XMD8-92 inhibits pancreatic tumor xenograft growth via a DCLK1-dependent mechanism

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Abstract

XMD8-92 is a kinase inhibitor with anti-cancer activity against lung and cervical cancers, but its effect on pancreatic ductal adenocarcinoma (PDAC) remains unknown. Doublecortin-like kinase1 (DCLK1) is upregulated in various cancers including PDAC. In this study, we showed that XMD8-92 inhibits AsPC-1 cancer cell proliferation and tumor xenograft growth. XMD8-92 treated tumors demonstrated significant downregulation of DCLK1 and several of its downstream targets (including c-MYC, KRAS, NOTCH1, ZEB1, ZEB2, SNAIL, SLUG, OCT4, SOX2, NANOG, KLF4, LIN28, VEGFR1, and VEGFR2) via upregulation of tumor suppressor miRNAs let-7a, miR-144, miR-200a-c, and miR-143/145; it did not however affect BMK1 downstream genes p21 and p53. These data taken together suggest that XMD8-92 treatment results in inhibition of DCLK1 and downstream oncogenic pathways (EMT, pluripotency, angiogenesis and anti-apoptotic), and is a promising chemotherapeutic agent against PDAC.

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related deaths in the U.S. The cancer is associated with <5% of 5-year survival rate after diagnosis, and a median survival of approximately 6 months [20,28]. The prognosis of advanced pancreatic cancer remains appalling despite improvements in chemotherapeutic strategies. The high rate of mortality due to PDAC is primarily due to early metastasis and local invasion, leaving most patients at a devastating unresectable stage (approximately 85% unresectable at time of diagnosis) [10,60]. Despite more than 10 years of FDA-approved therapies and marked improvements in medical and surgical care, there has been no significant improvement in PDAC patient survival [21]. Identification of new molecular targets and optimization of drug delivery systems against these targets are needed in order to improve therapeutic outcomes for this disease, particularly against the drug-resistance PDAC phenotypes [35]. A number of novel therapeutic agents targeting tumor cells, tumor vasculature, or stromal responses are currently under various stages of evaluation in clinical trials for pancreatic cancer [8,35,40].

A growing body of evidence suggests that stem cells may play a decisive role in the development and progression of cancer [9,23]. A tumor stem cell (TSC) or cancer stem cell (CSC) is defined as a cell within a tumor that is able to self-renew and to produce the heterogeneous lineages of cancer cells that comprise the tumor [9]. CSCs are often resistant to chemotherapy and radiation therapy; this may explain why current treatments do not cure PDAC or prevent recurrences [12,18,22,45,47]. These cells promote tumor growth and progression through a number of mechanisms, including initiation of the tumor, differentiation into bulk tumor cells, metastasis, and alteration of adjacent stroma (reviewed in [1]). Similar to CSCs of other organs, pancreatic CSCs can be distinguished from bulk tumor cells on the basis of unique surface markers, abilities to form spheres 3-D culture conditions, the ability to develop tumor xenografts in mice. For example, a subpopulation of pancreatic cells expressing cell surface markers such
as ALDH1, SOX2, or a combination of multiple proteins such as CD44, CD24, and epithelial-specific antigen (ESA) (designated as CD44*CD24*ESA*) exhibit high level of tumorigenic potential [17,29]. Although CD44, CD24, and ESA are markers of pancreatic CSCs, their functional significance is unclear. CSCs have also been linked to epithelial-to-mesenchymal transition (EMT) in various solid tumors including PDAC. Cancer cells that undergo EMT exhibit loss of epithelial polarity and markers (e.g. E-cadherin), and in turn acquire invasive properties and stem cell-like features. These properties are believed to prelude metastasis. In fact, prior to dissemination into circulation, the PDAC cells acquire mesenchymal traits. Abrantly expressed SOX2 contributes to PDAC proliferation, stemness, and dedifferentiation through the regulation of some EMT gene drivers such as SNAI, ZEB1, ZEB2 and TGBj2 [17].

Recently, a number of reports have identified the miR-200 family of miRNAs as important markers and regulators of EMT [14]. Our extensive investigations and those by others (reviewed in [15]) have revealed doublecortin-like kinase 1 (DCLK1) as another important regulator of these miRNAs, stemness of cancer cells, and EMT. DCLK1 is a TSC marker in intestine [38] and also marks quiescent stem cells that are activated after radiation injury [32,33]. Furthermore, the Dclk1<sup></sup><sup>−/−</sup> cell population demonstrates enriched expression of many of these and other TSC markers including CD133, CD24/CD44/ESA, and ALDH [3,26,38]. The protein is over-expressed in cancers derived from pancreas, liver, colon, esophagus and intestines [25,30,32–34]. Previously, we demonstrated that siRNA-led inhibition of DCLK1 results in tumor growth arrest in cancer xenograft models [52]. It also results in upregulation of key tumor suppressor microRNAs (let-7a, mir-200a, and mir-144) that regulate critical oncogenic pathways (e.g. c-MYC, KRAS, NOTCH1), and several EMT-related transcription factors (e.g. TWIST, ZEB1, ZEB2, SNAI, and SLUG) [50,52].

A novel small molecule kinase inhibitor (XM8D-92) has been synthesized as a potent inhibitor of Mitogen-activated protein kinase 7 (MAPK7/BMK1; K<sub>d</sub> = 80 nM) [11,62]. BMK1 kinase activity was demonstrated to inhibit PML-dependent activation of the tumor suppressors p21 and p53. Inhibition of BMK1 phosphorylation by XM8D-92 resulted in inhibition of breast and ovarian cancer cell proliferation and tumor xenograft growth [62]. XM8D-92 also can inhibit the kinase activity of DCLK1 and is well tolerated in mice [62]. DCLK1 is one of the few kinases with greater than 90% displacement by this inhibitor [11,62]. Although BMK1, DCLK1, TNK1, and PLK4 are displaced more than 90% by this inhibitor, the binding affinity for TNK1 (K<sub>d</sub> = 890 nM) and PLK4 (K<sub>d</sub> = 600 nM) is much weaker, and XM8D-92 has no significant effect on TNK1 and PLK4 activity in vitro or in vivo [62]. In this report, we wanted to elucidate whether XM8D-92 inhibits DCLK1 and its downstream target in pancreatic cancer.

We found that treatment of AsPC-1, human pancreatic cancer cells derived tumor xenografts with XM8D-92; this resulted in tumor growth arrest, downregulation of DCLK1, and increased expression of tumor suppressor miRNAs miR-143/145, miR-200a-c, let-7a, and miR-144. A subsequent inhibition of factors that promoted pluripotency, angiogenesis, EMT, c-MYC and NOTCH1 was also observed. These data taken together indicate that XM8D-92 demonstrates anti-cancer activity by inhibiting DCLK1 in pancreatic cancer. Thus, this novel DCLK1 inhibitor is likely to be a candidate therapeutic agent for various cancers including PDAC.

Materials and methods

Reagents

XM8D-92 was purchased from Tocris Bioscience (Minneapolis, MN). All cell culture reagents were purchased from Sigma Aldrich (St. Louis, MO). For the in vitro analysis, cells were treated with XM8D-92 (0.78-25 μM). For the in vivo tumor xenograft experiments, 50-mg/kg body weight of XM8D-92 was injected via i.p. dissolved in DMSO and Corn oil [62].

Cell culture

Human pancreatic AsPC-1 cells were obtained from the American Type Culture Collection and propagated in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin in a humidified chamber at 37°C and 5% CO<sub>2</sub>.

Cell proliferation assays

Cells (10<sup>4</sup> cells per well) were seeded into a 96-well tissue culture plate in triplicate. The cells were cultured in the presence of XM8D-92 with DMSO as a vehicle at 0, 0.78, 1.56, 3.13, 6.25, 12.50 and 25 μM. 48 h post treatment, 10 μl of TACS MITT Reagent (RND Systems) was added to each well and the cells were incubated at 37°C until dark crystalline precipitate became visible in the cells. 100 μl of 266 mM NH<sub>4</sub>OH in DMSO [53] was then added to the wells and placed on a plate shaker at low speed for 1 min. After shaking, the plate was allowed to incubate for 10 min protected from light and the OD<sub>550</sub> for each well was read using a microplate reader. The results were averaged and calculated as a percentage of the DMSO (vehicle) control +/- the standard error of the mean.

Xenograft tumor model

NOD/SCID mice were purchased from the Jackson Laboratory (Bar Harbor, Maine) and housed in pathogen-free conditions. They were cared for in accordance with guidelines set forth by the American Association for Accreditation of Laboratory Animal Care and the U.S. Public Health Service Commissioned Corps’ “Policy on Human Care and Use of Laboratory Animals”. All studies were approved and supervised by the University of Oklahoma Health Sciences Center’s Institutional Animal Care and Use Committee (IACUC). AsPC-1 cells (1 x 10<sup>6</sup>) were injected subcutaneously into the flanks of 4- to 6-wk-old mice (n = 5). Tumors were measured using a caliper and the volume was calculated as [length x width<sup>2</sup>] / 2. The tumors were palpable 30 days after injection of cells. XM8D-92 was reconstituted in sterile corn oil and injected intraperitoneally (50 mg/kg body weight). Each animal bearing the tumor was injected with XM8D-92 or corn oil (vehicle control) on days 30–44 (15 doses, 1 dose/day). All mice were killed on day 45.

Immunohistochemical analysis

Heat-induced epitope retrieval was performed on 4-μm formalin-fixed, paraffin-embedded sections utilizing a pressurized Decloaking Chamber (Biocare Medical LLC, Concord, CA) in citrate buffer (pH 6.0) at 99°C for 18 min. Brightfield: slides were incubated in 3% hydrogen peroxide at room temperature for 10 min. After incubation with primary antibody [KLF4, OCT4, SOX2, NANOG and Activated NOTCH1 (Abcam Inc., Cambridge, MA), c-MYC (Santa Cruz Biotechnologies Inc., Santa Cruz, CA) or VEGFR1, VEGFR2, NOTCH1 (Santa Cruz Biotechnologies Inc. Overnight at 4°C, slides were incubated in Promark peroxidase-conjugated polymer detection system (Biocare Medical LLC) for 30 min at room temperature. After washing, slides were deoviled with Diaminobenzidine (Sigma–Aldrich). Microscopic Examination: Slides were examined utilizing a Nikon 80i microscope and DXM1200C camera for brightfield analysis. Images were captured utilizing NIS-Elements software (Nikon).

Real-time reverse transcription-polymerase chain reaction analyses

Total RNA isolated from tumor xenografts and cancer cells was subjected to reverse transcription using Superscript<sup>®</sup> II RNase H-Reverse Transcriptase and random hexanucleotide primers (Invitrogen, Carlsbad, CA). The complementary DNA (cDNA) was subsequently used to perform real-time polymerase chain reaction (PCR) by SYBR<sup>™</sup> chemistry (SYBR Green I, Molecular Probes, Eugene, OR) for specific transcripts using gene-specific primers and JumpStart<sup>™</sup> Taq DNA polymerase (Sigma–Aldrich). The crossing threshold value assessed by real-time PCR was noted for the transcripts and normalized with β-actin messenger RNA (mRNA). The quantitative changes in mRNA were expressed as fold-change relative to control with ±SEM value.
The following primers were used:

- **β-actin:** forward: 5'-GGTATCCACATCTGCTGGAA-A', reverse: 5'-ATCCATGCTCCTTCTACAGG-3';
- **DCLK1:** forward: 5'-CAGCAACAGGATTGATGTTGA-A', reverse: 5'-CTcaacggatggaggag-3';
- **c-MYC:** forward: 5'-CACACATACAGCAACTCGCA-A', reverse: 5'-TTCAGCCTCTTGAGCCGAC-3';
- **NOTCH1:** forward: 5'-CGCTTCACAGTCATTGAA-A', reverse: 5'-GTGTATGTTGTCGCACC-3';
- **KRAS:** forward: 5'-GACGATAACAGCTTACCTCAC-3';
- **miR-200a-c:** forward: 5'-CGCTTGGACGAGTGCTCTG-3';
- **ZEB1:** forward: 5'-AGCCGATCTGCGGTGATGCC-3';
- **SNAIL:** forward: 5'-GTCTTCAAGGACACAT-3';
- **SLUG:** forward: 5'-GCAGAGGCTTGAGCCGAC-3';
- **VEGFR2:** forward: 5'-ACCCATGCTCCTCCTTCCTG-3';
- **miRNAs:** forward: 5'-GGATGGGATTGTTGATGCACG-3';
- **pri-U6:** forward: 5'-AGGGCCAGCAGCAGGC-3';
- **pri-let-7a:** forward: 5'-GCCGTGGCCATCTTACTGG-3';
- **pri-miR-143/145:** forward: 5'-TTCCACAGCAGCCCCTG-3';
- **pri-miR-200b:** forward: 5'-GCCATGAGTTCCATGCCACTT-3';
- **pri-miR-200c:** forward: 5'-GCTGGGATATCATCATATACTG-3';
- **KLF4:** forward: 5'-CGATCGTCTTCCCCTCTTTG-3';
- **RREB1:** forward: 5'-TTGCCTGAGTCTGTTTTCC-3';
- **miR-200a-c:** forward: 5'-CGGTTCATCATTACCAGGCAG-3';
- **pri-miR-143/145:** forward: 5'-CGGACTAGTACATCATCTATACTG-3';
- **pri-miR-200b:** forward: 5'-AACGCTTCACGAATTTGCGT-3';
- **pri-miR-200c:** forward: 5'-CTACGTTTCAGAGGAGATGGA-3';
- **pri-U6:** forward: 5'-ATCCTCACCATCATCACACTGG-3';
- **miRNAs:** forward: 5'-CGGATCGTCTTCCCCTCTTTG-3';
- **pri-miR-143/145:** forward: 5'-AGGGCCAGCAGCAGGC-3';
- **pri-miR-200b:** forward: 5'-GCCGTGGCCATCTTACTGG-3';
- **pri-miR-200c:** forward: 5'-TTCCACAGCAGCCCCTG-3';

**miRNA analysis**

Total RNA isolated from tumor xenografts was subjected to reverse transcription with SuperScript II RNase H-Reverse Transcriptase and random hexanucleotide primers (Invitrogen). The cDNA was subsequently used to perform real-time PCR by SYBR chemistry for pri-let-7a, pri-mir-144, pri-mir-200a-c and pri-mir-143/145 transcripts using specific primers and JumpStart Taq DNA polymerase. The crossing threshold value assessed by real-time PCR was noted for transcripts using specific primers and JumpStart Taq DNA polymerase. The crossing threshold value assessed by real-time PCR was noted for transcripts using specific primers and JumpStart Taq DNA polymerase.

**Statistical analysis**

All experiments were performed in triplicates. Results are reported as average ± SEM unless otherwise indicated. Data were analyzed using the Student’s t-test. Results were considered statistically significant when p < 0.01.

**Results**

**XM8D-92 inhibits DCLK1, c-MYC, KRAS and NOTCH1 mRNA in AsPC-1 cells in vitro**

Based on the previously published report, XM8D-92 is known to inhibit BMK1 kinase activity and also bind to DCLK1 [11,62]. Taking this into consideration, we treated AsPC-1 human pancreatic cancer cells with doses up to 25 mM of XM8D-92 for 48 h. Proliferation of cancer cells was assessed using standard MTT assay. Total RNA isolated were subjected to quantitative real-time RTPCR analysis for p53 and p21, DCLK1, c-MYC, KRAS and NOTCH1. We observed a dose-dependent significant downregulation of AsPC-1 cancer cell proliferation (Fig. 1A). Following the RTPCR analysis, we did not observe increase in expression of tumor suppressor genes p21 or p53 mRNA following the treatment (Supplementary Fig. 1A and B). Subsequently, we observed significant dose-dependent downregulation of DCLK1 mRNA and protein (by Western blot analysis) following treatment with 10 and 15 μM of XM8D-92 (Fig. 1B and C). Furthermore, we also observed nearly 60% reduction in c-MYC, KRAS and NOTCH1 mRNA in AsPC-1 cells treated with XM8D-92 (Fig. 1D). These data taken together demonstrate that treatment AsPC-1 cells with XM8D-92 in vitro results in downregulation of DCLK1, c-MYC, KRAS and NOTCH1 mRNA.

**XM8D-92 inhibits pancreatic tumor xenograft growth**

Pancreatic tumor xenografts were generated by injecting AsPC-1 cells subcutaneously into the lower flanks of NOD/SCID mice. Tumors were allowed to develop for 30 days. When tumors were palpable, mice were treated with either XM8D-92 (50 mg/kg body weight) in DMSO and sterile corn oil (i.p.) or Control (injected with DMSO and corn oil) (n = 5 animals in each group). Treatments were given every day for 15 days and tumor volumes were measured every third day. Tumors were excised at day 45, and tumor volumes are represented in Fig. 2A. Control or vehicle-treated tumors grew exponentially throughout the experiment, whereas treatment with XM8D-92 not only arrested the tumor growth but resulted in decrease in the tumor volume compared to initial no treatment (day 0) (Fig. 2A). Treatment with XM8D-92 resulted in a significant (>80%) reduction (p < 0.01) in tumor volume compared to control tumors. We also observed more than 2-fold decrease in the tumor weight following treatment with XM8D-92 (Fig. 2B). miRNA analysis demonstrated a significant downregulation (p < 0.01) of DCLK1 mRNA in tumor tissue from XM8D-92 compared to control tumors (Fig. 2C). Following immunohistochemical analysis, we observed significant downregulation of DCLK1 protein in tumors treated with XM8D-92 compared to control tumors (Fig. 2D and E). Similar to PDAC cell lines, in tumor xenografts following treatment with XM8D-92, we did not observe increase in expression of BMK1 downstream tumor suppressor genes p21 and p53 mRNA indicating that BMK1 related activity is no affected in pancreatic cancer (Supplementary Fig. 1C and
These data taken together demonstrate that XMD8-92 inhibits AsPC-1 tumor xenograft growth and inhibits DCLK1 mRNA and protein. XMD8-92 treatment inhibits pluripotency in pancreatic tumor xenografts

Pluripotency factors KLF4, OCT4, SOX2 and NANOG are upregulated in various aggressive cancers and in cancer stem cells [6,42,48,54,55]. In our previous studies, following knockdown of DCLK1 using siRNA, we observed decreased expression of these pluripotency factors via miR-143/145 miRNA cluster-dependent mechanism [51]. In this study, we wanted to determine whether treatment with XMD8-92 also resulted in regulation of the pluripotency factors via miR-143/145. In XMD8-92 treated tumors, we observed significant (p < 0.01) upregulation of miR-143/145 cluster compared to control tumors (Fig. 3A). Furthermore, we observed significant downregulation of pluripotency factors KLF4, OCT4, SOX2, and NANOG mRNA (Fig. 3B) and protein (Fig. 3C) in tumors treated with XMD8-92 compared to control tumors. Ras-responsive element binding protein 1 (RREB1) represses miR-143/145 promoter activity, which indicates that repression is an early event in pancreatic cancer initiation and progression [24]. Additionally, KRAS and RREB1 are targets of miR-143/145, demonstrating a feed-forward mechanism that potentiates RAS signaling-mediated PDAC tumor progression [24]. It has been recently demonstrated that ectopic expression of miR-143/145 results in repressed metastasis and increased adhesion of pancreatic cancer cells [41]. Earlier, we have demonstrated that following knockdown of DCLK1 results in decreased expression of RREB1 via miR-143/145. Similarly, in this study, we observed >50% reduction in RREB1 mRNA following treatment with XMD8-92 compared to control tumors (Fig. 3B).

XMD8-92 inhibits EMT and Angiogenesis via miR-200 in tumor xenografts

Invasive cancers are often characterized by EMT, a process in which immobile epithelial tumor cells can transform into highly

Fig. 1. XMD8-92 inhibits DCLK1 is AsPC-1 pancreatic cancer cells. (A) Proliferation of AsPC-1 following treatment with XMD8-92. (B) The expression of DCLK1 mRNA in the AsPC-1 cells following treatment with XMD8-92 quantitated by real-time RT-PCR. (C) DCLK1 protein estimated using Western blot in Control and following treatment with XMD8-92. (D) Quantitative real-time RT-PCR analysis of c-MYC, KRAS and NOTCH1 mRNA following treatment with XMD8-92 in AsPC-1 cells. Values are given as average ± SEM, and asterisks denote statistically significant differences (p < 0.01) compared with Control (vehicle treated).
metastatic and proliferative mesenchymal cells. EMT plays a key role in cancer invasion and metastasis. EMT-type cells in pancreatic cancer have increased expression of the stem cell markers CD24, CD44, and ESA, and increased sphere-forming capacity, suggesting a link between EMT and CSCs. EMT in CSCs may play a critical role in tumorigenesis in general, and PDAC in particular. EMT is initiated by transcription factors ZEB1 and ZEB2, and subsequently by SNAIL and SLUG. These transcription factors are upregulated in various cancers and have poor prognosis for the disease. Vascular endothelial growth factor (VEGF) and their two-tyrosine kinase receptors (VEGFR1 and VEGFR2) are known to promote tumor vasculature and endothelial proliferation, and are also involved in tumor metastasis. Inhibition of VEGFR1 and VEGFR2 results in inhibition of tumor angiogenesis and metastasis in pancreatic tumor mouse models. Recently, studies have shown that miR200a-c (miR-200) regulates EMT by targeting ZEB1 and ZEB2, and angiogenesis by targeting VEGFR1 and VEGFR2. We have previously observed that knockdown of DCLK1 results in inhibition of ZEB1, ZEB2, VEGFR2 and VEGFR2 via miR-200. In this study, we observed significant upregulation (>1.5-fold) of miR-200a, miR-200b, and miR-200c in tumors treated with XMD8-92 compared to control tumors (Fig. 4A). Subsequently, we observed significant downregulation of EMT transcription factors ZEB1, ZEB2, SNAIL and SLUG (Fig. 4B) in XMD8-92 treated tumors. We also observed significant downregulation (>60%) VEGFR1 and VEGFR2 mRNA (Fig. 4C) and protein (Fig. 4D) in the tumors treated with XMD8-92 compared to control tumors. These data taken together demonstrate that similar to DCLK1 knockdown, treatment of XMD8-92 results in downregulation of EMT and angiogenesis via miR-200 in pancreatic tumor xenografts.

**Fig. 2.** XMD8-92 treatment inhibits pancreatic tumor xenograft growth and inhibits DCLK1. (A) AsPC-1 human pancreatic cancer cells were subcutaneously injected into the flanks of NOD/SCID mice to generate tumors. At day 30, XMD8-92 (50 mg/kg in corn oil) was injected subcutaneously every day (n = 5 animals in each group). After 15 injections, tumors were excised at day 45 and are represented above. Tumor volume was measured every 3 days. Values are given as average ± Standard Deviation (SD), and asterisks denote statistically significant differences (*p < 0.01) compared with Control. (B) Bar graph represents the average tumor weight excised from animals treated with vehicle (Control) or XMD8-92. (C) The expression of DCLK1 mRNA in the tumors quantitated by real-time RT-PCR. (D) Immunohistochemical analysis of DCLK1 (brown) protein in the tumors. (E) Western blot analysis of DCLK1 protein in the tumors. Values in the bar graphs are given as average ± SEM, and asterisks denote statistically significant differences (*p < 0.01) compared with Control.

**XMD8-92 treatment results in inhibition of Let-7a downstream targets c-MYC, KRAS and LIN28B in pancreatic tumor xenografts**

It has been previously demonstrated that following siRNA-mediated knockdown of DCLK1 results in increased expression...
of tumor suppressor miRNA let-7a and downregulation of let-7 downstream targets c-MYC, KRAS and LIN28B. In this study, following treatment with XMD8-92, we observed significant (p < 0.01) upregulation of miRNA let-7a (Fig. 5A) and more than 60% reduction in c-MYC mRNA (Fig. 5B) and protein (Fig. 5B inset and 5C). We also observed significant downregulation of KRAS mRNA (>50%) (Fig. 5D – left panel) and LIN28B mRNA (>80%) (Fig. 5D – right panel). These data indicate that XMD8-92 treatment regulates oncogenes c-MYC, KRAS and LIN28B via let-7a. These data also indicate these actions are mediated via downregulation of DCLK1 in pancreatic tumor xenografts.

**XMD8-92 treated xenografts have less NOTCH1**

Notch signaling is upregulated in various cancers, including that of pancreatic cancer. Previous reports have indicated that NOTCH1 is downstream of DCLK1, and DCLK1 regulates NOTCH1 via miR-144 miRNA [49,50]. siRNA-mediated knockdown of DCLK1 results in upregulation of pri-miR-144 and subsequently downregulated NOTCH1 mRNA [49,50]. Similar to the above observation, in this study, following treatment with XMD8-92 a significant upregulation (>2.0 folds) of pri-miR-144 (Fig. 6A) and more than 60% reduction in NOTCH1 mRNA (Fig. 6B) and protein (Fig. 6C). Furthermore, we also observed significant downregulation of activated NOTCH1...
These data indicate that XMD8-92 treatment results in decreased expression of NOTCH1 via downregulation of DCLK1 in pancreatic tumor xenografts. Additionally, all the data has been summarized and is represented in a graphical format (Fig. 6E).

Discussion

DCLK1, a putative marker of intestinal and pancreatic stem cells, is upregulated in various solid tumors including colorectal, pancreatic, breast and prostate compared to paired normal tissues [15,49,50]. Furthermore, the role of Dclk1 in PDAC initiation is strengthened by results using lineage-tracing and oncogenic cancer stem cell-initiating mouse models (Dclk1\(^{Cre}\); Kras\(^{G12D}\)) [56–59]. It was confirmed in a recent study that Dclk1 marks a morphologically distinct and functionally unique population of pancreatic cancer-initiating cells using different mouse models of pancreatic cancer (Pdx1\(^{Cre}\); KRAS\(^{G12D}\); P53\(^{f/f}\) and Mst1\(^{Cre}\); KRAS\(^{G12D}\)) [3]. Dclk1 + cells had cancer stem cell-like properties, and both pre-invasive and invasive pancreatic cancer depend on this sub-population of Dclk1-expressing cells [3].

Very recent reports indicate that DCLK1 can be used as a prognostic factor in colorectal cancer [15] and a potential methylation marker in cholangiocarcinoma [2]. Recently published work established that Dclk1 marks TSCs that continuously produce tumor progeny in the polyps of Apc\(^{Min/+}\) mice polyps [38]. In that study, they demonstrated that specific ablation of Dclk1 + TSCs resulted in a marked regression of polyps without apparent damage to the normal intestine [38]. Furthermore, we have demonstrated that siRNA-mediated knockdown of DCLK1 results in upregulation of miR-200a, an inhibitor of EMT, and corresponding downregulation of ZEB1 and ZEB2 with subsequent rescue of E-cadherin in both human pancreatic and colorectal cancer cells [27,49,50,61]. Therefore siRNA-mediated DCLK1 blockade results in EMT inhibition in both human pancreatic and colorectal cancer cells [49,50]. Recently, we have observed that angiogenic factors (VEGFR1 and VEGFR2), downstream of miR-200a-c [7,43], are regulated by DCLK1 [51]. Additionally, we found that DCLK1 knockdown induces the miR-143/145 tumor suppressor miRNA cluster following treatment with XMD8-92 (Fig. 6D). These data indicate that XMD8-92 treatment results in decreased expression of NOTCH1 via downregulation of DCLK1 in pancreatic tumor xenografts. Additionally, all the data has been summarized and is represented in a graphical format (Fig. 6E).
that regulates KRAS, a key oncogene that is mutated in more than 95% of PDACs [31,51] and pluripotency factors OCT4, SOX2, NANOG and KLF4 [51]. These exciting data illustrate the importance of targeting DCLK1 in cancer.

Though siRNA-based therapy provides many advantages, there are certain challenges that need to be overcome as a potential new drug. These challenges include off-target effect, immune stimulation, reduced uptake by cells, short half-life, and toxic effect of saturation of RNAi [36]. In order to overcome these limitations, we wanted to test certain small molecule kinase inhibitors that have binding affinity towards DCLK1 as an alternate approach to inhibit DCLK1 activity. Based on the recent publications, we wanted to test the efficacy and mechanism of XMD8-92 in PDAC tumor inhibition. XMD8-92 is an inhibitor of MAP7/BMK1 kinase activity [11,62]. BMK1 has been demonstrated to inhibit PML-dependent activation of the tumor suppressors p21 and p53, resulting in inhibition of breast and ovarian cancer cell proliferation and tumor xenograft growth [62]. Based on the affinity binding studies, DCLK1 is one of the few kinases with greater than 90% displacement by XMD8-92 [11,62]. Since XMD8-92 has similar binding affinity to BMK1 and DCLK1 kinases, we wanted to elucidate whether XMD8-92 inhibits DCLK1 and or BMK1 and its downstream target in PDAC.

In AsPC-1 cells and tumor xenografts treated XMD8-92, we observed significant downregulation of DCLK1 and their downstream targets similar to the effects of DCLK1 siRNA treatment. But, we did not observe activation of BMK1 downstream tumor suppressor p21 or p53 following treatment with XMD8-92 indicating that BMK1-PML activities might not robust in PDAC. At this point, this is a speculation that requires further studies to verify it. Nevertheless, this is the first report that XMD8-92 inhibits DCLK1 and affected downstream oncogenes and tumor suppressors in PDAC. Following treatment with XMD8-92, we observed significant AsPC-1 tumor growth inhibition and downregulation of DCLK1, c-MYC, KRAS, NOTCH1, ZEB1, ZEB2, SNAIL,Slug, OCT4, SOX2, LIN28, OCT4A, KLF4, VEGFR1 and VEGFR2. We also found upregulation of tumor suppressor miRNAs (downstream of DCLK1) e.g. let-7a, miR-144, miR-200a-c and miR-143/145 in tumors treated with XMD8-92 in a mechanism similar to siRNA-mediated knockdown of DCLK1 (Fig. 6E).

In the tumor xenograft study (following treatment with XMD8-92), we observed an increase in miR-143/145 expression and downregulation of downstream targets including KLF4, OCT4, SOX2, OCT4A, KLF4, and RREB1. In addition to these targets, it has been demonstrated that miR-143/145 cluster also regulates extracellular signal-regulated kinase 5 (ERK5), MYC, and insulin receptor substrate-1 (IRS-1) [4,13,24,44,46]. This indicates a higher probability that XMD8-92 treatment also downregulates/affects these oncogenes. Furthermore, let-7a is known to target interleukin-6 (IL-6), which is an inducer of NF-kB activity [19,24]. In a recent study, it has been demonstrated that induced expression of let-7a in PDAC cells decreased phosphorylation of signal transducer and activator of transcription 3 (STAT3); this resulted in decreased EMT.
migration, and growth of PDAC cells [39]. It can be assumed that following treatment with XMD8-92, we will observe a decreased expression of IL-6 as well as a related decrease in NF-κB activity. Furthermore a reduction in STAT3 phosphorylation, and migration of PDAC cells may also be expected following treatment with XMD8-92.

A study has demonstrated that enhance of zeste homolog 2 (EZH2), a histone methyltransferase, is overexpressed in aggressive PDACs. Suppression of EZH2 inhibited PDAC cell growth and also inhibited liver metastasis of PDAC in vivo [5]. These data taken together indicate that EZH2 is a potential target for PDAC therapy. More recently, Guo et al., demonstrated that treatment with miR-144 inhibitor promotes bladder cancer cell proliferation, whereas miR-144 overexpression inhibits cell proliferation [16]. Furthermore, EZH2 is a target gene of miR-144. Downregulation of miR-144 induces the expression of EZH2, which results in activation of Wnt/beta-catenin signaling and subsequent cancer cell proliferation [16]. These data indicate the notion that induction of miR-144 following treatment with XMD8-92 may also target EZH2, ultimately resulting in inhibition of PDAC tumor growth. All of these collective studies indicate possible alternative pathways affected by the tumor suppressor miRNAs that are upregulated following treatment with XMD8-92.

Furthermore, studies have demonstrated that XMD8-92 is well-tolerated and the mice appeared healthy with minimal distress with a plasma concentration up to 10 μM (50 mg/kg dose) during the treatment periods [62]. Similar to that study, we did not find any distress in mice following treatment with XMD8-92 for 15 days. All of these studies indicate that XMD8-92 is an ideal small molecule kinase inhibitor with robust anti-cancer activity. Our studies clearly implicate that XMD8-92 inhibits DCLK1 and DCLK1-related oncogenes via upregulation of tumor suppressor miRNAs and results in inhibition of pluripotency, tumorigenesis, EMT and angiogenesis in PDAC.

**Conflict of interest**

CWH is cofounder of COARE Biotechnology, Inc. The other authors disclosed no potential conflicts of interest.

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