Doublecortin-like kinase 1 is a therapeutic target in squamous cell carcinoma

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Abstract
Doublecortin like kinase 1 (DCLK1) plays a crucial role in several cancers including colon and pancreatic adenocarcinomas. However, its role in squamous cell carcinoma (SCC) remains unknown. To this end, we examined DCLK1 expression in head and neck SCC (HNSCC) and anal SCC (ASCC). We found that DCLK1 is elevated in patient SCC tissue, which correlated with cancer progression and poorer overall survival. Furthermore, DCLK1 expression is significantly elevated in human papilloma virus negative HNSCC, which are typically aggressive with poor responses to therapy. To understand the role of DCLK1 in tumorigenesis, we used specific shRNA to suppress DCLK1 expression. This
1 | INTRODUCTION

Squamous cell carcinoma (SCC) originates in the mucosal epithelium at several sites including the oral cavity, oropharynx, and anus. Head and neck SCC (HNSCC) is the sixth most common cancer worldwide. Etiologic factors associated with HNSCC include tobacco and alcohol abuse, and human papilloma virus (HPV) infection. Despite therapeutic advances, HNSCC remains difficult to treat; due, in part, to late-stage tumor presentation, as well as high rates of local recurrence and distant metastases that contribute to poor 5-year survival. Anal SCC (ASCC) is a rare cancer, which like HNSCC is associated with HPV infection, and its incidence is increasing. Accordingly, the identification of novel targets and therapeutics is needed to improve SCC treatment outcomes. There remains a paucity of preclinical descriptions of ASCC biology or potential drug targets.

Recently, doublecortin like kinase 1 (DCLK1) was found to be highly expressed in salivary gland tumors and is associated with low overall and disease-free survival. DCLK1, encodes a member of the doublecortin family and protein kinase superfamily. DCLK1 contains two N-terminal doublecortin domains and a C-terminal kinase domain homologous to Ca2+/calmodulin-dependent kinases (CamK). Doublecortin domains participate in microtubule polymerization, whereas the serine/threonine protein kinase domain and a serine/proline-rich domain, mediate multiple protein–protein interactions. The activity of each domain is independent of one another. DCLK1 is expressed in various cell types including neurons, osteoblasts, and tuft cells in the colon. Initial reports by our group have reported DCLK1 expression is observed in a small percentage of cells (<5%) in colon and pancreatic adenocarcinomas. Further, it has been established as a marker of tumor-initiating cells. The role of DCLK1 in SCCs remains unknown.

Presently, we determine that DCLK1 expression is upregulated in SCC. Further, DCLK1 regulates HNSCC proliferation, invasion, and migration. We also report for the first time that DiFiD (3,5-bis [2,4-difluorobenzylidene]–4-piperidone) binds to DCLK1 with high selectivity. Moreover, DiFiD mediated suppression of DCLK1 led to G2/M arrest and apoptosis and significantly suppressed tumor growth of HNSCC xenografts and ASCC patient derived xenografts, supporting that DCLK1 is critical for SCC growth.

KEYWORDS

anal SCC, DCLK1, DiFiD, HNSCC, patient derived xenograft, therapy

2 | METHODS AND MATERIALS

2.1 | Cell lines, patient tissues, and reagents

Well-characterized HNSCC cell lines UM-SCC-1 (from Dr. Tom Carey, University of Michigan), OSC19 (from Theresa Whiteside, University of Pittsburgh), HN5 (from Dr. Jeffrey Myers, The University of Texas MD Anderson Cancer Center), and FaDu (ATCC) were used in this study. Het-1A, an immortalized noncancerous esophageal squamous epithelial cell line was obtained from ATCC. Established cell lines were authenticated by short tandem repeat profiling at Johns Hopkins in 2018 using the Promega GenePrint 10 kit and analyzed using GeneMapper v4.0 software. FaDu were maintained in EMEM (Corning) with 10% heat inactivated FBS. All other cell lines were maintained in DMEM (Corning) with 10% heat-inactivated FBS (Sigma-Aldrich) without antibiotics. Cells were incubated at 37°C in the presence of 5% CO2. Tissue Microarray (#HN483) was purchased from US Biomax, Inc. Sections from paraffin embedded blocks of 17 deidentified ASCC patient samples were obtained from the Department of Pathology, University of Kansas Medical Center. After review, the institutional IRB determined that this study (STUDY00140502) was not research involving human subjects. DiFiD and EF24 were acquired as previously reported. KN-62 was purchased from Tocris Bioscience.

2.2 | Cloning of DCLK1 DCX domain

The DCLK1 DCX domain was amplified by PCR. cDNA was run on 2% agarose gels and bands were purified using the GeneJET gel purification kit (Thermo Fisher Scientific) according to the manufacturer’s protocol. DCLK1 DCX DNA was cloned into the N-Terminal pFLAG 3 vector (Addgene) by restriction digest using NotI and BamHI.
restriction enzymes. FaDu cells were transfected and selected using 400 µg/ml G418 sulfate salt. Primers used for DCX Domain:

DCLK1 NotI FP: 5′-GATCGGCGCGATGTCCTCGCAGA-3′
DCLK1 BamHI DCX RP: 5′-GATCGGATCCCTAGCCATCGTTCTC-3′

2.3 | DCLK1 kinase assay

Purified recombinant DCLK1, CamKIIα, CamKIIβ, and CamKIV (150 ng; SignalChem) were incubated in reaction buffer (Invitrogen) with 9 nM peptide substrate (Gs peptide), 10 µM ATP (Invitrogen), and either vehicle DMSO (dimethyl sulfoxide), EF24, DiFiD, or KN-62 (a pan-CAMK inhibitor) for 30 min at 37°C. We used Gs peptide because it has been used as a substrate for CamK assays.18,19 Our assays were adapted based on these studies. Fold change was calculated relative to ATP counts in the control. Consequently, 100 µl of luciferin-luciferase mixture (ATP determination kit; Invitrogen) was added into each well and luminescence was monitored at 560 nm by using Synergy™ NEO microplate reader.

2.4 | Molecular docking

All docking calculations were carried out with AutoDock Vina software (Molecular Graphics Lab; Scripps Research Institute, http://vina.scripps.edu/) to analyze DiFiD interactions with the 3D structure of kinase domains of DCLK1 (Protein Data Bank ID: 5JZN). Default parameters on Autodock tools were used to analyze the docking. Total Kollman and Gasteiger charges were added to the protein and the ligand before docking. We used Lamarckian GA to find the best conformations and chose approximately 10 conformations for further analyses. The most stable compound conformation was selected based on the scoring function and the lowest binding energy, and visualized using Pymol (https://pymol.org/2/).

2.5 | Surface plasmon resonance (SPR)

SPR analysis of DiFiD-DCLK1 binding affinity was performed at Ametek Reichert Technologies, using Reichert4SPR instrumentation. A dextran sensor chip (Reichert part# 13206066) was activated for 240 s with the mixing 400 mM EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) and 100 mM NHS (N-hydroxysuccinimide) at a flow rate of 10 µl/min. Recombinant DCLK1 protein (SignalChem) was dissolved in running buffer (10 mM HEPES, 138 mM NaCl, 2.7 mM KCl, 0.05% Tween-20, 5% DMSO, pH 7.4) and injected to the sample channel at a flow rate of 10 µl/min to reach immobilization. The chip was deactivated by 1 M ethanolamine hydrochloride-NaOH at a flow rate of 25 µl/min for 240 s. DiFiD was diluted in running buffer at a flow rate 25 µl/min with an association phase of 120 s, followed by 120 s dissociation. The K_D value of DiFiD against DCLK1 protein were obtained by Biacore 8k evaluation software.

2.6 | Cellular thermal shift assay (CETSA)

The ability of DiFiD to interact with and stabilize DCLK1 in cells was determined using CETSA.22 Briefly, cells (8 × 10^6) were treated with media containing DMSO or DiFiD (5 µM) for 4 h. After treatment, the cells were aliquoted into PCR tubes and exposed to a temperature gradient. Subsequently, cells were lysed using three repeated freeze-thaw cycles in liquid nitrogen followed by centrifugation. The resultant lysates were then utilized in downstream western blot analyses.

2.7 | Drug affinity responsive target stability (DARTS)

The ability of DiFiD to interact with and stabilize DCLK1 in cells was studied by using DARTS.23 FaDu cells were cultured and grown up to 70%–80% confluency. Cells were washed three times with ice-cold PBS and lysed using M-PER lysis buffer supplemented with protease inhibitor cocktail tablet (Roche) and collected by scraping off with a cell scraper. Cells were lysed on ice for 10 min, clarified by centrifugation, and protein estimated using BCA method. Cell lysates were divided into equal concentration aliquots and incubated with DMSO or DiFiD (5 µM) for 30 min with gentle shaking. Following incubation, lysates were treated with pronase (10 mg/ml stock) in 1:1/100, 1:1/200, 1:1/400, 1:1/800, 1:1/1600, 1:1/3200, 1:1/6400 protein:pronase ratio for 15 min. The protease digestion was stopped using 20X protease inhibitor cocktail (Roche) for 10 min on ice. The resultant protein samples were diluted with 4X Laemmli buffer, heated at 70°C for 10 min and loaded on to 10% SDS-PAGE gel, transferred to PVDF membrane and incubated with DCLK1 antibody at a concentration of 1:1000. Protein levels on western blot were evaluated by Bio-Rad ChemiDoc™-XR5+ instrument and analyzed by image lab software. Band intensity was calculated relative to the lowest dilution of pronase.

2.8 | Animal studies

All protocols were approved by the Institutional Animal Care and Use Committee at the University of Kansas Medical Center. To assess DCLK1 expression at various stages of disease progression, 4-nitroquinoline-oxide (4-NQO, 100 ppm in sterile drinking water ad libitum) was administered for 16 weeks to C3H mice (n = 20) using a previously reported protocol.24 Mice were then given sterile drinking water for 3 weeks, at which time animals were killed and tongues excised. Immunohistochemistry was then used to assess DCLK1 expression.

Five-week-old female Foxn1/nude mice (Charles River Laboratory) were injected with 1 × 10^6 HNS or FaDu cells in the flank. One week following implantation, mice were randomized into two groups
with 7 mice per group in the HNS injected mice and 10 mice per group in the FaDu treated mice. Animals were treated with either vehicle control (2.5% DMSO in water) or DiFiD (2 mg/kg body weight), administered intraperitoneally daily for 15 days. Tumor growth was measured every 2–3 days by a blinded observer measuring tumor diameters using vernier calipers and volume was calculated (Tumor volume = longest dimension x shortest dimension^2 x 0.52) as previously described. At the end of treatment, animals were euthanized, and the tumors were collected, weighed, and processed for downstream analytical assays.

Additionally, we established a patient-derived xenograft from an anal SCC that metastasized to the liver. The tumor was passaged twice through NOD SCID gamma (NSG) mice. ASCC tumors from the third passage were implanted subcutaneously into flanks of 8-week-old mice. Mice were randomized based on the tumor volume into two groups with 10 mice (5 female and 5 male) in the control group and 14 mice (9 female and 5 male) in the DiFiD treatment group. Average tumor volumes across both groups were 50 ± 8.9 mm^3. Mice were treated daily with either vehicle control (DMSO in water) or DiFiD (2 mg/kg body weight) intraperitoneally daily for 15 days. Tumor growth was measured, and the volume calculated as mentioned before. Fractional tumor volumes were calculated as per previously published protocols.

### 2.9 Statistical analysis

Data are reported as mean ± SEM. Parametric, one-tailed t-test with Welch correction was used to assess significance in all experiments unless stated. Outliers were detected using Graphpad software. Significant outliers were identified using the Grubb's test with α = 0.05. Significant outliers were removed from further statistical analysis. For in vivo studies, repeated measures analysis of variance test was employed to assess the level of significance in tumor volumes between treatment arms. For The Cancer Genome Atlas (TCGA) survivorship comparison, log-rank (Mantel–Cox) test assessed differences between curves. To generate a best expression cut off, patients were stratified into two groups and association between survival and reads per kilobase (RPKM) was examined. The RPKM value that yields the maximum difference between survival of the two groups at the lowest log-rank p-value determined best expression cut off. From this, a high/low expression cut off (0.37) was applied. All statistical calculations were performed on GraphPad Prism software (version 6.03), with significance determined by p < 0.05.

### 3 RESULTS

#### 3.1 DCLK1 is upregulated in squamous cell cancers

DCLK1 is associated with pro-survival signaling in various cancers, including colorectal and pancreatic cancers. Its expression is elevated in HNSCC tumor samples compared to normal oral epithelial tissue (Figure 1AB, p < 0.01). TCGA analysis show that increased DCLK1 expression correlated with increased tumor histological grade (Supporting Information: Figure S1A), and increase expression was higher in HPV-negative patients (Supporting Information: Figure S1B).

Kaplan–Meier survival curves generated from TCGA HNSCC mRNA expression data sets demonstrated a trend toward lower overall survival in patients with DCLK1 high expressing tumors (n = 192) compared to DCLK1 low expressing tumors (n = 307) (Figure 1C). Patients with DCLK1 low expressing tumors survived on average 4.827 years post diagnosis; patients with DCLK1 high expressing tumors had a shorter median survival of 2.995 years.

To assess correlation of DCLK1 with disease progression, we evaluated the expression of DCLK1 in a cDNA panel (Origene), from normal oral mucosa (n = 8), benign (n = 10), and HNSCC (n = 16) patient tissues. DCLK1 was significantly upregulated in cancerous tissue compared to normal (Figure 1D, p < 0.01). To evaluate DCLK1 expression through various stages of tumor progression, tongue tissue from 4NQO treated mice was collected (n = 20). Mice developed various stages of disease with 7/20 mice developing low grade squamous intraepithelial lesions, 10/20 developing high grade squamous intraepithelial lesions, and 3/20 developing invasive HNSCC. Levels of DCLK1 expression progressively increased in dysplastic and invasive lesions, with highest expression observed in HNSCC (Figure 1E,F). We then evaluated DCLK1 expression in HNSCC cell lines; OSC19, HN5, UM-SCC-1, and FaDu and Het-1A, a noncancerous immortalized esophageal epithelial line. DCLK1 was differentially expressed with the highest expression observed in HN5 and FaDu cells, and lowest expression in Het-1A cells (Figure 1G).

Immunofluorescence revealed DCLK1 to be expressed in HN5 spheroids (Supporting Information: Figure S1C). Therefore, HN5 and FaDu were used for subsequent studies. These data suggest that DCLK1 is elevated in HNSCC compared to normal oral mucosa and is associated with tumor progression. Further, ASCC patient samples (n = 17) were stained for DCLK1 expression. DCLK1 was more significantly expressed in ASCC tumor compared to normal anal mucosa (Supporting Information: Figure S1D and E). We observed increased nuclear staining along the tumor invasive front (Supporting Information: Figure S1D).

#### 3.2 DCLK1 suppression inhibits HNSCC growth

To evaluate the antitumor efficacy of targeting DCLK1 in HNSCC, we attenuated DCLK1 levels using two shRNA constructs, sh3 and sh4. DCLK1 mRNA and protein were significantly reduced (Figure 2A,B) in FaDu cells stably transduced with sh3 or sh4 compared to scrambled controls. DCLK1 has been shown to play a critical role in anchorage independent growth in vitro. Hence, we assessed the effects of DCLK1 knockdown on spheroid formation. DCLK1 knockdown suppressed spheroid formation in FaDu cells compared to scrambled controls (p < 0.01, Figure 2C). DCLK1 knockdown also resulted in stunted colony formation (Figure 2D). Functionally, DCLK1 shRNA
knockdown reduced HNSCC migration and invasion in vitro (Figure 2F,G, respectively). To demonstrate that DCLK1 is an important driver of cancer in vivo, FaDu cells stably transduced with shRNA targeting DCLK1 (sh3 and sh4), or a scrambled control shRNA were subcutaneously injected into NSG mice. Suppression of DCLK1 resulted in slower growing tumors compared to scrambled controls (Figure 2I). Tumor lysates were then analyzed by immunoblot for DCLK1, cyclin D1, and Bax (Figure 2H and Supporting Information: Figure 2). Expectedly, DCLK1 expression was diminished in both shRNA groups. Cell cycle regulator, cyclin D1 expression was...
FIGURE 2  DCLK1 suppression inhibits tumor growth, invasion, and migration. (A) Fold change in mRNA expression of DCLK1 in FaDu cells stably transduced with shRNA targeting DCLK1 (termed: sh3 and sh4) relative to cells transduced with a scrambled control shRNA. Data represent cumulative results from three independent experiments with ±SEM. (B) Western blot of DCLK1 protein in control and stably knocked down DCLK1 sh3 and sh4 FaDu clones. GAPDH served as a loading control. (C) Quantification of spheroid number from spheroid formation assay. Data represent cumulative results from two independent experiments with ±SEM. (D) Representative images of colony formation assays for DCLK1 control, sh3 and sh4 FaDu clones. (E) Quantification of colony number. Data represents cumulative results from three independent experiments with ±SEM. (F) Migration and (G) invasion assays are normalized for differences in proliferation rates over the duration of the assay. Data represents cumulative results from three independent experiments with ±SEM. (H) Tumor volumes from NSG mice that were inoculated subcutaneously with 1 × 10^6 FaDu cells stably transduced with shRNA targeting DCLK1 (sh3 and sh4) or scrambled control shRNA into their right flank (n = 10 animals per group). Tumor measurements began 7 days postinjection of cells. Error bars represent ±SEM. (I) Quantification of western blot analysis from lysates of a cohort of xenograft tumors. Error bars represent ±SEM (western blots shown in Supporting Information: Figure S5A) *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. DCLK1, doublecortin like kinase 1; NSG, NOD SCID gamma mice. [Color figure can be viewed at wileyonlinelibrary.com]
significantly reduced, while proapoptotic, Bax expression was greatly increased in DCLK1 knockdown tumors. Collectively, these data indicate DCLK1 may help drive cancer progression, and DCLK1 suppression may lead to cell cycle arrest and apoptosis.

### 3.3 | DiFiD binds to DCLK1 and inhibits its activity

The small molecule, 3,5-bis (2,4-difluorobenzylidene)-4-piperidone (DiFiD) was previously shown to suppress the growth of pancreatic cancer cells in vitro and in vivo. However, the precise target for DiFiD remained unknown. DCLK1 is implicated in pancreatic and colorectal cancer progression. Therefore, we first determined compound–protein interaction. Molecular docking predicted DiFiD interacts with DCLK1 by forming hydrogen bonds with aspartic acid 533, with a binding energy of ~7.9 kcal/mol, as shown in ribbon and surface views (Figure 3A,B,C,D).

Additionally, the binding kinetics of DiFiD and DCLK1 were studied via SPR. We observed dissociation constants of $K_{D1} = 71 \text{nM}$ and $K_{D2} = 902 \text{nM}$ (Figure 3E). Taken together this data shows that DiFiD binds DCLK1 with high affinity. To confirm that DCLK1 is a binding target of DiFiD in the cells, we performed CETSA to assess protein stability following thermal denaturation. FaDu cells were treated with 5 µM DiFiD at 37°C for 4 h. Cells were aliquoted into equal volumes, subjected to a thermal gradient and DCLK1 expression was then evaluated by western blot. Thermal denaturation of DCLK1 occurred at 54°C in the DMSO treated control group, which increased to 58°C in the presence of DiFiD (Figure 3F). To further validate DiFiD binding with DCLK1, we performed the DARTS assay. Briefly, FaDu cell lysates were incubated with DMSO or DiFiD (5 µM) for 30 min. We then incubated cells with increasing protein:pronase ratio. We observed that DiFiD protected DCLK1 from protease-mediated degradation, as DCLK1 expression was extended to 1:1/800 protein: pronase ratio compared to 1:1/1600 in the DMSO control arm (Figure 3G). Altogether, these data suggest that DiFiD binds to DCLK1.

To confirm DiFiD specifically interacts with the kinase domain of DCLK1, we performed CETSA analyses. For this, we stably expressed the N-terminal DCX domain (lacking the kinase domain) in HNSCC cells (Supporting Information: Figure S3A and S3B). To detect DCX domain fragments in FaDu cells, we utilized DCLK1 antibody produced using residues near the N-terminus. This specifically recognizes DCLK1 protein isoforms containing 82 kDa DCX sequences. We found that cells expressing the predicted 51 kDa N-terminal DCX domain did not exhibit thermal stabilization when treated with DiFiD (Supporting Information: Figure S3C). This suggests that DiFiD does not stabilize DCLK1 lacking the kinase domain. Therefore, we conclude that DiFiD preferentially binds to the kinase domain of DCLK1, further confirming molecular docking predictions.

DCLK1 belongs to the family of kinases with homology to calmodulin kinases, but it does not depend on Ca2+/calmodulin for its kinase activity. Since DiFiD interacts with the kinase domain, we next assessed the effect of DiFiD on DCLK1 kinase activity by performing an in vitro kinase assay. Here, we incubated recombinant DCLK1 with GS peptide as substrate to assess the ability of DCLK1 to phosphorylate the peptide. Along with DiFiD, we also tested the ability of EF24 (IKK inhibitor) and KN62, a pan-CaMKII inhibitor. We observed greater inhibition of DCLK1-mediated ATP consumption following incubation with DiFiD, than with either EF24 or KN-62 (Figure 3H). To determine the specificity of DiFiD to DCLK1, we also performed studies with CaMKIIα, CaMKIIβ, and CaMKIV. We observed that DiFiD did not affect the activities of these CaMK proteins. These data demonstrate a higher selectivity and specificity of DiFiD for DCLK1.

Since DiFiD has favorable binding to DCLK1, inhibits its kinase activity, and is relatively ineffective at reducing Het1A proliferation (Supporting Information: Figure 4B), we hypothesized that cells expressing lower levels of DCLK1 would be resistant to DiFiD. Consequently, we performed hexosaminidase assays with cells where DCLK1 was knocked down using specific shRNA. There was an increase in their respective half maximal inhibitory concentration (IC$_{50}$) values from 1.3 µM to greater than 11 µM compared to control cells (Figure 3I). These data demonstrate DiFiD activity is dependent upon the presence of DCLK1, and DiFiD has poor activity against cells with low DCLK1 expression. These data further suggest that DCLK1 is a highly specific direct target of DiFiD.

### 3.4 | DiFiD demonstrates potent cytotoxicity in HNSCC in vitro

Since DiFiD inhibits DCLK1 activity, we sought to determine the effect of DiFiD on HNSCC cancer cell viability. We observed that DiFiD inhibits HNSCC viability in a dose and time-dependent manner (Figure 4A and Supporting Information: Figure S3A). We identified an effective dose to assess the mechanism of action. The IC$_{50}$ was measured by hexosaminidase assay and observed within 48 h at concentrations of 750 nM and 1.5 µM in HN5 and FaDu cell lines, respectively. In addition, we tested toxicity of DiFiD on an immortalized noncancerous cell line, Het1A (Figure 1G). We observed that the IC$_{50}$ for Het1A at 48 h was 9 µM, a 6–12-fold increase compared to FaDu and HN5 cells, respectively (Supporting Information: Figure S4B). In addition, we observed that DiFiD attenuates spheroid growth in both HN5 and FaDu cell lines when treated at IC$_{50}$ concentrations, suggesting that it affects anchorage independent growth (Figure 4B). We next determined the effect of DiFiD on clonogenicity of HNSCC cells. Initial studies with IC$_{50}$ doses demonstrated complete suppression of colony formation. However, when treated at lower doses of 93.75 nM (1/8th IC50) and 187.5 nM (1/4th IC50) for 48 h, we observed a dose dependent decrease in both the number and size of colonies for HN5 cells (Figure 4C,D, p < 0.01). Similarly, FaDu cells treated with a 1/4th (375 nM) and 1/8th (187.5 nM) IC$_{50}$ dose of DiFiD exhibited a significant inhibition in colony number and size.
These data suggest that DiFiD has potent cytotoxic effects on HNSCC that are long lasting.

**3.5 | DiFiD induces G2/M arrest and apoptosis**

To further characterize the effects of DiFiD treatment on HNSCC proliferation, we used flow cytometry to evaluate cell cycle progression. HN5 and FaDu cell lines were treated with DiFiD. Within 24 h, DiFiD treatment increased the percentage of cells arrested at the G2/M phase (Figure 5A and Supporting Information: Figure S5). At 48 h, there was an increase in the sub G0, or fragmented DNA stage following treatment, suggesting increased cell death. To confirm G2/M arrest, western blot analysis was performed for G2/M associated proteins cyclin B1 and cell division cycle protein 2 (CDC2). HN5 and FaDu cancer cells were treated with IC50 doses of DiFiD for 24 h, in which time dependent increases in cyclin B1 and simultaneous decreases in CDC2 expression were observed in the treatment arm (Figure 5B,C). We also performed western blot analysis for pro- and cleaved-PARP protein. DiFiD treatment induced
PARP cleavage in both HN5 and FaDu cell lines (Figure 5B,C). This suggests that DiFiD further induces apoptosis in HNSCC cancer cell lines associated with a G2/M arrest. This was confirmed by Annexin V/PI staining that demonstrated an increased percentage of DiFiD treated cells were in the apoptotic and dead fractions compared to vehicle control (Figure 5D). To assess the role of caspases in the induction of apoptosis by DiFiD, we performed caspase 3/7 assay to assess effector caspase activity. DiFiD induced a significant upregulation in the activity of the effector caspases (Figure 5E, p < 0.001).

Taken together, these data confirmed that DiFiD mediated suppression of DCLK1 activity contributes to mitotic catastrophe of HNSCC.

3.6 | DiFiD has antitumor effects in vivo

To determine the in vivo antitumor activity of DiFiD, we treated FaDu and HN5 subcutaneous tumors in Foxn1nu/nu mice. Briefly, HN5 or FaDu cells were injected subcutaneously into the flanks of nude mice and were subsequently treated with DiFiD at 2 mg/kg/day for 15 days (Figure 6A). DiFiD treatment significantly inhibited tumor growth in HN5 xenografts compared to vehicle control (DMSO) treated tumors (n = 7, p < 0.05, Figure 6B and Supporting Information: Figure 6A). Similarly, with FaDu xenograft subcutaneous tumors, DiFiD treatment significantly reduced tumor growth compared to the vehicle control.
treatment arm \((n = 10, p < 0.05, \text{Figure 6C})\) and Supporting Information: Figure 6E). Lastly, the antitumor effect of DiFiD was tested in an ASCC patient-derived xenograft model. DiFiD treatment significantly reduced PDX tumor growth as evidenced by tumor volume and weight of ASCC PDX tumors when compared to the control group \((p < 0.05, \text{Figure 6D})\) and \(p < 0.001, \text{Figure 6E}\). These preclinical data indicate that DiFiD is a promising, well-tolerated, therapeutic agent for the management of SCC.

To elucidate the molecular mechanism whereby DiFiD exerts its antitumor effects on HNSCC, we analyzed xenograft tumors using...
western blot analysis. Tumor samples were subjected to electrophoresis, and subsequently, expression of Bax, cyclin D1, and DCLK1 were determined. DiFiD significantly induced the expression of Bax in HN5 (Figure 6F and Supporting Information: Figure 6B) and FaDu (Figure 6G and Supporting Information: Figure 6F) tumor samples relative to vehicle control. Additionally, DiFiD significantly reduced expression of cyclin D1 (Figure 6F and Supporting Information: Figure 6C) and DCLK1 (Figure 6F and Supporting Information: Figure 6D) in HN5 tumors, suggesting inhibition of proliferation and cell cycle entrance. FaDu tumor xenografts also demonstrated decreased expression of cyclin D1 (Figure 6G and Supporting Information: Figure 6G) and DCLK1 (Figure 6G and Supporting Information: Figure 6D) in FaDu tumors.
Information: Figure 6H), further substantiating our observations in vivo. Taken together, these studies demonstrate that DiFiD inhibits tumor growth by targeting DCLK1 and inducing cellular apoptosis.

4 | DISCUSSION

SCCs of the head and neck and anus are aggressive malignancies with high rates of local recurrence, distant metastasis, and poor clinical outcomes, including reduced survival. Current standard of care include surgery followed by chemoradiotherapy. However, despite aggressive treatment, the survival rate remains low highlighting a significant unmet medical need in SCC patients. In this article, we demonstrate that DCLK1 is a clinically relevant target for HNSCC and ASCC. DCLK1 is well characterized as a reserve, stress induced stem cell marker in pancreatic and colorectal cancers.5,6 Using multiple platforms, we demonstrate DCLK1 expression is significantly upregulated in HNSCC compared to normal oral mucosa of patient tissues. Furthermore, high expression of DCLK1 correlates with poor clinical outcomes. These findings agree with a recent report associating high DCLK1 levels with poor HNSCC patient survival.4

Genetic knockdown of DCLK1 has demonstrated promising findings in neuroblastoma, colorectal, and pancreatic tumors.29,30 DCLK1 knockdown triggers apoptosis and inhibits proliferation, mitochondrial function, and ATP synthesis in neuroblastoma cells.30 TCGA analysis has shown DCLK1 to be positively correlated with NOTCH signaling in HNSCC.31 Additionally, in vitro inhibition of DCLK1 via siRNA or inhibitor (LRRK2-IN-1) in HNSCC cell lines showed decreased levels of downstream NOTCH effectors, HEY1, HES1, and HESS.31 Furthermore, DCLK1 siRNA nanoparticle delivery to colorectal and pancreatic tumor xenografts resulted in the significant inhibition of tumor growth with seemingly high tolerance.29 Our data presented herein supports these previous findings, as we observed decreased spheroid growth, colony formation, migration, and invasion following knockdown of DCLK1. As such, due to the high expression of DCLK1 in HNSCC tumor tissues and cell lines, we postulated that DCLK1 may be a potential therapeutic target for HNSCC.

Currently there is a DCLK1 inhibitor, DCLK1-IN-1, which has been reported to inhibit DCLK1 activity with high selectivity.32 Nevertheless, DCLK1-IN-1 has not been tested in vivo. This compound was derived from the 5, 11-dihydro-6H-benzo[e]pyrimido[5,4-b]1,4 diazepin-6-one scaffold, which includes the LRKK2 and LRRK2-IN-1 inhibitors. Weygant et al.,33 in targeting leucine-rich repeat kinase 2 (LRRK2), with the small molecule inhibitor LRRK2-IN-1, reported inhibition of DCLK1, and subsequent attenuation of HCT116 (colon) and AsPC-1 (pancreatic) growth, and invasiveness. However, direct binding of DCLK1 by LRRK-IN-1 was not examined in this study and the effects are most likely due to the indirect effects of LRRK inhibition on DCLK1 activity.34 Furthermore, several kinase inhibitors with antitumor activity, such as XMD8-92 (MAPK7 inhibitor), BI-2536 (PLK1 inhibitor), and TAE-684 (ALK inhibitor), demonstrate nonspecific activity, with a comparable affinity toward DCLK1 as much as their target kinases.35 Therefore, while inhibition of DCLK1 may play a role in the therapeutic activity of these compounds, it is highly valuable to identify a specific DCLK1 inhibitor for future clinical applications that has potent antitumor activity.

Previously, we reported that DiFiD shows antitumor activity toward pancreatic cancer cells.13 However, the direct target for DiFiD was not elucidated. We used an in vitro kinase assay to demonstrate that DiFiD effectively inhibits DCLK1 kinase activity, but not related CaMK family members, suggesting that the compound is a specific competitive inhibitor of DCLK1. We further confirmed a direct interaction through in vitro binding assays and SPR analysis. The CETSA and DARTs binding assays involved the uptake of the compound by cells before thermal or enzymatic denaturation, respectively.22 We showed that DCLK1 was robustly stabilized by DiFiD. Additionally, cells stably transfected with expression plasmids containing only the DCX domain did not exhibit thermal stabilization as was observed with full length DCLK1 containing the kinase domain. Interestingly, we found that DCX domain required higher temperatures to denature compared to full length protein. This is likely due to enhanced microtubule association and stabilization of the domain, as this domain in DCLK1 is critical for its microtubule binding and polymerization activity.7,36 SPR analysis, which is the gold standard for target interactions, identified strong binding of DiFiD to DCLK1 with low nM concentration equilibrium dissociation constants (KD). Taken together, these data demonstrate that DiFiD has high selectivity for DCLK1 with binding sites located in the C-terminal kinase domain.

We demonstrated that DiFiD induces cell-cycle arrest and apoptosis, attenuating HNSCC proliferation and colony formation in vitro, and tumor growth in vivo. Het1A, noncancerous cells expressing low amounts of DCLK1 in comparison to the HNSCC cell lines, HN5 and FaDu, demonstrated lower DiFiD mediated toxicity. When we knocked-down DCLK1 in FaDu cells using shRNA, we observed a 10-fold decrease in DiFiD toxicity, further demonstrating the specificity of DiFiD for DCLK1. DiFiD increased cell cycle arrest in the G2/M phase resulting in mitotic catastrophe, data consistent with previously published studies.14,33 This is further supported by our observation of a significant increase in the sub-G0 population following treatment with DiFiD. Moreover, we observed an upregulation of the G2/M associated protein, cyclin B1, and a decrease in CDC2, proteins that are abnormally regulated when cells undergo mitotic catastrophe.37,38 These data corroborate our previous reported results demonstrating the induction of p21 and a reduction in cyclins A1 and D1 following DiFiD treatment in pancreatic cancer.14

ASCC has an incidence rate of 0.2–4.4 per 100,000 people per year which has risen worldwide over the last three decades, especially in homosexual men (35 per 100,000 per year) and those with HIV (75–135 per 100,000 per year).39,40 Despite these rising numbers, the standard of care treatment for this cancer comprised of fluorouracil and mitomycin C has remained essentially unchanged since its inception.41 The addition of intensity-modulated radiation therapy and cisplatin has shown similar efficacy to fluorouracil and
mitomycin C in a large, randomized trial. The relative rarity of ASCC presents challenges in conducting pivotal clinical trials. In addition, validated preclinical models of ASCC that accurately replicate clinical observations are limited. Existing preclinical models of ASCC include a cell line derived from a lymph node metastasis, two transgenic mouse models and a xenograft from a single patient. Here we present a patient derived xenograft model and provide preliminary evidence of a promising, new therapeutic target for the management of ASCC.

In our studies, we observed significant antitumor effects in SCC preclinical models following treatment with DiFiD. DCLK1 is expressed in various normal cell types, including neurons, osteoblasts, and colon stem cells, and is involved in physiological processes, including retrograde transport, neuronal migration, and neurogenesis. The data presented in this article suggest that DiFiD is well tolerated at doses that demonstrated antitumor activity, as mice maintained normal weight gain and ambulation. DiFiD tolerance was observed in an earlier study, further supporting the notion that it is well tolerated at the doses used to inhibit cancer growth.

5 | CONCLUSION

DCLK1 is critical for HNSCC and anal SCC growth. For the first time we demonstrated that DiFiD binds to DCLK1 and inhibits SCC growth in vitro and in vivo. Thus, our results strongly establish DCLK1 as a clinically relevant therapeutic target for SCC.

AUTHOR CONTRIBUTIONS

David Standing: curated, analyzed, and interpreted data, and was a major contributor in writing the manuscript. Levi Arnold: curated, analyzed, and interpreted data, and was a major contributor in writing the manuscript. Prasad Dandawate: curated data for in silico binding, DARTS and CETSA binding assays, and contributed to manuscript writing and editing. Brendan Ottmann: curated and analyzed data regarding DCLK1 knockdown in HNSCC cells and animal models. Vusala Snyder: curated and analyzed spheroïd formation and viability assay data, and assisted in writing of the manuscript. Sivapiya Ponnurangam: curated western blots for animal studies. Afreen Sayed: assisted in curation, and analysis of TCGA data sets and manuscript writing/editing. Dharmalingam Subramaniam: curated, analyzed, and interpreted data for cDNA array. Pugazhendhi Srinivasan: curated data for IHC staining, assisted with lentiviral production, and manuscript editing. Sonali Choudhury: assisted with animal data curation and colony maintenance. Jacob New: assisted with animal data curation and colony maintenance. Deep Kwatra: curated and analyzed data for DCLK1 kinase activity. Prabhu Ramamoorthy: curated IF staining of HNSCC spheroid cultures. Badal C. Roy: assisted with DCLK1 cloning and truncation experiments. Melissa Shadoin: assisted with curation of data for viability assays and migration and invasion data sets. Raed Al-Raji: study consultant. MO IHC scoring Sumedha Gunewardena: statistics consultant. John Ashcraft: tissue collection. Shahid Umar: study consultant. Scott J. Wei: study consultant related to drug delivery. Ossama Tawfik: secondary IHC scoring consultant. Subhash B. Padhye: oversaw production of DiFiD and drug study consultant. Bernhard Biersack: drug study consultant. Shrinkant Anant: interpreted data sets, major contributor in writing manuscript, oversaw study design. Sufi Mary Thomas: interpreted data sets, major contributor in writing manuscript, oversaw study design. All authors read and approved the final manuscript.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION
Additional supporting information can be found online in the Supporting Information section at the end of this article.

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