Development of Cell-based High Throughput Luminescence Assay for Drug Discovery in Inhibiting OCT4 and DNA-PKcs Interaction

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Abstract

Amplification-independent c-MYC overexpression is suggested in multiple cancers. Targeting c-MYC activity has therapeutic potential, but efforts thus far have been mostly unsuccessful. To find a druggable target to modulate c-MYC activity in cancer, we identified two kinases, MAPKAPK2 and the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), which phosphorylate the S111 and the S93 residues of OCT4, respectively, to transcriptionally activate c-MYC. Using these observations, we present here a novel cell-based luminescence assay to identify compounds that inhibit the interaction between DNA-PKcs and OCT4. After screening approximately 80,000 compounds, we identified 56 compounds ("hits") that inhibited the luminescence reaction. Using a custom antibody specific for pOCT4S[93], the "hits" were validated for their effect on OCT4 phosphorylation and activation. Seven compounds were selected for the second step of validation, which focused on the interaction between kinase and substrate. After further characterization, we identified two compounds that significantly impaired the ability of DNA-PKcs to bind to and phosphorylate OCT4. The compounds demonstrate a significant ability to kill cancer cells in the nanomolar range. In conclusion, we developed a cell-based luminescence assay to identify novel inhibitors targeting c-MYC transcriptional activation, and have found two compounds that may function as lead compounds for further development.

Keywords

Drug discovery, c-MYC, DNA-PK, protein-protein interaction, small molecule inhibitor, kinase

Introduction

There are over 500 protein kinases in humans (Wilson et al., 2018). Dysregulation of kinases by mutations are frequently associated with cancer initiation, proliferation, progression, and recurrence. These protein kinases have become crucial targets for developing drugs in the treatment of various cancers, and thus far, the FDA has approved over 50 kinase inhibitors for cancer therapy (Roskoski, 2020). Protein kinases that have been successfully targeted are ALK, BCR-Abl, B-Raf, BTK, CDK's, c-Met, EGFR, JAK, MEK1/2, PDGFR, RET, Src, and VEGFR (Roskoski, 2020; J. Zhang, Yang, & Gray, 2009). This class of drugs led a transformation from conventional chemotherapy to targeted cancer treatment and has overcome the normal cell toxicities of traditional chemotherapy. Although kinase inhibitors have shown activity in various types of cancers, there are several challenges to overcome, including drug resistance, unwanted toxicities, and compromised efficacy.

Kinase inhibitors are categorized based on the mechanisms of catalyzing the transfer of the terminal phosphate of ATP to the substrates. Type I kinase inhibitors are defined as small molecules that bind to the active conformation of a kinase in the ATP pocket. They represent ATP-competitors that mimic the purine

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ring of the adenine moiety of ATP (J. Zhang et al., 2009). Despite their clinical activity, these drugs display relatively low selectivity because the ATP-binding pocket is highly preserved among kinases. This low selectivity for specific target kinases is a possible reason for the cardiotoxicity associated with Type I kinase inhibitors (Bhullar et al., 2018; Force & Kolaja, 2011). Type II inhibitors reversibly bind to an inactive conformation (usually Asp-Phe-Gly (DFG)) of a kinase, and this interaction is proposed to exhibit better selectivity and lower toxicity than Type I kinase inhibitors (Garuti, Roberti, & Bottegoni, 2010; Kufareva & Abagyan, 2008). Type III kinase inhibitors bind outside the ATP-binding site and modulate kinase activity in an allosteric manner. Consequently, these inhibitors have the potential to be highly selective, as bindings sites targeted by allosteric inhibitors tend to be exclusive to particular kinases (Fabbro, 2015; Panicker, Chattopadhaya, Coyne, & Srinivasan, 2019). As allosteric inhibitors are anticipated to overcome limitations of the previous version of kinase inhibitors, several investigational agents are in the early stages of development (Fasano et al., 2014). Inhibitors that bind reversibly outside the ATP binding pocket in the kinase substrate-binding site are classified as Type IV kinase inhibitors. These are ATP-noncompetitive inhibitors that offer higher selectivity (Cox, Shomin, & Ghosh, 2011). Finally, type V kinase inhibitors are small molecules that form an irreversible covalent bond with the target enzyme and target a catalytic nucleophile cysteine within the active site of the enzyme (Bhullar et al., 2018; Cox et al., 2011).

The majority of the FDA-approved kinase inhibitors target ATP binding sites of kinases (Breen & Soellner, 2015; Huang, Zhou, Lafleur, Nevado, & Caflisch, 2010). Despite their anti-tumor activity, the ongoing challenges for clinical activity of kinase inhibitors are to enhance their selectivity and to overcome drug resistance (Fabbro, 2015). As described above, several efforts are underway to investigate ways to improve kinase inhibitors while minimizing their off-target effects (Mohiuddin & Kang, 2019). Here, we provide an approach to identify inhibitors with enhanced selectivity by targeting kinase-substrate interactions of DNA-PKcs. In our previous studies, we have demonstrated that OCT4 binds to the promoter/enhancer region to activate c-MYC transcriptionally, and one of the two kinases identified to phosphorylate OCT4 in this process is DNA-PKcs (Mohiuddin, Wei, & Kang, 2020; Wei et al., 2020). To overcome the limitation of currently available kinase inhibitors, we developed a cell-based assay to identify compounds that selectively inhibit the interaction of OCT4 with DNA-PKcs. Using the assay, we screened a chemical library of compounds to identify "hits" that inhibit the ability of DNA-PK to bind to OCT4. Subsequently, we validated the compounds identified from the chemical library. The purpose of this paper is to describe a novel cell-based assay to identify novel modulators of the interaction between DNA-PKcs and OCT4 and the method of hit validation employed to supplement the assay that may be applicable across many kinase-substrate interactions.

1. **Methods**
2. **Mammalian cell culture and transduction**

HEK293FT (ThermoFisher) cells were cultured in DMEM (ThermoFisher) supplemented with 10% FBS, 2 mM Glutamine, 100 units/mL Penicillin, 100 μg/mL Streptomycin Sulfate and 1 mM Sodium Pyruvate (ThermoFisher). The HEK293FT cells were plated at a cell dose of 1×10^7 on a 10-cm tissue culture dish and incubated at 37°C 5% CO2 incubator until the cells reach 80% confluence. The HEK293FT cells were co-transfected either lentiviral ORFs, the constructs shown in Fig. 2a, along with Lenti-vpak Packaging Kit (OriGene) using the transfection reagent MegaTran 1.0 (OriGene). After 48-72 h transfection, the virus-containing medium was collected, spun down, filtered (0.45 μm), and used for targeting into NCI-H82 by infection. The virus-infected stable clones were obtained after at least 2-3 weeks of selection in 10% FBS/RPMI-1640 with 0.5 μg/mL of Puromycin (Sigma-Aldrich). Protein lysates extracted from the stable clones were analyzed by SDS-PAGE/IB using specific antibodies to confirm their protein expression. The methods were performed in accordance with relevant guidelines and regulations and approved by the Institutional Biosafety Committee at Texas Tech University Health Sciences Center. The current study did not involve animals or human subjects.

Chemical Compounds

A total of 79,671 compounds were provided by the Targeted Therapeutic Drug Discovery & Development

Program at The University of Texas at Austin (Cho et al., 2018). The compounds were compiled from the following compound libraries: NIH clinical collection (674 compounds; Evotec, San Francisco, CA), Natural product or Natural product-like (3,280 compounds; MicroSource Discovery, Gaylordsville, CT and LifeChem, Niagara-on-the-Lake ON, Canada), Lopac (1,280 compounds; Sigma-Aldrich), fragment sets (18,143 compounds) obtained from Chembridge (San Diego, CA) and ChemDiv (San Diego, CA), kinase set (11,250 compounds; Chembridge), and diversity sets (43,158 compounds) obtained from NCI, ChemDiv, LifeChem, and Maybridge (ThermoFisher). Additionally, two other libraries were interrogated: 1) A kinase-focused library (600 compounds), custom selected by the Texas Screening Alliance for Cancer Therapeutics (TxS-ACT) from various vendors with known activity against approximately 100 kinases, and 2) An academic collection (2,000 unique molecules) with diverse pharmacophores deposited from chemists at The University of Texas at Austin and the University of Kansas. Compounds were plated in 384-well plates dissolved in 100% DMSO at 10 mM concentration.

Identification of compounds interfering kinase-substrate binding ("Hit ID")

HEK293FT cells stably expressing SmBiT-tagged DNA-PKcs and LgBiT-tagged OCT4 were suspended at 1 x 10^6 cells/mL of Opti-MEM® cell culture medium with reduced serum and seeded 9 μL of the suspension per well in a sterile black 384-well plate (Greiner, Cat #788086). Then, the cells were incubated for ~4 hours, and then 1 μL of the compounds (10 mM stock, 1:1000 dilution with Opti-MEM®) were added to wells to make the final concentration of compounds at 1 μM. After 6 hours of incubation with the compounds, Nano-Glo® Live Cell Assay (Promega) reagent was prepared as instructed by the company and added 1.3 μL to each well. Then, the plates were incubated for 20-30 minutes at room temperature before luminescence was measured by SpectraMax iD3 microplate reader (Molecular Devices). The cell counts per well, incubation time and serum content in the culture medium were optimized before the screening. All pipetting utilized the BenchSmart 96 semi-automated pipetting system (Rainin).

Luminescence was measured from each plate, and the data were collected in numerical values. For statistical analyses to identify a significant reduction in signals by compounds, data normality was tested by using a Shapiro–Wilk test and also visually examined by using a Q-Q normal plot. A box-cox transformation was performed when necessary. Compounds with a value of two standard deviations below the mean are considered outliers ("Hits"), i.e., inhibition of kinase-substrate binding, inhibition of kinase activity, or direct cell kill effect. The initial screening will identify the compounds with any of these three effects.

Custom polyclonal phospho-OCT4^{S93} antibody production.

The anti-human phospho-OCT4^{S93}(anti-pOCT4^{S93}) rabbit antibody was produced by GenScript Biotech. The pOCT4^{S93} polyclonal antibody was prepared by immunizing two New Zealand rabbits three times with an NH_2-terminal KLH (keyhole limpet hemocyanin)-conjugated phosphopeptide GLETSQPEGEAGVG as an antigen. The phospho-specific antibody was affinity-purified through a phosphopeptide-conjugated Sepharose CL-4B column. Eluted IgG was then passed through the corresponding non-phosphorylated peptide (GLETSQPEGEAGVG) column to deplete any IgG that was not specific to pOCT4^{S93}.

Validation of "hits" by immunoblotting and immunoprecipitation

The stable cell line was prepared for immunoblotting and co-immunoprecipitation by infecting NCI-H82, a small cell lung cancer cell line, with a doxycycline-inducible *pCW57.1-POU5F1-mycDDK* construct using a lentiviral system as previously described (Wei et al., 2020; Y. Zhang et al., 2014). The cell lines were prepared for immunoblotting and co-immunoprecipitation to validate the effect on kinase-substrate binding. Unless otherwise specified, cells grown in a T75 flask were first dissociated by PUCKs or Trypsin-EDTA and washed once with ice-cold 1× PBS. Cells were lysed on ice with modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 μg/mL Leupeptin, 1 μg/mL Aprotinin, 1 μg/mL Pepstatin A, 1 mM PMSF, 1 mM Na_3VO_4 and 1 mM NaF), followed by centrifugation at 14,000 × g for 15 min at 4°C. Protein concentration was determined by BCA assay (Pierce). An equal amount of proteins from different samples were electrophoretically separated on 4-12% SDS-PAGE, transferred to Hybond membrane (GE Healthcare), blocked with 1% BSA or 5% skim milk, immunoblotted with the

indicated primary antibodies, and incubated with 1:3,000 HRP-conjugated mouse or rabbit IgG secondary antibodies followed by detection with enhanced chemiluminescence (GE Healthcare). Primary antibodies used were: anti-DNA-PKcs (MBL International), anti-Ku80 (Cell Signaling Technology), anti-Ku70 (Cell Signaling Technology), anti-c-MYC (EMD Millipore), anti-p53 (BD Biosciences), anti-phospho-p53^{S15} (Cell Signaling Technology), anti-OCT4 (Abcam), anti-pOCT4^{S93} (Gentech), anti-pOCT4^{S111} (Gentech), anti-HSP27 (BD Biosciences), anti-pHSP27^{S78}, anti-MK2 (Cell Signaling Technology), anti-GAPDH (Santa Cruz Biotechnology). For EZView Red anti-FLAG pull-down, 500 μg of protein lysates as prepared above were pulled down at 4°C overnight with 40 μL EZview Red anti-FLAG M2 affinity gels (Sigma-Aldrich), washed 4 times with modified RIPA, and then eluted with an excess of 3× FLAG peptide (100 μg/mL). Immuno-complexes were resolved by 4-12% SDS-PAGE and immunoblotted with the indicated antibodies (anti-HA antibody). For pull-down studies using C8- and D8-conjugated agarose beads, 1 mg of protein lysates as prepared above for pulled down at 4°C overnight with 50 μL C8- or D8-conjugated agarose beads (CellMosaic). Beads were washed 4 times with modified RIPA, then eluted using glycine buffer elution. Beads were incubated with 100 μL of 0.1 M HCl + Glycine (pH = 3.5) at room temperature for 20 minutes. Ten μL of 0.5 M Tris-HCl (pH = 7.4) was added to neutralize the acidification. Immuno-complexes were resolved, as described above.

In vitro DNA-PKcs kinase activity and ADP-Glo^{TM} assays

100 Units of purified DNA-PKcs (Promega) or 200 ng His-MK2 was incubated at 30°C for 30 minutes in 20 μL kinase buffer containing 40mM Tris (pH 7.5), 20 mM MgCl$_2$, 0.1 mg/mL BSA, activation buffer (100 μg/mL calf thymus DNA in 1X TE buffer), 150 μM ATP, and inhibitors of interest. Following the 30-minute incubation, 1 μg of bacterially-derived OCT4 (ProteinOne), p53 (Creative BioMart), HSP27 (Enzo) protein substrates were added to the reaction. Samples were incubated again for 30 minutes at 30°C, and then the reactions were quenched with the addition of 4x NuPAGE LDS and 100 mM dithiothreitol (DTT) prior to proceeding with immunoblotting, as described above.

For the ADP-Glo^{TM} assays, a 25 μL kinase reaction was prepared to consist of 100 Units of purified DNA-PKcs or 200 ng His-MK2, 40mM Tris (pH 7.5), 20 mM MgCl$_2$, 0.1 mg/mL BSA, activation buffer (100 μg/mL calf thymus DNA in 1X TE buffer), 150μM ATP, 0.2 μg BSA, peptide substrate (amino acid sequence: EPPLSQEAFADLWKK; Promega), and inhibitors of interest or DMSO (control). Following a 30-minute incubation period at 30°C, 25 μL of ADP-Glo^{TM} reagent was added to stop the reaction and deplete unconsumed ATP. Samples were incubated at room temperature for 40 minutes. Fifty μL of kinase detection reagent was added to the samples to introduce luciferase and luciferin to detect the presence of ATP. After 60 minutes of incubation, luminescence was measured by SpectraMax iD3 microplate reader (Molecular Devices). Three reactions per treatment were done, and the luminescence measurements were averaged. DMSO (control) treatments were standardized to 100% kinase activity.

In vitro cytotoxicity assays

Human small cell lung cancer cell lines (NCI-H417, NCI-H82, NCI-H2171, NCI-H847, NCI-H1048, NCI-H146, NCI-H510A, NCI-H1963, NCI-H1876) were kindly provided by Dr. Adi Gazdar at University of Texas Southwestern. Cells were cultured in RPMI (GE Lifesciences) supplemented with 10% heat-inactivated fetal bovine serum. Cell lines were tested for and free of mycoplasma, and cell line identities were verified using short tandem repeat genotyping as compared with the original primary sample material within the CCcells database: www.CCcells.org. Small cell lung cancer cells (2 x 10^6 cells) were plated in 96-well plates for 24 hours prior to treatment with inhibitors (0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10 μmol/L). Six replicates of each drug concentration were used. After 96 hours of drug incubation, cellular viability was measured using the DIMSCAN assay, as outlined in previous studies(Kang et al., 2011; C. Zhang et al., 2012).

Results

Assay Development and Optimization

Having demonstrated that DNA-PKcs binds to and phosphorylates OCT4 at its Ser93 residue, we developed

a luminescence-based drug screening assay through the use of the NanoBiT® Protein: Protein Interaction system, which utilizes two subunit promoters (LgBiT and SmBiT) fused to genes of interest. Once these gene products are translated, fusion between the two subunits generates a luminescent signal. We stably expressed both the region of OCT4 necessary for *MYC* transcription (the NTD and POUs domains) tagged with the LgBiT subunit and the region of DNA-PKcs essential for OCT4 binding and phosphorylation tagged with the SmBiT subunit in the same vector through the use of the porcine teschovirus-1 2A (P2A) self-cleaving peptide (Figure 1). The P2A system follows a "stop-carry on" mode of translation, wherein ribosomes pause translation at the C-terminus of the 2A peptide before resuming translation at the end of the 2A sequence. This mode of translation generates two peptide fragments that are expressed in equal amounts within the transduced cell.

To determine incubation time, we continuously monitored luminescence up to 45 minutes after adding Nano-Glo® Live Cell Reagent into NCI-H82 cells stably transduced with the vectors shown in Fig. 1. Also, three different cell seeding densities (2,500, 5,000, and 10,000 cells/well) and two FBS content in the culture medium (10% FBS in RPMI or Opti-MEM®) were examined. It is noted that the cells in culture medium with reduced serum generates 2-3-fold higher luminescence compared with RPMI supplemented with 10% FBS in both NCI-H82 cells (Figure S1A) and HEK-293 FT cells (Fig. S1b). In addition, the cell count of 10,000 cells/well (10,000 cells in 10 µL of reaction volume: 1 x 10^6 cells/mL) displayed the highest luminescence signals relative to 5,000 or 2,500 cells/well regardless of the FBS content (Figure S1A&B). The luminescence signals were reduced in NCI-H82 cells cultured with Opti-MEM® containing medium at 10,000 cells/well over 45 minutes (34% reduction). In contrast, the signal reduction over incubation time was not seen with lower cell counts or in cells cultured in RPMI (Figure S1). The luminescence in HEK-293FT (DNA-PKcs-OCT4 interaction assay) gradually increased over the initial 15 minutes of incubation. Thus, 15-20 minutes of incubation was employed for the screening assays. The luminescence signals from NCI-H82 cells transduced with the empty vector were <10 in all three cell counts regardless of the FBS content in culture medium over 45 minutes (Figure S1C).

Compounds Identified from Chemical Library through the Assays

After establishing stable transduction of our two desired fragment constructs, we screened a drug library of ~80,000 compounds to identify potential inhibitors of the interaction between DNA-PKcs and OCT4 (Figure 2). Cells were seeded in plates containing Opti-MEM® culture media with reduced serum for 4 hours. Cells were then incubated with 1 µM of each compound for 6 hours prior to completion of the addition of the Nano-Glo® Live Cell Assay reagent (Figure 2A). Following the analysis of the luminescence data, outliers (decreased signal) were identified as potential inhibitors of the DNA-PKcs-OCT4 interaction (representative heatmap in Figure 2A). Compounds that inhibited the luminescence reaction by a factor of 1.96 x standard deviation were deemed to be hits (Figure 2B). Of the library of compounds tested, we identified 56 compounds for the DNA-PKcs-OCT4 interaction that significantly reduced the luminescence in the corresponding cell lines.

Effect on pOCT4^{S93}

Using mass spectrometry data, we previously demonstrated that OCT4 interacts with DNA-PKcs, and confirmed these findings through subcellular fractionation and co-immunoprecipitation. Further, the PhosphoMotif Finder software predicted that DNA-PKcs phosphorylates OCT4 at Ser93. To validate these anticipated findings, we first developed a custom phospho-antibody specific to OCT4 Ser93(Wei et al., 2020). Thus, the inhibition of the phospho-OCT4 levels was used as the first step of validation. To validate the effect of our compounds on the phosphorylation of OCT4, we infected a high c-MYC expressing small cell lung cancer (SCLC) cell line, NCI-H82, with a doxycycline-inducible lentiviral vector expressing *POUF51* tagged with mycDDK. Using a custom-produced phospho-OCT4 antibody, we determined the effect of the previously identified "hits" on the phosphorylation of OCT4 at its Ser93 residue in our OCT4-overexpressing NCI-H82 construct (Fig. S2a). After DOX-induction for 12 hours, we treated these cells with 1 µM of each compound. After 8 hours of treatment, immunoblot analysis was performed to determine the expression levels of pOCT4^{S93}. We identified six candidate compounds from the "hits" that significantly impaired

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phosphorylation of OCT4 at its Ser93 residue (Figure S2A&B). We also selected one compound as a negative control. Two compounds, C8 and D8, demonstrated both a remarkable inhibition of OCT4 Ser93 phosphorylation and decreased OCT4 expression.

Effect on DNA-PKcs-OCT4 Interaction

After validating the effects of the "hits" on OCT4 phosphorylation, we sought to better categorize these novel inhibitors as either impairing the catalytic activity of DNA-PKcs or targeting the binding interaction between DNA-PKcs and OCT4. To this end, we used the stably infected, DOX-inducible *POU5F1* tagged with mycDDK (FLAG) constructs in NCI-H82 and treated these cells with our validated hits. We then pulled down the FLAG-tagged OCT4 protein and detected the presence of DNA-PKcs. Of the seven validated hits, two compounds: C8 and D8, decreased expression of DNA-PKcs after OCT4 pull-down (Figure 3A). In line with the findings from the first hit validation step, treatment with C8 and D8 decreased the amount of OCT4 protein that was pulled down.

Given that these inhibitors impaired the pull-down of both DNA-PKcs and OCT4, we sought to elucidate their mechanism of action further. To this end, we produced a custom D8-conjugated agarose gel that would allow us to pull down proteins from our DOX-inducible OCT4-overexpressing SCLC cell line that were bound to D8 and detect their presence via immunoblot. Our pull-down studies demonstrated that D8 binds specifically to DNA-PKcs, not OCT4, thereby disrupting the DNA-PKcs-OCT4 interaction (Figure 3B). To better characterize the interaction between DNA-PKcs and its substrates after treatment with D8, we performed *in vitro* Ni-NTA pull-down studies using His-tagged OCT4 and p53. After 30 minutes of treatment, we observed that D8 significantly impaired the interaction between DNA-PKcs and OCT4 in a dose-dependent manner but did not affect binding between DNA-PKcs and p53 (Figure 3C).

Further characterization of compounds inhibiting DNA-PKcs catalytic activity

Having established that C8 and D8 significantly inhibited the interaction between DNA-PKcs and OCT4, but noting that C8 and D8 impaired OCT4 pull-down, we sought to characterize our validated hits better. We first determined the effect of our novel inhibitors on c-MYC expression in our DOX-inducible OCT4-overexpressing SCLC cell line (Figure 4A). In line with our proposed mechanism, inhibition of OCT4 phosphorylation at Ser93 correlated to a decrease in c-MYC expression. Notably, C8 and D8 demonstrated remarkable reductions in c-MYC and OCT4 expression.

Next, we focused on *in vitro* kinase assays to assess our inhibitors' specific activity against DNA-PKcs-mediated phosphorylation. The ADP-Glo^{TM} assay utilizes a luciferase reaction where the amount of luminescence measured correlates to the amount of ATP consumed by the kinase reaction. All seven of the inhibitors validated in the first step of hit validation demonstrated activity against DNA-PKcs-mediated phosphorylation of the peptide substrate (Figure 4B). C8, D8, and G5 showed drastic reductions in DNA-PKcs kinase activity. E10, the positive control selected, showed a modest decrease in kinase activity. We then performed *in vitro* DNA-PKcs kinase activity assays by utilizing two known DNA-PKcs substrates: OCT4 and p53. After incubating the validated compounds with DNA-PKcs for 30 minutes, bacterially-derived (lacking post-translational modification) OCT4 and p53 proteins were added to the kinase reaction. We then detected the presence of phosphorylated pOCT4[S93] and phospho-p53[S78] by IB (Figure 4C). C8 and D8 treatment inhibited phosphorylation of both OCT4 and p53, reinforcing their consideration as novel DNA-PKcs inhibitors with widespread activity. Interestingly, G5 did not significantly impair OCT4[S93] phosphorylation but did inhibit p53[S78] phosphorylation. One possible explanation is that G5 localizes and binds to a region of DNA-PKcs crucial for binding to p53 and the peptide substrate, but not OCT4. To assess whether C8 and D8 had activity against other kinases, we performed the same set of *in vitro* assays using MK2 as the targeted enzyme and OCT4 and HSP27 as its substrates. C8 and D8 are not active against MK2, suggesting that they are specific inhibitors against DNA-PKcs activity (Figure S3A&B).

Taken together, the pull-down and *in vitro* assays also demonstrate that the decreases in pOCT4[S93] are not due to degradation of OCT4; instead, these compounds act directly on DNA-PKcs. To address whether the decrease in total OCT4 protein by C8 and D8 treatment is due to the proteasomal degradation, we

treated the DOX-inducible OCT4-overexpressing SCLC cell line with C8 and D8. Although OCT4 expression decreased in a dose-dependent manner, pretreatment of bortezomib prevented the decrease in OCT4 protein level (Figure S3C). These results suggest that while C8 and D8 act primarily to bind to DNA-PKcs, thereby preventing its ability to phosphorylate substrates, they also serve to upregulate the degradation of its substrates.

In vitro **cytotoxicity**

We tested the *in vitro* cytotoxic activity of C8 and D8 in five *MYC* -amplified and five *MYC* -nonamplified cell lines to determine their efficacy as single agents. Both compounds demonstrated notable activity in both the *MYC* -amplified and *MYC* -nonamplified cell lines. C8 was more cytotoxic (mean IC_{50} = 3.30 nM in *MYC*- amplified, = 2.70 nM in *MYC* -nonamplified, = 3.00 nM in both) in SCLC cell lines relative to D8 cytotoxic (mean IC_{50} = 25.92 nM in *MYC*- amplified, = 15.04 nM in *MYC* -nonamplified, = 20.48 nM in both) (Figure 5A&B). These data indicate that both compounds have potential as single-agent therapies in SCLC. Further testing with current standard-of-care regiments may also be warranted. Recent studies have shown that SCLC tumors with high c-MYC expression are particularly susceptible to aurora kinase A inhibition (Mollaoglu et al., 2017). C8 and/or D8 in conjunction with aurora kinase inhibitors, such as alisertib, may prove to be an effective targeted therapy.

Discussion

Aberrant kinase activity has been implicated in tumor development and progression in a variety of cancers (J. Zhang et al., 2009). Thus, the development of kinase inhibitors has emerged as a therapeutic strategy in the treatment of malignancy. To date, over 50 kinase inhibitors have received FDA approval for the treatment of a variety of malignancies (Bhullar et al., 2018). These novel compounds have shown great promise, but are limited in their overall efficacy because of off-target toxicities and the development of chemoresistance. Off-target toxicities are common in type I inhibitors, given that the ATP binding pocket is well-conserved throughout the kinome (Bhullar et al., 2018). Chemoresistance to kinase inhibitors often develops rapidly through a variety of mechanisms: 1) mutations to the targeted kinase that reduce the binding affinity of the compound, 2) the activation of malignant downstream targets that circumvent kinase activity, 3) upregulation of parallel signaling pathways, 4) mutations in drug uptake and transport, 5) epigenetic changes to cellular processes (Camidge, Pao, & Sequist, 2014; Gross, Rahal, Stransky, Lengauer, & Hoeflich, 2015; Holohan, Van Schaeybroeck, Longley, & Johnston, 2013; Niederst & Engelman, 2013; Tam & Weinberg, 2013). These limitations highlight the necessity to develop new kinase inhibitors with higher specificity towards their targets that can be paired with other therapeutics to overcome drug resistance.

We have developed a novel cell-based drug screening assay that explicitly identifies inhibitors of the kinase-substrate interaction between DNA-PKcs and OCT4 that ultimately target aberrant c-MYC expression in SCLC. In our experiments, we identified the regions of DNA-PKcs that are necessary for OCT4 phosphorylation. We then achieved stable expression of these regions of *PRKDC* (labeled with the LgBiT subunit) and the domains of *POU5F1* that are critical to drive c-MYC expression (labeled with the SmBiT subunit) by utilizing the P2A self-cleavage system. We then screened ˜80,000 compounds and identified 56 compounds that inhibited the luminescence reaction between the LgBiT and SmBiT subunits, thereby potentially impairing the interaction between DNA-PKcs and OCT4. Given that we have applied the same principles to develop a similar system targeting the interaction between MK2 and OCT4, this cell-based drug screening assay can apply to a multitude of kinase-substrate interactions.

Our first set of validation experiments indicated that 6 of the 56 compounds inhibited DNA-PKcs-mediated phosphorylation of OCT4 at its Ser-93 residue. One compound (E10) was used as a positive control. We then confirmed that two of these compounds, C8 and D8, significantly impair the ability of DNA-PKcs to bind to OCT4 through our pull-down studies and led to the degradation of OCT4. To further characterize our inhibitors, we confirmed that these inhibitors act on our novel DNA-PKcs/OCT4/c-MYC pathway by inhibiting c-MYC expression in an SCLC cell line. C8, D8, and G5 significantly inhibited DNA-PKcs kinase activity, DNA-PKcs-mediated phosphorylation of OCT4, and DNA-PKcs-mediated phosphorylation of p53.

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Although our initial aim was to identify specific inhibitors of the DNA/OCT4 interaction, we have identified C8 and D8 as potent inhibitors of DNA-PKcs kinase activity across multiple substrates. An increase in efficacy may balance this perceived loss in specificity.

After establishing C8 and D8 as novel DNA-PKcs inhibitors, we conducted *in vitro* cytotoxicity assays to determine their effect on numerous low and high c-MYC expressing SCLC cell lines. We demonstrated that C8 and D8 are cytotoxic to SCLC cell lines (IC_{50} = 3.0 nM and 20.5 nM, respectively). c-MYC expression in SCLC is prevalent in chemo-refractory disease (Kim et al., 2006), and clinical studies have focused on targeting its activity through upstream mediators (Hook et al., 2012; Owonikoko et al., 2016; Sos et al., 2012). Given that our data show that these compounds are effective in targeting aberrant c-MYC expression in SCLC and that they are cytotoxic to SCLC cell lines, the further study focused on their *in vivo* activity as both single agents and in combination with other therapies is necessary. These compounds have the potential to serve as lead compounds that may be further developed to target DNA-PKcs with higher affinity and potency.

Acknowledgments

Conflict of Interest Statement

The authors have no conflict of interest to declare.

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Figure Legends

Figure 1. A schematic overview of the initial screening and two-step hit validation is presented. Initial screening was done after the co-transfection of wild-type *POU5F1* tagged with LgBiT and fragment 6 of *PRKDC* tagged with SmBiT. Once stable clones were established, cells were seeded in a 96-well plate and were treated with an inhibitor. Decreased luminescence indicated a positive hit. Screening and hit identification yielded 56 compounds that were selected for further study. These compounds were validated via determining their effect on phosphorylation of OCT4 Ser93, kinase activity, and the protein-protein interaction between OCT4 and DNA-PKcs. 2 compounds were confirmed to inhibit pOCT4^{S93} and c-MYC expression, impair DNA-PKcs kinase activity, and prevent the protein-protein interaction between OCT4 and DNA-PKcs.

Figure 2. Compound screening and identification of "hits." a Graphical representation of plates used to identify hits as potential inhibitors of DNA-PKcs-mediated phosphorylation of OCT4. A blank plate (top) was used to standardize readings. Compounds that demonstrated significant decreases in luminescence are shown in red (middle and bottom). **b** Compounds were deemed as hits (red triangles) if they demonstrated a reduction in luminescence greater than 1.96 x the standard deviation from the mean as calculated in the blank plate panel (top).

Figure 3. Hit Validation #2. Inhibition of the DNA-PKcs-OCT4 interaction following inhibitor treatment. a Stable clones of NCI-H82 cells expressing a DOX-inducible OCT4-mycDDK construct were treated with doxycycline for 18 hours to induce OCT4-mycDDK expression. Cells were then treated with the inhibitors at the concentrations indicated for 6 hours. Protein lysates were pulled down using EZView Red ANTI-FLAG Affinity Gel. Immunoblotting was done to determine the effects of inhibitor treatment on the DNA-PKcs-OCT4 interaction. Two compounds, C8 and D8, decreased the expression of both DNA-PKcs and OCT4. **b**Stable clones of NCI-H82 cells expressing a DOX-inducible OCT4-mycDDK construct were treated with doxycycline for 18 hours to induce OCT4-mycDDK expression. Protein lysates were pulled-down using D8-conjugated agarose. D8 bound to DNA-PKcs, not OCT4. **c**In vitro Ni-NTA pull down assays. DNA-PKcs was incubated with D8 for 30 minutes before the addition of bacterially-derived OCT4 and p53 (His-tagged). His-tagged substrates were pulled down using Ni-NTA purification. D8 treatment impaired the interaction between DNA-PKcs and OCT4 in a dose-dependent manner (left) but did not affect the interaction between DNA-PKcs and p53 (right).

Figure 4. A second-step validation was conducted to validate hits and to evaluate the effect on the kinase activity of the hits. a Hit validation demonstrating that two compounds: C8 and D8, inhibit OCT4, pOCT4^{S93} and c-MYC expression in a DOX-inducible OCT4-overexpressing SCLC cell line (NCI-H82 pCW57.1-OCT4-mycDDK).**b** ADP-Glo^{TM} assay demonstrating that C8, D8, and G5 drastically impair DNA-PKcs kinase activity. NU7441 (brown), a known DNA-PKcs inhibitor, was used as a positive control.

c *In vitro* kinase activity assays. DNA-PKcs was incubated with inhibitors for 30 minutes prior to the addition of bacterially-derived OCT4 (His-tag) and p53 protein. After a second 30 minute incubation, IB demonstrates that C8 and D8 decrease OCT4^{S93}phosphorylation. C8, D8, and G5 decrease p53^{S15}phosphorylation. NU (NU7441) was used as a positive control. Compounds (concentration in μM): C8 (0.625), D8 (1.25), E10 (2.5), G2 (1.25), G5 (1.25), G10 (2.5), NU (2.5).

Figure 5. *In vitro* **cytotoxicity of the compounds was evaluated, and the dose-response curves are presented. a** Calculated IC$_{50}$ values for C8 (left) and D8 (right) in 10 high and low c-MYC-expressing SCLC cell lines. **b** Dose-response curves of D8 in 10 SCLC cell lines are presented. The cells were treated with vehicle (DMSO, 0.1% as final concentration), 1 nM, 3 nM, 10 nM, 30 nM, 100 nM, 300 nM, 1 μM, 3 μM, 10 μM for 96 hours before viability was assessed. Each condition was tested in 6 replicates. Symbols: mean, error bars: standard deviation.

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