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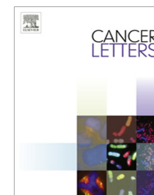

XMD8-92 inhibits pancreatic tumor xenograft growth via a DCLK1-dependent mechanism

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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 devastatingly 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

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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. Aberrantly expressed SOX2 contributes to PDAC proliferation, stemness, and dedifferentiation through the regulation of some EMT gene drivers such as *SNAIL*, *ZEB1*, *ZEB2 and TGBβ2* [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 $Dclk1^{+}$ 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 overexpressed 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, SNAIL and SLUG) [50,52].

A novel small molecule kinase inhibitor (XMD8-92) has been synthesized as a potent inhibitor of Mitogen-activated protein kinase 7 (MAPK7/BMK1; Kd = 80 nM)[11,62]. BMK1 kinase pathway is one of the MAP kinase cascades, which play an important role in oncogenic signaling during tumorigenesis. Activated BMK1 has been demonstrated to inhibit PML-dependent activation of the tumor suppressors p21 and p53. Inhibition of BMK1 phosphorylation by XMD8-92 resulted in inhibition of breast and ovarian cancer cell proliferation and tumor xenograft growth [62]. XMD8-92 also can the inhibit 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 (Kd = 890 nM) and PLK4 (Kd = 600 nM) is much weaker, and XMD8-92 has no significant effect on TNK1 and PLK4 activity *in vitro* or *in vivo* [62]. In this report, we wanted to elucidate whether XMD8-92 inhibits DCLK1 and its downstream target in pancreatic cancer.

We found that treatment of AsPC-1, human pancreatic cancer cells derived tumor xenografts with XMD8-92; 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 XMD8-92 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

XMD8-92 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 XMD8-92 (0.78–25 μM). For the *in vivo* tumor xenograft experiments, 50-mg/kg body weight of XMD8-92 was injected via *i.p.* dissolved in DMSO and Corn oil [62].

Cell culture

Human pancreatic cancer 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_2.

Cell proliferation assays

Cells (10^4 cells per well) were seeded into a 96-well tissue culture plate in triplicate. The cells were cultured in the presence of XMD8-92 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 MTT 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 NH4OH 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 OD550 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 (1×10^7) 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 $\times$ width^2) $\times$ 0.5. The tumors were palpable 30 days after injection of cells. XMD8-92 was reconstituted in sterile corn oil and injected intraperitoneally (50 mg/kg body weight). Each animal bearing the tumor was injected with XMD8-92 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)] 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 devolved 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™ 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™ chemistry (SYBR Green I, Molecular Probes, Eugene, OR) for specific transcripts using gene-specific primers and JumpStart™ 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.

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