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

An Ning Cheng,1 Yu-Kang Lo,1 Yi-Sheng Lin,1 Tswen-Kei Tang, Chun-Hua Hsu,1,2,3,4 John T.-A. Hsu,5 Alan Yueh-Luen Lee,⁎

National Institute of Cancer Research, National Health Research Institutes, Miaoli 35053, Taiwan
Department of Nursing, National Quemoy University, Kinmen 89250, Taiwan
Genome and Systems Biology Degree Program, National Taiwan University and Academia Sinica, Taipei 10617, Taiwan
Department of Agricultural Chemistry, National Taiwan University, Taipei 10617, Taiwan
National Health Research Institutes, Institute of Biotechnology and Pharmaceutical Research, Miaoli 35053, Taiwan
Department of Biotechnology, College of Life Science, Kaohsiung Medical University, Kaohsiung 80708, Taiwan

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 chemotherapay and radiation for current cancer therapy.

1. Introduction

Cdc7 is a highly conserved serine/threonine kinase from yeast to human and also known as Dbf4/Drfl-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-
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 (RLuc-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 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. In addition, we found that dequalinium chloride and clofocitol 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.

2. Materials and methods

2.1. Construction of plasmids

Fragments (Luc1:1-110aa; Luc2:111-310aa) of the RLuc 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 RLuc-PCA fragments (Luc1 or Luc2; pcDNA3%).

2.2. Cell culture, reagents, and immunoblot analysis

293T cell lines were plated into 6-well plate 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 (Thermo-Fisher) 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 Maestrofentin transfection reagent (Omicnic 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. RLuc 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 μA.

2.7. In vitro kinase assay

In vitro kinase assay was performed using 293T 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 MgCl₂, 1 mM DTT, 10 μM ATP) and in the presence of 10 μCi [γ-32P]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) 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 × 10⁶); bearing a palpable tumor (100–150 mm³) 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 (mm³) = D² 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 Rluc-PCA for screen chemicals to interrupt the interaction between Cdc7 and Dbf4. We chose to generate a Rluc-PCA, a widely used bioluminescence reporter, because of its simplicity and sensitivity [30]. Split 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 unstructured regions [23,31]. The structure of Rluc, 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, split 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 (Rluc-PCA) for the study of Cdc7-Dbf4 interaction. (A) Rluc is a 310 a.a. protein and contains two major domains, Cap domain and α/β hydrolase domain. Rluc 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 Rluc fragments to study the interaction between Cdc7 and Dbf4. The association of Cdc7 and Dbf4 induces the assembly of two Rluc fragments, leading to increasing Rluc-PCA activity. A linker peptide was inserted to connect Luc1 and Cdc7 as well as Luc2 and Dbf4.](image-url)
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 Rluc-PCA Cdc7-Dbf4 sensor fusing complementary fragments of Rluc 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 Rluc 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 Rluc 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 Rluc-PCA reporter, we first designed a peptide named Cdc7-Dbf4

![Image](36x179 to 551x584)
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 Rluc-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 Rluc-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 rebuilt 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 Rluc-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 Rluc-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-DBD4 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 Clofloctol (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 commercially 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 β1- and β2-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 progresses 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 S3, the substrate of Cdc7-Dbf4 kinase (2,31) (Fig. 4A). In vitro kinase experiments show 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 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 chemotherapeutic drugs is not a new science, the toxicity of 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 ionizing 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 the inhibition 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 (RLuc-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 RLuc-PCA have been described in previous reports [30,31]. This approach provides the ability to record and quantify
repeatedly live changes of protein-protein interaction in cell populations. In contrast to bioluminescent resonance energy transfer and fluorescence approaches, Rluc-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 Rluc 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. 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), Clofocetol (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 are transiently transfected with FLAG-tagged Dbf4 and Cdc7 plasmid. Anti-FLAG immunoprecipitates were incubated with GST-MCM2 (aa1-169) in the presence of [γ-32P]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 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 GST-MCM2 (aa1-169) and the input of GST-MCM2 (aa1-169) were shown by autoradiogram (top) and Coomassie Blue-stained gel (bottom), respectively. (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. 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 Cdc7 antibody (Thermo-Fisher) for 2 h at 4 °C followed by capturing the protein by protein A/G-agarse beads at 4 °C for 3 h. Then the beads were washed and applied for Western blot analysis as indicated (Right panel).]
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

| Drug               | Ratio | CI Value | CI Description |
|--------------------|-------|----------|----------------|
| CDDP+Cpd7          | 1:1   | 0.70051  | Moderate synergism |
| CDDP+Cpd7          | 1:5   | 1.58997  | Antagonism      |
| CDDP+Cpd9          | 1:1   | 1.62308  | Antagonism      |
| CDDP+Cpd9          | 1:5   | 0.78036  | Moderate synergism |

* 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.
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 Rluc-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. 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 Clofocitol (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 Clofocitol 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.

**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 toxicity testing and clinical trial. We screened a library collection of the US FDA-approved drug compounds using the RLuc-PCA reporter. After several rounds 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 lipopholic 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.

Funding source
This work was supported by grants from the Ministry of Science and Technology (NSC98–2311–B–400–003–MY3, MOST102–2302–B–400–014, and MOST105–2628–B–400–003–MY3), Taiwan;Ministry of Health and Welfare (MOHW106–T212–122,015), Taiwan; National Health Research Institutes (105/106A1-CA-PP-07), Taiwan to A. Y.-L. Lee.

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 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.

References
[1] Scalfani RA. Cdc7p-Dbf4p becomes famous in the cell cycle. J Cell Sci 2000;113( Pt 12):2111–7.
[2] Labib K. How do Cdc7 and cyclin-dependent kinases trigger the initiation of chromosome replication in eukaryotic cells? Genes Dev 2010;24:1208–19.
[3] Bonte D, Lindvall C, Liu H, Dykema K, Forge K, Weinreich M. Cdc7-Dbf4 kinase over-expression in multiple cancers and tumor cells line is correlated with p53 inactivation. Biochim Biophys Acta 2008;1784:590–7.
[4] Montagnoli A, Moll J, Colotta F. Targeting cell division cycle 7 kinase: a new approach for cancer therapy. Clin Cancer Res 2010;16:4500–8.
[5] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646–74.
[6] Kulkarni AA, Kingsbury SR, Tadzarova S, Hong HK, Loddo M, Rashid M, et al. Cdc7 kinase is a predictor of survival and a novel therapeutic target in epithelial ovarian carcinoma. Clin Cancer Res 2009;Vol. 15:2417–25.
[7] Chochiczhik M, Lebeau A, Marx AH, Tharun L, Terracciano L, Heilenkott J, et al. Overexpression of cell division cycle 7 homolog is associated with gene amplification frequency in breast cancer. Hum Pathol 2010;41:358–65.
[8] Datta A, Ghatak D, Das S, Banerjee T, Paul A, Butti R, et al. p53 gain-of-function mutations increase Cdc7-Dbf4-dependent replication initiation. EMBO Rep 2017;18:3020–50.
[9] Cheng AN, Jiang SS, Fan CL, Le YK, Kuo CY, Chen CH, et al. Increased Cdc7 expression is a marker of oral squamous cell carcinoma and overexpression of Cdc7 contributes to the resistance to DNA-damaging agents. Cancer Lett 2013;337:218–25.
[10] Namdari S, Mirmohammadseghad A, Hassan M, Mota R, Marini A, Alouei A, et al. Identification and functional characterization of ASK-D644, a novel cell survival gene in cutaneous melanoma with prognostic significance. Carcinogenesis 2007;28:2501–7.
[11] Gonzalez MA, Tachibana KE, Laskey RA, Coleman N. Control of DNA replication and its potential clinical exploitation. Nat Rev Cancer 2005;5:135–41.
[12] Srods W, Mahalingam D, O’Dwyer P, Santocanale C, Kelly K, Carew J, et al. Cdc7 kinase—a new target for drug development. Eur J Cancer 2010;46:33–40.
[13] Menichincheri M, Alasseur A, Caccione C, et al. Cdc7 inhibition reveals a p53-dependent replication checkpoint that is defective in cancer cells. Cancer Res 2004;64:7110–6.
[14] Im JS, Lee JK. ATR-dependent activation of p28 MAP kinase is responsible for apoptotic cell death in cells depleted of Cdc7. J Biol Chem 2008;283:25171–7.
[15] Kolton ES, Tshuako AL, Brown DS, Aay N, Arcasal A, Chan V, et al. Discovery of XL413, a potent and selective Cdc7 inhibitor. Bioorg Med Chem Lett 2012;22:3727–31.
[16] Menichincheri M, Valasaina R, Cheng AN, Menichincheri M, Rainoldi S, Marchevski V, et al. A Cdc7 kinase inhibitor restricts initiation of DNA replication and has antitumor activity. Nat Chem Biol 2008;4:357–65.
[17] Ermoli A, Bargiotti A, Brasca MG, Civolella A, Colombo N, Fachin G, et al. Cell division cycle 7 kinase inhibitors: 1H-pyrrolo[2,3-b]pyridines, synthesis and structure-activity relationships. J Med Chem 2009;52:4380–90.
[18] Kurasaawa O, Oguro Y, Miyazaki T, Homma M, Morii K, Iwai K, et al. Identification of a new class of potent Cdc7 inhibitors designed by putative pharmacophore model: Synthesis and biological evaluation of 3,3-dihydrobromo[3,2-g]pyridine-5(1H)-ones. Bioorg Med Chem 2017;25:2133–47.
[19] Irie T, Asami T, Sawa A, Uno Y, Hanada M, Taniyama C, et al. Discovery of novel furanone derivatives as potent Cdc7 kinase inhibitors. Eur J Med Chem 2017;130:406–18.
[20] Reichelt A, Bails JM, Bartberger MD, Yao G, Shu H, Kaller MR, et al. Synthesis and structure-activity relationship of trisubstituted thiadiazoles as Cdc7 kinase inhibitors. J Med Chem 2014;60:364–82.
[21] Menichincheri M, Bargiotti A, Berthelsen J, Bertrand JA, Bossi R, Ciavolella A, et al. First Cdc7 kinase inhibitors: pyrrolyridopyridines as potent and orally active antitumor agents. 2. Lead discovery. J Med Chem 2009;52:293–307.
[22] Johnson N, Varshavsky A. Split ubiquitin as a sensor of protein interactions in vivo. Proc Natl Acad Sci U S A 1994;91:10340–4.
[23] Michnick SW, Remy I, Campbell-Valois FX, Vailee-Belisse A, Pelletier JN. Detection of protein-protein interactions by protein fragment complementation strategies. Methods Enzymol 2000;328:206–30.
[24] Kubara A, Kawai Y, Sato M, Otsawa T, Umezawa Y. Locating a protein-protein interaction in living cells via split Renilla luciferase complementation. Anal Chem 2003; 75:4716–87.
[25] Luker KE, Smith MC, Luker GD, Gammon ST, Pwinnca-Worms H, Pwinnca-Worms D. Kinetics of regulated protein-protein interactions revealed with_firefly luciferase complementation imaging in cells and living animals. Proc Natl Acad Sci U S A 2004;101:12288–93.
[26] Munirgan R, Garnbith SS. Monitoring protein-protein interactions using split synthetic renilla luciferase protein-fragment-assisted complementation. Anal Chem 2003;75:1584–9.
[27] Lee AF, Chia T, Truong LN, Cheng AN, Do J, Cho MJ, et al. Db4 is direct downstream target of ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) protein to regulate intra-S-phase checkpoint. J Biol Chem 2012;287: 2531–41.
[28] Gamboa-Vujicic G, Emma DA, Liao SY, Fuchter C, Manetta A. Toxicity of the mitochondrial poison dequalinium chloride in a murine model system. J Pharm Sci 1993; 82:231–5.
[29] Alessandri MG, Ducchi M, Scalori V, Danesi R, Del Tacca M, Bernardini MC, et al. The pharmacokinetic profile of dequalinium in rat plasma and tissues after oral and rectal administration. Drugs Exp Clin Res 1986;12:343–7.
[30] Michnick SW, Ear PH, Manderson EN, Remy I, Stefan E. Universal strategies in research and drug discovery based on protein-fragment complementation assays. Nat Rev Drug Discov 2007;6:569–82.
[31] Stefan E, Aquin S, Berger N, Landry CR, Nyfeler B, Bouvier M, et al. Quantification of dynamic protein complexes using Renilla luciferase fragment complementation and functional characterization of ASK-D644, a novel cell survival gene in cutaneous melanoma with prognostic significance. Cancer Res 2007;28: 2501–7.
Hughes S, Elustondo F, Di Fonzo A, Leroux FG, Wong AC, Snijders AP, et al. Crystal structure of human CDC7 kinase in complex with its activator DBF4. Nat Struct Mol Biol 2012;19:1101–7.

Chin CC, Li JM, Lee KF, Huang YC, Wang KC, Lai HC, et al. Selective beta2-AR Blockade Suppresses Colorectal Cancer Growth through Regulation of EGFR-Akt/ERK1/2 Signaling, G1-phase arrest, and Apoptosis. J Cell Physiol 2016;231:459–72.

Capodanno D, Dharmashankar K, Angiolillo DJ. Mechanism of action and clinical development of ticagrelor, a novel platelet ADP P2Y12 receptor antagonist. Expert Rev Cardiovasc Ther 2010;8:151–8.

Doggrell SA. Ticagrelor, a platelet aggregation inhibitor for the potential prevention and treatment of arterial thrombosis and acute coronary syndromes. J Drugs 2009;12:309–17.

Mendling W, Weissenbacher ER, Gerber S, Prasauskas V, Grob P. Use of locally delivered dequalinium chloride in the treatment of vaginal infections: a review. Arch Gynecol Obstet 2016;293:469–84.

Wang M, Shim JS, Li RJ, Dang Y, He Q, Das M, et al. Identification of an old antibiotic clofocotol as a novel activator of unfolded protein response pathways and an inhibitor of prostate cancer. Br J Pharmacol 2014;171:4478–89.

Cheng AN, Fan CC, Lo YK, Kuo CL, Wang HC, Lien IH, et al. Cdc7-Cdk9-mediated phosphorylation of HSP90-S164 stabilizes HSP90-HCLK2-MRN complex to enhance ATR/ATM signaling that overcomes replication stress in cancer. Sci Rep 2017;7:17024.

Specenier PM, Vermorken JB. Current concepts for the management of head and neck cancer: chemotherapy. Oral Oncol 2009;45:409–15.

Natici A, Murillo IS, Kliszczak AE, Catherwood MA, Montagnoli A, Samali A, et al. Mechanisms of action of a dual Cdc7/Cdk9 kinase inhibitor against quiescent and proliferating CLI cells. Mol Cancer Ther 2011;10:1624–34.

Akritopoulou-Zanze I, Hajduk PJ. Kinase-targeted libraries: the design and synthesis of novel, potent, and selective kinase inhibitors. Drug Discov Today 2009;14:291–7.

Lamba V, Ghosh I. New directions in targeting protein kinases: focusing upon true allosteric and bivalent inhibitors. Curr Pharm Des 2012;18:2536–45.

Pantziaraka P, Bouché G, Meheus L, Sukhatme V, Sukhatme VP, Vikas P. The Repurposing Drugs in Oncology (ReDO) Project. CancerMedicineScience, vol. 8; 2014:442.

Skirle L, Forde HE, Netland IA, Leiss L, Skeie BS, Enger PO. Drug repositioning in cancer. Pharmacol Res 2017;124:74–91.

Huang J, Zhao D, Liu Z, Liu F. Repurposing psychiatric drugs as anti-cancer agents. Cancer Lett 2018;419:257–65.

Padyk BM, Gupta YK. Drug repositioning: re-investigating existing drugs for new therapeutic indications. J Postgrad Med 2011;57:153–60.

Hugo WB, Frier M. Mode of action of the antibacterial compound dequalinium acetate. Appl Microbiol 1969;17:118–27.

D'Auria FD, Simonetti G, Strippoli V. Antimicrobial characteristics of a tincture of dequalinium chloride. Annali di igiene 1989;1:1227–41.

Yablonsky F. Alteration of membrane permeability in Bacillus subtilis by clofocotol. J Gen Microbiol 1983;129:1089–95.

Schneider Berlin KR, Ammini CV, Rowe TC. Dequalinium induces a selective depletion of mitochondrial DNA from HeLa human cervical carcinoma cells. Exp Cell Res 1998;245:137–45.

Sancho P, Galeano E, Nieto E, Delgado MD, Garcia-Perez AL. Dequalinium induces cell death in human leukemia cells by early mitochondrial alterations which enhance ROS production. Leuk Res 2007;31:969–78.

Smith KM, Dutti A, Fujitani M, Grinshtein N, Zhang L, Morozova O, et al. Selective targeting of neuroblastoma tumour-initiating cells by compounds identified in stem cell-based small molecule screens. EMBO Mol Med 2010;2:371–84.

Chou TC. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Reviews 2006;58:621–81.