Dbf4, and A102, L105 of Cdc7 (Fig. 2F). The results showed that point mutations within V327 of Dbf4 and L105 of Cdc7 reduce bioluminescence signals in the *R*luc-PCA reporter, but not in the mutation in I330 of Dbf4 (Fig. 2G). In addition, the double mutation in L105 and V327 (L105E/V327E) and L105 and I330 (L105E/ I330E) almost abolished the bioluminescence signals of the *R*luc-PCA reporter. Taken together, our data indicate that bioluminescence signals in the Rluc-PCA reporter specifically reflect the interaction between Cdc7 and Dbf4.

3.4. The Rluc-PCA reporter-based high-throughput screening for compounds targeting Cdc7-Dbf4

To identify drugs that selectively target Cdc7-Dbf4 *via* interrupting their interaction, we screened a library collection of >1170 the US Food and Drug Administration (FDA)-approved drug compounds from a commercial supplier according to their effects on the intracellular bioluminescence signals of 293 T cells reported by the Rluc-PCA Cdc7-Dbf4 sensor. The bioluminescence signal (relative luminescence units, RLU) was used to describe the interaction variation between Cdc7 and Dbf4 induced by different compounds. While not all of these compounds have impact on the bioluminescence, even increase, we did identify 165 compounds that significantly decreased the RLU in 293 T cells (RLU < 50%, 14.1% of total library) and 88 compounds that cause a decrease in the RLU < 40% (Fig. 3A; Table S1). To exclude the decrease in bioluminescence is caused by cytotoxicity and to identify the

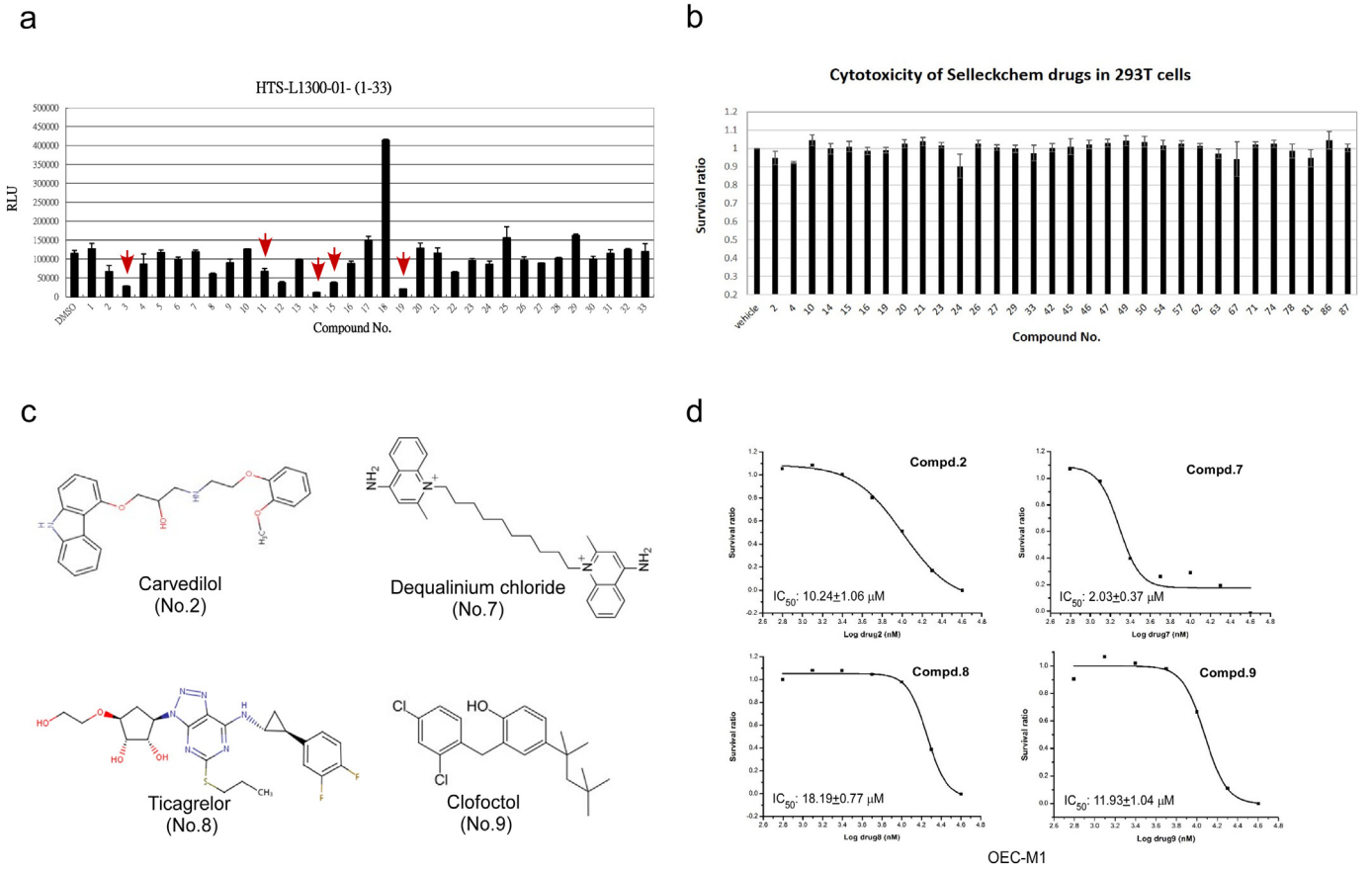

Fig. 3. Rluc-PCA-based high-throughput screening for compounds affecting the interaction of Cdc7 with Dbf4. (A) Representative results of Rluc-PCA-based high-throughput screening targeting the interaction of Cdc7 with Dbf4. 293 T cells expressing Cdc7-Dbf4 Rluc-PCA reporter were incubated with compounds and luciferase signals were measured. The luciferase signals (RLU) that below 50% of control were chosen as the red arrow pointed in the first round. (B) Specificity of Rluc-PCA-based high-throughput screening targeting the interaction of Cdc7 with Dbf4. The compounds that screened by the first round (RLU below 40% of control) were examined their cytotoxicity on 293 T cells. The 32 compounds without cytotoxicity were shown. (C) Chemical structure of potential inhibitors targeting Cdc7-Dbf4 interaction. (D) IC50 plot of the compounds on OEC-M1 cancer cells. Carvedilol (No. 2), Dequalinium chloride (No. 7), Ticagrelor (No. 8), and Clofoctol (No. 9) were chose to examine the cytotoxic effect on OEC-M1 cells by MTS assay. IC50 determination and the plot of the compounds on OEC-M1 cells were created and fitted by Origin8 software.

compounds that indeed decreased the RLU, we used 88 compounds to examine the cytotoxicity of 293 T cells transfected by Rluc-PCA Cdc7-Dbf4 sensor. The results showed that 32 of 88 compounds exhibited no significant 293 T cell toxicity ($p < 0.05$, Student's *t*-test) (Fig. 3B; Table S2 and S3). Considering the compounds that are non-anticancer drug, cheap, and commercial available, we chose 10 of 32 compounds (Table S4) to examine the cytotoxicity of cancer cells. The results showed that 4 of 10 compounds exhibited significant cytotoxicity in FaDu cancer cells (Fig. S1; Table S4). The four compounds we found are Carvedilol (No. 2), Dequalinium chloride (No. 7), Ticagrelor (No. 8), and Clofoctol (No. 9) (Fig. 3C). Carvedilol was known to block β-1 and β-2 adrenergic receptor (AR) and used in the treatment of mild congestive heart failure. In addition, β2-AR blockage is a potential therapeutic strategy for combating the progressions of β2-AR-dependent colorectal cancer [34]. Ticagrelor is a platelet aggregation inhibitor that does not require metabolic activation [35,36]. Dequalinium chloride and Clofoctol are known as antimicrobial agents. The former is an antimicrobial agents with a broad bactericidal and fungicidal activity [37] and the latter is a bacteriostatic antibiotic that is used in the treatment of respiratory tract, nose, and throat infections caused by Gram-positive bacteria [38]. The results we observed are supported by the previous study that some compounds exhibited anti-cancer effect [39,40].

We further confirmed the cytotoxicity of the four compounds on OEC-M1 cancer cells. The half inhibiting concentration (IC50) is listed as followed: Carvedilol (10.24 ± 1.06 μM), Dequalinium chloride (2.03 ± 0.37 μM), Ticagrelor (18.19 ± 0.77 μM), and Clofoctol (11.93 ± 1.04 μM) (Fig. 3D). To validate the compounds we found that indeed inhibit Cdc7-Dbf4 activity, we chose the best three compounds, Carvedilol (No. 2), Dequalinium chloride (No. 7), and Clofoctol (No. 9), to examine the effect on the function of Cdc7-Dbf4 by using Western blotting, *in vitro* kinase experiment, and BrdU incorporation assay. First, we found that Carvedilol (10 μM), Dequalinium chloride (2 μM), and Clofoctol (10 μM) inhibit Cdc7 activity in OEC-M1 cells to different extents judged by the intensity of MCM2 phosphorylation at S53, the substrate of Cdc7-Dbf4 kinase (2,31) (Fig. 4A). *In vitro* kinase experiments shows that Dequalinium chloride and Clofoctol obviously inhibit the phosphorylation of MCM2, even at 1 μM (Fig. 4B). Consistently, bromodeoxyuridine (BrdU) incorporation assay indicates that Dequalinium chloride and Clofoctol significantly block DNA replication (S phase) and delay cell cycle progression (G2/M phase) (Fig. 4C). To further validate the inhibition of Dequalinium chloride and Clofoctol on Cdc7-Dbf4 activity is through interfering with their interaction, we mixed Dequalinium chloride and Clofoctol with Cdc7-Dbf4 proteins to examine their interaction *in vitro* by using co-immunoprecipitation assay. The results indicated that the interaction between Cdc7 and Dbf4 is affected by the addition of Dequalinium chloride and Clofoctol (Fig. 4D). In conclusion, we found that Dequalinium chloride (No. 7) and Clofoctol (No. 9) that interrupt the interaction of Cdc7-Dbf4 indeed inhibit the kinase activity of Cdc7.

3.5. Dequalinium chloride and Clofoctol sensitize the therapeutic effect of cisplatin and radiation in oral cancer cells

We found that Dequalinium chloride and Clofoctol have cytotoxicity on OEC-M1 oral cancer cell line (Fig. 3D and Fig. S2) and inhibit the kinase activity of Cdc7-Dbf4 (Fig. 4). Since Cdc7-Dbf4 interacts with and phosphorylates HSP90-MRN complex to enhance ATR/ATM checkpoint signaling and DNA damage tolerance for the survival of cancer cells [41], the kinase inhibitor becomes an excellent anti-cancer agent that not only blocks DNA synthesis at the beginning but also sensitizes cancer cells to DNA damage agents. Although the resistance development of chemo- and radio-therapy, cis-diamminedichloroplatinum (II) (CDDP, cisplatin) and radiation are still widely used for the treatment of various solid tumors, including oral cancers [42]. Thus we tried to address whether Cdc7-Dbf4 inhibitors, Dequalinium chloride and Clofoctol, are promising therapeutic strategies to enhance the therapeutic effect of chemotherapy and radiation. We first confirmed the cytotoxic effect of Dequalinium chloride and Clofoctol and determined IC50 of cisplatin (5.58 μM) for OEC-M1 cells (Fig. 5A). However, the cytotoxicity of cisplatin on FADU cells was far to the results on OEC-M1 cells even without significant inhibition at 2 μM (Fig. 5B), suggesting that FADU cells are more resistant to cisplatin treatment than OEC-M1 cells. The combination experiment using FADU and OEC-M1 cells was performed. The results showed that Dequalinium chloride but not Clofoctol is able to enhance the effect of cisplatin on FADU cells (Fig. 5B, right panel). Consistently, the combination index (CI) data showed that Dequalinium chloride combined with CDDP at ratio 1:1 has synergistic effect and Clofoctol combined with CDDP at the same ratio has no synergistic effect on OEC-M1 cells (Fig. 5C and Table 1). And Dequalinium chloride combined with CDDP at ratio 2:1 has synergistic effect and Clofoctol has no synergistic effect at the same ratio on FADU cells (Fig. 5D). The results indicated that Dequalinium chloride has a better synergistic effect with cisplatin than Clofoctol with cisplatin.

We then examined whether Dequalinium chloride and Clofoctol enhance the cytotoxic effect of ionic radiation (IR) on OEC-M1 cells. The half inhibiting dose (ID50) of IR for OEC-M1 cells is determined to be 6.5 Gy (Fig. 5E and S3). Thus we used 2 Gy and 5 Gy of IR to combine different concentrations of Dequalinium chloride and Clofoctol and treated OEC-M1 cells. We found that treatment of lower IR (2 Gy) has no significant inhibition and IR of 5 Gy has around 20% inhibition on OEC-M1 cells but is effective only when combined with Dequalinium chloride and Clofoctol in a dose-dependent manner (Fig. 5F). Consistently, the combination of IR (5 Gy) and Dequalinium chloride or Clofoctol enhances the activation of apoptosis in OEC-M1 cells (Fig. 5G).

3.6. Dequalinium chloride suppresses tumor growth In vivo

The potential of Dequalinium chloride and Clofoctol as an anticancer drug *in vivo* was evaluated in nude mice carrying subcutaneous implanted tumors derived from the oral cancer OEC-M1 cell line. Tumor-bearing mice were given twice a week with either Dequalinium chloride or Clofoctol by oral administration at 15 mg/kg and 250 mg/kg, respectively. After oral administration of Dequalinium chloride for six consecutive weeks, a reduction in tumor volume with respect to vehicle-treated mice was observed (Fig. 6A). Tumor growth inhibition, calculated the day after the end of treatment, was about 90% at the dose of 15 mg/kg, where evidence of tumor regression in four out of five animals was observed (Fig. S4). In addition, Dequalinium chloride did not cause significant body weight loss in mice, suggesting that at the dose the compound appeared to be well tolerated (Fig. 6B). Unexpectedly, Clofoctol did not significantly reduce tumor volume in mice although the compound also cause no significant body weight loss in mice (Fig. 6C and D). Altogether these results indicate that Dequalinium chloride, a Cdc7-Dbf4 kinase inhibitor, has antitumor activity *in vivo* in preclinical cancer models.

4. Discussion

In the present study, we described a *Renilla* Luciferase Protein Fragment Complementation assay (*R*Luc-PCA) system that can be used to analyze protein-protein interaction between Cdc7 and Dbf4. We identified the drugs that selectively target Cdc7-Dbf4 *via* interrupting their interaction after we screened a library collection of >1170 the US Food and Drug Administration (FDA)-approved drug compounds according to their effects on the Rluc-PCA Cdc7-Dbf4 sensor. Our results indicate that Dequalinium chloride, a *bona fide* Cdc7-Dbf4 kinase inhibitor, has antitumor activity *in vivo* in preclinical cancer models (Fig. 7), suggesting that the Rluc-PCA Cdc7-Dbf4 sensor is a good platform to the development of anticancer drugs that targets on Cdc7 interaction with Dbf4 of cancer cells.

The advantages of the *R*luc-PCA have been described in previous reports [30,31]. This approach provides the ability to record and quantify

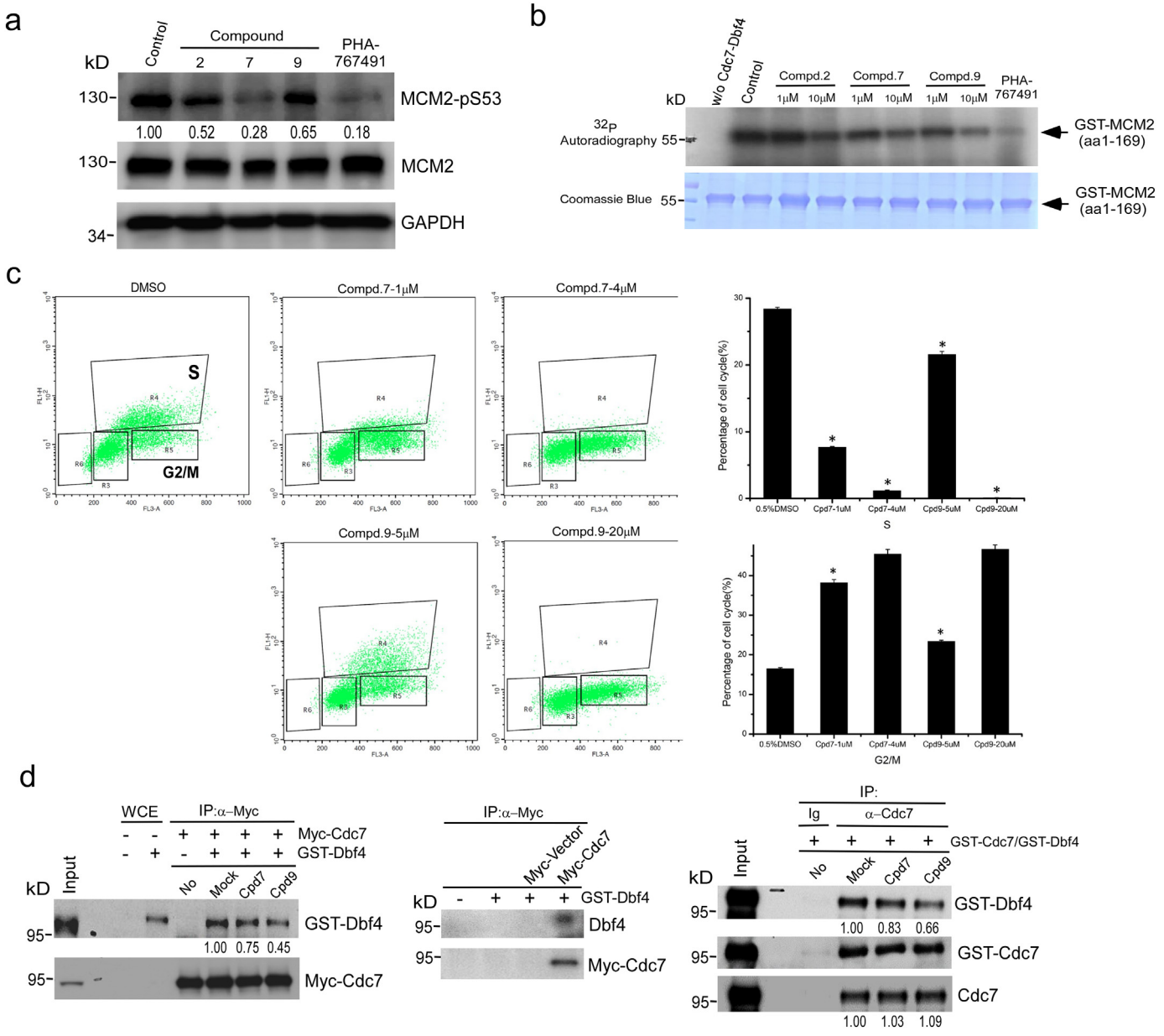

Fig. 4. Validation of the compounds screened by Rluc-PCA reporter by Cdc7-Dbf4 kinase activity. (A) The effect of the compounds on Cdc7-Dbf4 kinase activity. OEC-M1 cells were treated with Carvedilol (10 μM), Dequalinium chloride (2 μM), Clofoctol (10 μM) or PHA-767491 (2 μM) for 48 h. Protein extracts were prepared and analyzed by Western blot using the indicated antibodies. MCM2-pSer53 is a marker of Cdc7 activity. (B) The effect of the compounds on Cdc7-Dbf4 kinase activity shown by *in vitro* kinase assay. *In vitro* kinase assay was performed using 293 T cells that is transiently transfected with FLAG-tagged Dbf4 and Cdc7 plasmid. Anti-FLAG immunoprecipitates were incubated with GST-MCM2 (aa1-169) in the presence of [γ-^{32}P]ATP at 30 °C for 40 mins. Before incubation, each set of immunoprecipitates was treated with the compounds or PHA-767491. Phosphorylation of GST-MCM2 (aa1-169) and the input of GST-MCM2 (aa1-169) were shown by autoradiogram (*top*) and Coomassie Blue-stained gel (bottom), respectively. (C) The effect of the compounds on Cdc7-Dbf4 kinase activity shown by BrdU incorporation assay. OEC-M1 cells were treated with Carvedilol, Dequalinium chloride, or Clofoctol as the indicated concentration for 48 h. The treated cells were pulsed with 20 μM BrdU in culture medium at the last 2 h, and then conducted by FITC-BrdU Flow Kit (BD Biosciences). After pulse, the treated cells were fixed and dual-stained BrdU (Y-axis) and 7-AAD (X-axis) prior to flow cytometry analysis. FACS analysis was performed to demonstrate cell cycle distribution: S-phase (region R4) and G2/M (region R5) (gated area, left panel). The percentages of cells in S and G2/M phase are shown (right panel) (*: $p < 0.05$, Student's *t*-test). (D) The effect of the compounds on the interaction between Cdc7 and Dbf4 shown by co-immunoprecipitation assay. 293 T cells were transfected with plasmid encoding Myc-Cdc7 for 48 h. Then total cell lysates were collected and incubated with Myc-tag antibody (Millipore, 9E10) at 4 °C overnight followed by capturing the protein by protein A/G-agarose beads at 4 °C for 3 h. The beads were incubated with recombinant GST-Dbf4 protein (0.1 μg) and Cpd7 (5 μM) or Cpd9 (10 μM) at room temperature for 2 h. Then the beads were washed and applied for Western blot analysis as indicated (Left panel). Cell lysates from vector-transfected cells or GST-Dbf4 alone immunoprecipitated by Myc-tag antibody were used as a negative control (Middle panel). Recombinant GST-Cdc7 (2.5 μg) and GST-Dbf4 protein (2.5 μg) were incubated with Cpd7 (5 μM) or Cpd9 (10 μM) at room temperature for 2 h. Then the mixture was incubated with Cdc7 antibody (Thermo-Fisher) for 2 h at 4 °C followed by capturing the protein by protein A/G-agarose beads at 4 °C for 3 h. Then the beads were washed and applied for Western blot analysis as indicated (Right panel).

repeatedly live changes of protein-protein interaction in cell populations. In contrast to bioluminescent resonance energy transfer and fluorescence approaches, *R*luc-PCA is a readout for absolute values of protein complexes, which permits for the accurate quantification of even modest disruptions. In pharmacologic view, the very high signal-to-background ratio due to refolding of the *R*luc fragments in living cells allows more sensitive detection of protein-protein interaction dynamics by imaging single cells or simply by spectroscopic monitoring of whole cell populations that would be suitable for high-throughput drug screening applications.

Cdc7 is a serine/threonine kinase that plays an important role in the initiation of DNA replication and in S phase checkpoint control.

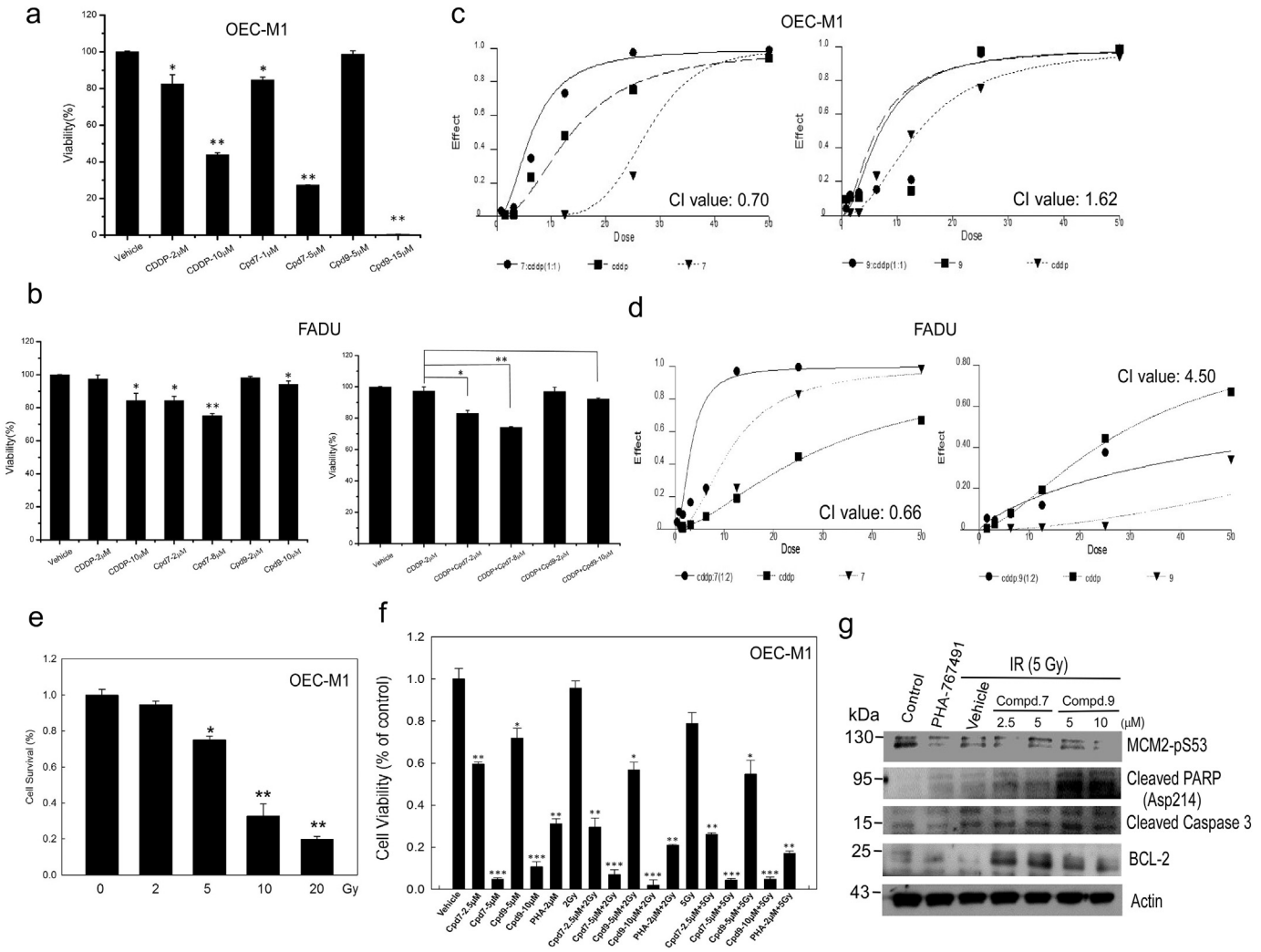

Fig. 5. Dequalinium chloride and Clofoctol sensitize the therapeutic effect of cisplatin and radiation in oral cancer cells. (A) The cytotoxic effect of cisplatin (CDDP), Dequalinium chloride, and Clofoctol on OEC-M1 cells. Effect of the compounds on OEC-M1 cell viability was analyzed by MTS assay. The concentration of cisplatin (CDDP), Dequalinium chloride, and Clofoctol was indicated. (B) The combination effect of CDDP and Dequalinium chloride or Clofoctol on FADU cells. Effect of the compounds (left panel) or the combination (right panel) on FADU cell viability was analyzed by MTS assay. The concentration of CDDP, Dequalinium chloride, and Clofoctol was indicated. (C) The determination of combination index of CDDP and the compounds in OEC-M1 cells. CDDP and the selected compounds were used at concentrations ranging 0.78125 to 50 μM. The treated cells were incubated for 48 h and determined the cytotoxicity using MTS assay. The combination index (CI) was calculated using CalcuSyn software (version 1.1.1). CI values were used to determine synergy (CI < 1), additivity (CI = 1), and antagonism (CI > 1) of the drug combinations tested. Experiments were conducted in triplicate ($n = 3$) with 7 serial concentrations at each drug. (D) The determination of combination index of CDDP and the compounds in FADU cells. CDDP and the selected compounds were used at concentrations ranging 0.78125 to 50 μM. The treated cells were incubated for 48 h and determined the cytotoxicity using MTS assay. The combination index (CI) was calculated using CalcuSyn software (version 1.1.1). Experiments were conducted in triplicate (n = 3) with 7 serial concentrations at each drug. (E) The cytotoxic effect of ionic radiation (IR) on OEC-M1 cells. Effect of IR on OEC-M1 cell viability was analyzed by MTS assay. The dose of IR treated was indicated. (F) The combination effect of IR and Dequalinium chloride or Clofoctol on OEC-M1 cells. Effect of the compounds or the combination on OEC-M1 cell viability was analyzed by MTS assay. The dose of IR treated and the concentration of Dequalinium chloride or Clofoctol were indicated. (G) Dequalinium chloride or Clofoctol enhances the effect of IR on OEC-M1 cells shown by apoptotic induction. OEC-M1 cells were exposed by IR or not and then treated with Dequalinium chloride or Clofoctol for 48 h. Apoptosis-associated proteins were detected by Western blot analysis using indicated antibodies. Anti-Actin antibody was used as loading control. The dose of IR treated was indicated. The concentration of Dequalinium chloride and Clofoctol was indicated. The concentration of PHA-767491 used was 2 μM.

Upregulation of Cdc7 has been observed in numerous tumor cell lines and tissues [3,4], making Cdc7 an attractive target for cancer therapy [4,12]. Moreover, the fact that differential killing activity of Cdc7 inhibition [13] has led to the development of small molecules targeting Cdc7 kinase for cancer therapy [4,15–17]. PHA-767491, the first nanomolar Cdc7 inhibitor [16], along with other inhibitors, is an ATP-competitive inhibitor that occupies the ATP-binding pocket of the kinase. However, targeting the ATP pocket of kinase for drug development has several weaknesses. First, the similarity of the ATP binding site across most kinase targets often results in problems with specificity, where one compound may potently inhibit multiple kinases. For example, the off-target effect of PHA-767491 on Cdk9 was observed [16,43]. Second, another consequence of the conserved nature of the ATP binding site is the highly congested intellectual property landscape for kinase inhibitors, making it difficult to discover a novel inhibitor that is chemically distinct from existing compounds. A recent report indicated that >10,000 patents and patent applications covering protein kinase

Table 1
Combination index (CI) of CDDP with the selected compounds in OEC-M1 cells.

Drug	Ratio	CI value	CI description[a]
CDDP+Cpd7	1:1	0.70051	Moderate synergism
	1:5	1.58997	Antagonism
CDDP+Cpd9	1:1	1.62308	Antagonism
	1:5	0.78036	Moderate synergism

[a] The combination index (CI) method is based on those described by Chou [56]. The ranges of CI are refined. CI < 1, = 1, and > 1 indicate synergism, additive effect, and antagonism, respectively.

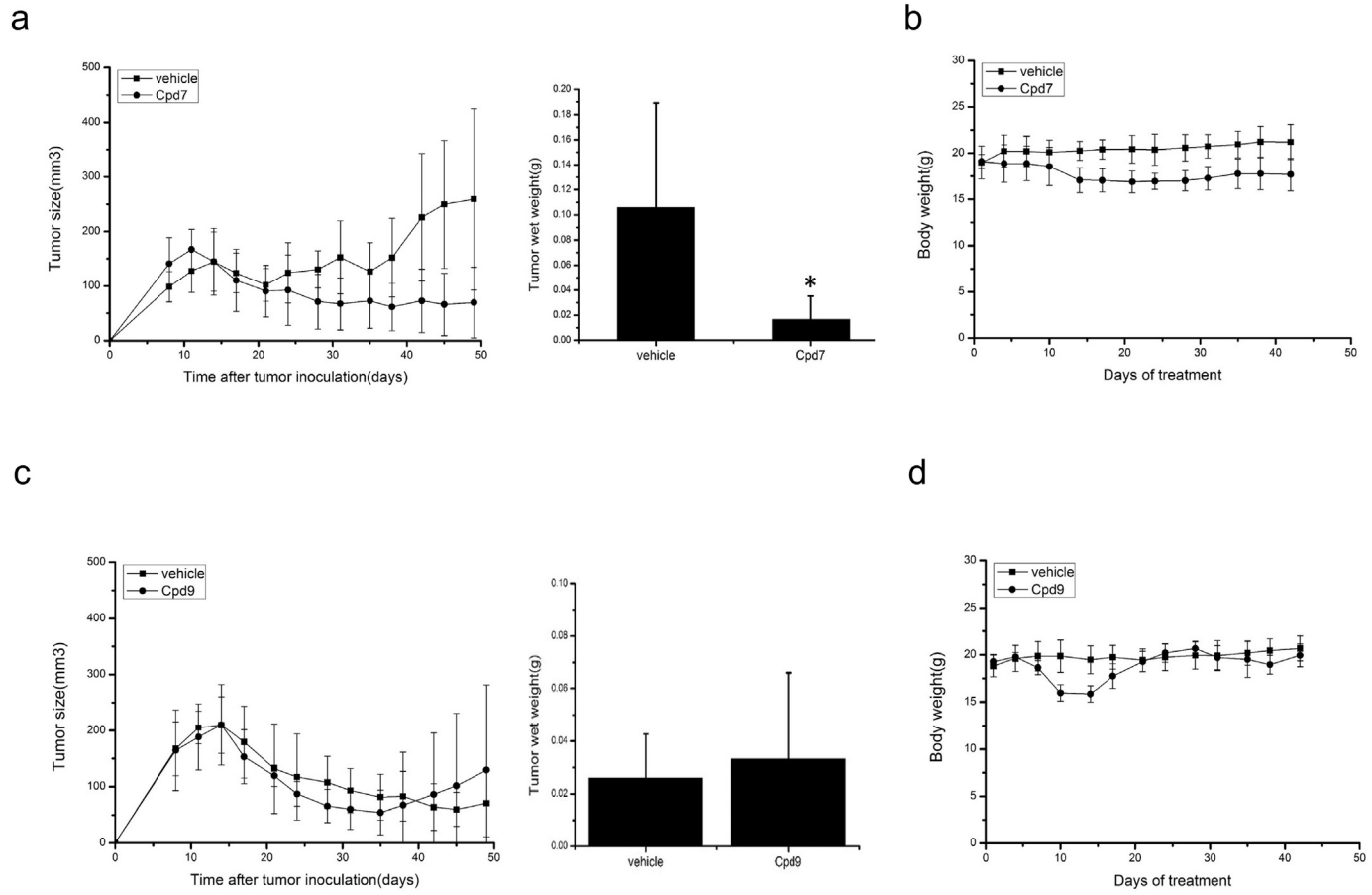

Fig. 6. Dequalinium chloride suppresses tumor growth *In vivo*. (A) BALB/c nude mice carrying subcutaneous OEC-M1 cell human tumors were treated with either vehicle or Dequalinium chloride (A and B) or Clofoctol (C and D) by oral administration. Tumor-bearing mice of 19.1 ± 1.1 g body weight (B and D) were given twice a week with either Dequalinium chloride or Clofoctol by oral gavage at 15 mg/kg and 250 mg/kg, respectively. Curves indicate tumor growth in vehicle-treated (filled squares) or the compounds-treated (filled circles) mice. Data are represented as mean ± s.e.m.

inhibitors have been published since 2001 [44]. Finally, since ATP is typically present at a concentration of 1 mM in the cell, it may be difficult to identify inhibitors with sufficient potency to effectively compete with endogenous ATP. Given these weaknesses, it is desperate to find alternative approaches to kinase inhibition in order to control selectivity and circumvent the need to compete with endogenous ATP [45]. Since Cdc7 is an important target of anticancer drug, the *R*luc-based PCA platform represents a widely applicable assay to develop new aspect of inhibitor in a high-throughput screening scale.

In this work, we report the identification of Cdc7 inhibitors that selectively target the interaction between Cdc7-Dbf4 *via* the strategy of drug repurposing. Drug repurposing or drug repositioning refers to

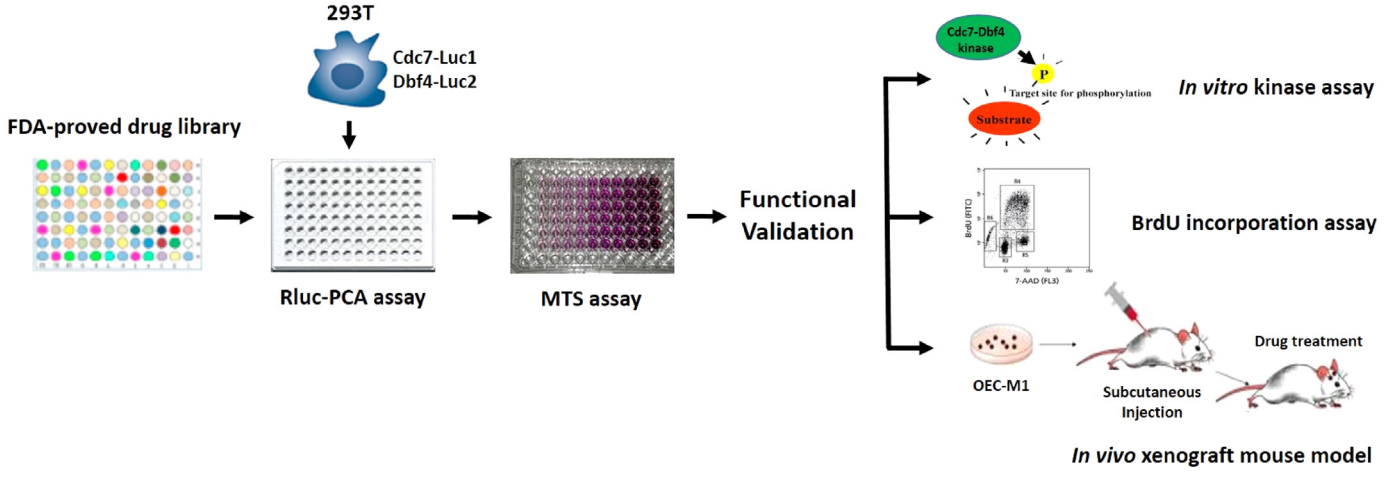

Fig. 7. Schematic of Rluc-PCA-based high-throughput screening for compounds affecting the interaction of Cdc7 with Dbf4. A library collection of >1170 the US FDA-approved drug compounds from a commercial supplier was used to screen drugs that selectively target Cdc7-Dbf4 interaction according to the bioluminescence signals reported by the Rluc-PCA Cdc7-Dbf4 sensor. 293 T cells expressing Cdc7-Dbf4 Rluc-PCA reporter were incubated with compounds and luciferase signals were measured. Functional validation includes *in vitro* kinase assay, BrdU incorporation assay, and *in vivo* xenograft mouse model.

the strategy of converting the indications of existing drugs from one therapeutic area to other diseases [46–49], which has received increasing interest as an alternative strategy for *de novo* drug development. A major advantage of drug repurposing is that it reduces the costs of drug development and shortens the time required for clinical application because of the existing results of toxicology testing and clinical trial. We screened a library collection of the US FDA-approved drug compounds using the Rluc-PCA reporter. After several round of screening and exclusion of traditional anti-cancer agents, we identified 2 compounds, Dequalinium chloride and Clofoctol, that are Cdc7-Dbf4 inhibitors and anti-cancer agents. Dequalinium chloride and Clofoctol are known as antimicrobial agents [38,50–52]. Dequalinium chloride, a quaternary ammonium compound, has a wide range of antimicrobial activity against bacteria, fungi and protozoa [37]. Its primary mechanism of action is the disruption of cell permeability and the subsequent loss of enzymatic activity [50]. In addition, Dequalinium chloride, as a positive charged lipophilic compound, exhibited anti-cancer activity based on selective mitochondrial accumulation and targeting mitochondrial DNA [39,53,54], and inhibiting cancer stem-like cells [55]. Therefore, Dequalinium chloride exhibited anti-cancer activity at least by alterations in mitochondrial function and inhibition of Cdc7 kinase activity in nucleus.

In conclusion, we demonstrated that Cdc7-Dbf4 Rluc-PCA reporter can be used as a platform to identify specific inhibitors of Cdc7-Dbf4 kinase through interrupting their interaction. We identify Dequalinium chloride and Clofoctol, known as antimicrobial agents, as potential inhibitors of Cdc7-Dbf4 kinase by using the strategy of drug repurposing. Dequalinium chloride inhibits cancer growth at least by the inhibition of Cdc7 kinase activity in nucleus and alterations in mitochondrial function. Thus, this approach paves the avenue to the identification and characterization of new aspects of Cdc7 inhibitor for cancer therapy.

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Conflict of interest

The authors have no conflicts of interest to declare.

Author contributions

A.-N.C., Y.-S.L., and A.Y.-L.L. conceived the project. A.-N.C., Y.-S.L., and Y.-K.L. designed the study and performed most of the experiments. C.-H.H. built the structure of the interface between Cdc7 and Dbf4. J.T.-A. H. provided the FDA-approved drug library and suggestions. T.-K.T. provided discussion and suggestions to the experiment or the manuscript. A.-N.C., Y.-K.L., and A.Y.-L.L. wrote the manuscript with input from all authors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2018.09.030.

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