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NEMO Peptide Inhibits the Growth of Pancreatic Ductal Adenocarcinoma by Blocking NF- κ B Activation

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Abstract

NF- κ B essential modulator (NEMO) binds and regulates I κ B kinase (IKK) and is required for NF- κ B activation. The NEMO-binding domain peptide (NBDP) of IKK was found to inhibit NF- κ B activation and promote apoptosis in cancer cells. Studies have shown that constitutive NF- κ B activation, one of the signature molecular alterations in pancreatic ductal adenocarcinoma (PDAC), is a potential therapeutic target. However, preclinical and therapeutic evidence that supports direct targeting of IKK activation in therapy is lacking. The aim of this study was to determine whether the combination of NBDP and gemcitabine would sensitize pancreatic cancer to the gemcitabine. We confirmed that NBDP inhibited NF- κ B activation and found that NBDP indeed promoted chemo-sensitivity to gemcitabine in PDAC. NBDP increased PARP and caspase 3 cleavage in the apoptosis pathway, increased apoptosis of PDAC cells, and suppressed PDAC cell growth in vitro. In addition, NBDP combined with gemcitabine significantly decreased levels

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AUTHOR CONTRIBUTIONS

Z.Z., J.L., and P.J.C. designed the project. Z.Z., H.Li, H.L., M.A., T.G., H.J., T.I., J.F., W.M., Y.L., and Y.S. perform various experiments. All authors discuss the results and participate in the revision of the manuscript.

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CONFLICTS OF INTEREST

The authors have no conflicts.

of NF- κ B activity and inhibited the growth of PDAC in vivo in an orthotopic xenograft mouse model. Mechanistic investigations showed that NBDP effectively competed with NEMO/IKK γ for binding to IKKs and thus inhibited IKK and NF- κ B activation, down-regulated expression levels of Erk, and decreased PDAC cell growth. Taken together, our current data demonstrate that NBDP sensitizes human pancreatic cancer to gemcitabine by inhibiting the NF- κ B pathway. NBDP is a potential adjuvant chemotherapeutic agent for treating pancreatic cancer.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the one of the most lethal malignancies in the United States, with a 5-year survival rate that has remained at 5% for the past 30 years [1]. With an estimated 53,070 new cases in 2016, it is projected to surpass breast, colorectal, and prostate cancers as the second leading cause of cancer-related deaths in the United States by 2030 [2]. Current therapy regimens are largely ineffective [3]. Novel and effective therapeutic targets and strategies for pancreatic cancer treatment remain to be identified.

A genetic profile of PDAC identifies several of its most commonly detected mutations and alterations as signatures, suggesting a unique set of defects in this disease [4, 5]. For example, oncogenic Kras is common in PDAC. Recent studies demonstrated that mutant Kras is required for initiation and maintenance of the tumorigenic phenotype of PDAC in genetically engineered mouse models, indicating an essential role of mutant Kras in PDAC development [6]. Our previous studies showed that NF- κ B was constitutively activated in about 70% of PDAC cases [7, 8]. Our more recent studies revealed that pancreas-targeted IKK2/ β inactivation in Kras^{G12D} and Kras^{G12D}; Ink4a/Arf^{F/F} mice inhibited NF- κ B activation and completely suppressed PDAC development, suggesting that constitutive NF- κ B activation is required for mutant Kras to induce PDAC development [9]. Importantly, our studies revealed that oncogenic Kras-activated AP-1 induces IL-1 α , which in turn activates NF- κ B and the NF- κ B downstream target genes IL-1 α and p62, to initiate IL-1 α /p62 feedforward loops for inducing and sustaining NF- κ B activity [9]. Therefore, our previous findings demonstrate the underlying molecular mechanism by which IKK2/ β /NF- κ B is activated by mutant Kras through dual feedforward loops of IL-1 α /p62 [9]. Furthermore, IL-1 α overexpression correlates with Kras mutation, NF- κ B activity, and poor survival in PDAC patients [9].

The activation of I κ B kinase (IKK) is a key event in NF- κ B signal transduction in response to many stimuli, and this complex consists of IKK α , IKK β , and a regulatory subunit, NF- κ B essential modulator (NEMO or IKK γ) [10, 11]. The ubiquitin-induced recruitment of A20 to NEMO is sufficient to block IKK phosphorylation by its upstream kinase TAK1 [12]. MCP1 induction serves as a negative feedback mechanism for attenuating NF- κ B activation in genotoxic response by mediating USP10-dependent deubiquitination of NEMO [13]. A number of studies demonstrate the inhibition of NF- κ B activation by the NEMO-binding domain peptide (NBDP) [14]. NBDP is synthesized in tandem with a protein transduction domain sequence from *Drosophila* Antennapedia to promote uptake of NBDP into the cytosol of target cells. Tanaka et al. showed that NF- κ B inhibition suppressed the metastasis of highly metastatic oral squamous cell carcinoma [15]. However, whether NF-

κ B inhibition by short-term use of various inhibitors is effective in suppression of tumor cell growth is still under ongoing study. Therefore, the aim of this study is to determine in a preclinical study whether the innovative approach of inhibiting constitutive NF- κ B activity by NBDP with or without gemcitabine, a standard chemotherapeutic drug in the clinic, is effective in treating PDAC. Our results show that NBDP increased chemo-sensitivity to gemcitabine and inhibited PDAC growth in orthotopic xenograft nude mouse models. Our finding indicates that inhibition of a key component that induces NF- κ B constitutive activation is a feasible therapeutic strategy in the suppression of pancreatic tumorigenesis and that NBDP could be useful as an adjuvant chemotherapeutic approach for treating PDAC.

RESULTS

Kras-induced NF- κ B activation was blocked by competitive inhibition with NBDP in PDAC cells

Previous studies from several laboratories including ours suggested that mutant Kras-activated NF- κ B activity is required for Kras-induced PDAC development in genetically engineered mouse models for PDAC, such as KPC and KIC models [9]. With IKK2 knockout in the pancreas, the mice remained free of PDAC for over 1 year, suggesting that IKK2 plays an essential role in PDAC development and is a potential therapeutic target. To determine whether IKK is an effective therapeutic target, we investigated the approach of inhibiting IKK to suppress tumorigenesis. A previous report demonstrating IKK inhibition via the NBDP targeting the NBD-NEMO interaction provided an approach to selectively inhibit NF- κ B activation-induced tumorigenesis [16]. NBDP consists of 11 amino acids from the carboxyl terminus of the catalytic IKK β subunit, which binds to the scaffold protein NEMO [16]. NBDP thus inhibits the interaction of both IKK α and IKK β with NEMO and prevents assembly of the IKK complex for activation of IKK [17].

The following experiments used the PDAC cell lines HPNE, HPNE/Kras^{G12V}, HPNE/Kras^{G12V}/p16sh, MiaPaca2, and Colo357. Except parental cell line, HPNE, all of which expressed high levels of NF- κ B activity associated with mutant Kras expression [18]. Figure 1 (A, B) shows phosphorylated or activated p65 NF- κ B in the PDAC cell lines. To determine whether NBDP could block NF- κ B activation, we treated HPNE/Kras^{G12V}/p16sh cells with predetermined optimal dose range (50, 100, and 200 μ M) of NBDP and found that phosphorylated p65 levels were decreased in nuclear extract (Fig. 1C), and these data were consistent with the results of a gel shift assay (Fig. 1D). We also demonstrated that in the cytoplasm of HPNE/Kras^{G12V}/p16sh, MiaPaca2, and Colo357 cells, NF- κ B activation was inhibited by NBDP (Fig. 1E–G), indicating that disruption of the interaction between IKK γ and IKK β in the IKK complex reduced NF- κ B activity.

To determine whether NBDP could be used as an antagonist of IKK γ in pancreatic cancer cells, we used an immunoprecipitation assay to show that the binding of NBDP with IKK β decreased the IKK γ and IKK β interaction, even with the stimulation of TNF- α (Fig. 2A). Then, we assessed the expression of the downstream target genes of NF- κ B and found that phosphorylated I κ B α and p65 were decreased with NBDP treatment in HPNE/Kras^{G12V}/p16sh cells (Fig. 2B). These results show NBDP inhibited the formation of IKK complex for

NF- κ B activation. To determine whether NBDP could decrease the activity of gemcitabine-induced NF- κ B activation, which is known to lead to chemo-resistance in some PDAC cells [16], we treated HPNE/Kras^{G12V}/p16sh, MiaPaca2, and Colo357 cell lines with NBDP alone or in combination with gemcitabine (10 μ M [17]) at various time points (0, 1, 2, 4, 6, and 12 h) (Fig. 2C–E). We found that levels of phosphorylated NF- κ B were increased at 2 h and at 4 h (Fig. 2C–E). These results showed that NBDP could significantly decrease phosphorylated p65 levels in HPNE/Kras^{G12V}/p16sh, MiaPaca2, and Colo357 cells (Fig. 2F). Thus, this approach may be considered as adjuvant therapy to decrease chemo-resistance to gemcitabine in pancreatic cancer treatment.

NBDP and gemcitabine decreased the proliferation and invasion of PDAC cells

To test whether directly blocking the NF- κ B pathway using NBDP would promote chemo-sensitization to gemcitabine in pancreatic cancer, we assessed the proliferation of HPNE/Kras^{G12V}/p16sh, MiaPaca2, and Colo357 cells upon treatment with NBDP and gemcitabine separately and in combination. We found that 200 μ M NBDP in HPNE/Kras^{G12V}/p16sh and MiaPaca2 cells and 100 μ M NBDP in Colo357 cells significantly inhibited proliferation (Fig. 3A). We also found that 1 μ M gemcitabine in HPNE/Kras^{G12V}/P16sh and Colo357 cells and 5 μ M gemcitabine in MiaPaca2 cells significantly inhibited proliferation (Fig. 3B). The half maximal inhibitory concentration (IC₅₀) values for NBDP in PDAC cells compared with control cells were as follows: 200 μ M in HPNE/Kras^{G12V}/P16sh cells, 200 μ M in MiaPaca2 cells, and 150 μ M in Colo357 cells (Fig. 3C). Treatment with NBDP (100 μ M) combined with gemcitabine (5 μ M) led to significant inhibition of proliferation in HPNE/Kras^{G12V}/P16sh, MiaPaca2, and Colo357 cells (Fig. 3D).

To confirm that NBDP inhibits PDAC cell proliferation and enhances the chemo-sensitivity of PDAC cells to gemcitabine, colony formation of HPNE/Kras^{G12V}/P16sh, MiaPaca2, and Colo357 cells was analyzed after treatment with NBDP alone or combined with gemcitabine. The number of colonies formed significantly decreased under treatment with NBDP in a dose-dependent manner in all three cell lines (Fig. 4A). NBDP also promoted chemo-sensitivity to gemcitabine, as colony formation decreased in all three cell lines under the combination treatment (Fig. 4B). Wound healing analysis showed that the invasion of PDAC cells was significantly impeded by NBDP (50, 100, and 200 μ M) in all three cell lines (Fig. 4C).

Competition inhibition of NF- κ B pathway by NBDP increased apoptosis in PDAC cells treated with gemcitabine

To determine whether blocking the NF- κ B pathway using NBDP would promote apoptosis in PDAC cells, we analyzed HPNE/Kras^{G12V}/P16sh cells treated with NBDP at 50, 100, and 200 μ M by flow cytometry. We found that NBDP did induce apoptosis (Fig. 5A). Furthermore, the ratio of apoptosis in HPNE/Kras^{G12V}/P16sh cells increased with increasing doses of NBDP (Fig. 5B). To determine whether chemo-sensitivity to gemcitabine would be promoted by NF- κ B inhibition-induced apoptosis in PDAC cells, we administered the combination of gemcitabine (10 μ M) and NBDP (100 μ M) to HPNE/Kras^{G12V}/P16sh, MiaPaca2, and Colo357 cells. There were significantly increased apoptosis in HPNE/Kras^{G12V}/P16sh and MiaPaca2 cells that received the combination treatment compared with

gemcitabine alone (Fig. 5C,D). In Colo357 cells, apoptosis was significantly increased in cells that received the combination treatment compared with control treatment but not compared with gemcitabine alone (Fig. 5C,D).

To confirm that NBDP induces apoptosis and increases chemo-sensitivity to gemcitabine by inducing apoptosis in PDAC cells, we examined the cleavage of caspase 3 and poly ADP-ribose polymerase (PARP) in the apoptosis pathway in response to NBDP and gemcitabine separately and in combination. Expression of cleaved PARP was increased in HPNE/Kras^{G12V}/P16sh cells treated with NBDP (Fig. 6A,C). Expression of caspase 3 and PARP also increased in response to NBDP combined with gemcitabine in HPNE/Kras^{G12V}/P16sh and MiaPaca2 cells (Fig. 6B,F,G). Expression of cleaved Parp was significantly increased in response to NBDP combined with gemcitabine in both HPNE/Kras^{G12V}/P16sh and MiaPaca2 cells but not in Colo357 cells (Fig. 6D,H,J). Expression of cleaved caspase 3 was significantly increased in response to NBDP combined with gemcitabine in both HPNE/Kras^{G12V}/P16sh and Colo357 cells but not in MiaPaca2 cells (Fig. 6E, I, K).

NBDP and gemcitabine inhibited tumorigenesis of PDAC cells in an orthotopic xenograft model *in vivo*

To demonstrate that inhibition of NF- κ B activation suppresses the tumorigenesis of PDAC cells *in vivo*, we determined whether NBDP cooperates with gemcitabine at clinically relevant doses to inhibit the growth of human PDAC cells in an orthotopic xenograft nude mouse model. HPNE/Kras^{G12V}/P16sh tumors in the mice were treated with NBDP and gemcitabine separately and in combination for 4 weeks and then separated from the pancreatic tissues. We found that the treated tumors were significantly smaller than untreated tumors (Fig. 7A,B). NBDP and gemcitabine significantly inhibited tumorigenesis individually, and the combination treatment inhibited tumorigenesis significantly more than either agent alone (Fig. 7C). The representative micrographs show the histopathologic features of HPNE/Kras^{G12V}/P16sh tumor (*a, b*), margin (*c*), and normal pancreatic tissue (*d*) by hematoxylin and eosin staining; (Figure 7D). Body weight was not significantly lost in any treated group (Fig. 7E) but did significantly increase in mice receiving the combination treatment compared with untreated mice over the 7-week observation period (Fig. 7F). One possible explanation is that PDAC in mice of untreated control group grew much faster and interfered digestive function of the mice. Thus, these mice lost the body weight. On the other hand, PDAC in treated group grew slower and did not interfere digestive function of the treated mice as much as that of control group. The body weight did not change that much. The PDAC in mice of untreated control group grew much faster and interfered digestive function of the mice. Thus, these mice lost the body weight. On the other hand, PDAC in treated group grew slower and did not interfere digestive function of the treated mice as much as that of control group. The body weight did not change that much. The combination treatment also increased apoptosis compared with either agent alone (Fig. 7G,H).

The excised tumors then underwent immunohistochemical analysis to evaluate disease stage and development of resistance. Among HPNE/Kras^{G12V}/P16sh tumors, untreated tumors were at advanced stages with high levels of Ki67 and cyclin D1 expression, while Ki67 and cyclin D1 were significantly suppressed in tumors treated with NBDP combined with

gemcitabine (Fig. 8B,C). We also observed chemo-resistance in tumors treated with gemcitabine, which increased phosphorylated Erk and phosphorylated P65 expression (Fig. 8A), but this chemo-resistance was suppressed by NBDP, which decreased phosphorylated Erk and phosphorylated P65 expression (Fig. 8D,E). We also confirmed that NBDP treatment could decrease phosphorylated P65 expression in tumor tissue of mice model (Fig. 8F,G). Figure 9 summarizes the signaling pathways that are targeted by NBDP.

DISCUSSION

Many inhibitors of the NF- κ B pathway have been identified, including a variety of natural compounds, such as antioxidants [109]. Several natural compounds may also be effective in cancer prevention and in treatment of cancer combined with conventional anticancer drugs, such as gemcitabine, which is a standard-of-care drug for patients with pancreatic cancer [19]. A key concern about using NF- κ B inhibitors in cancer therapy is the potential toxicity [3,19]. For example, given the critical role of NF- κ B in innate and adaptive immune responses, there may be a certain amount of risk due to immunodeficiency induced by the use of NF- κ B inhibitors. There are also issues of the specificity of the drug and off target effect and effective dose range. A major challenge is to develop an approach based on selective inhibition of the antiapoptotic activity of NF- κ B without affecting its functions in other cellular events such as immune responses. One way to do it is to identify and selectively inhibit the critical target genes. It is also technically very challenge to deliver NBDP into metastatic lesion and inhibit the metastasis of PDAC. Application of NBDP as a therapeutic approach is an important future research direction.

NBDP was previously shown to inhibit constitutive NF- κ B activity and decrease tumor growth of spontaneous activated B-cell-like diffuse large B-cell lymphoma in a canine model [21]. Furthermore, a phase I clinical trial in dogs showed the safety and efficacy of systemic NBDP in inhibiting constitutive NF- κ B signaling in spontaneous activated B-cell-like diffuse large B-cell lymphoma [22]. The aim of our study is to determine whether NBDP can also be used in pancreatic cancer treatment, whether it can inhibit NF- κ B constitutive activation, and whether there is severe cytotoxicity. We utilized an orthotopic xenograft nude mouse model of human PDAC for our experiments and intra peritoneum injection as therapeutic agent delivery approach for our pilot study as it provides us a much quick answer to our questions than using genetically engineered mouse models.

Our findings demonstrate that NBDP sensitizes human PDAC to gemcitabine. Chemo-resistance may often occur due to a constitutively activated NF- κ B pathway in PDAC cells [20]. Thus, inhibiting the NF- κ B pathway could potentially be used as an adjuvant chemotherapeutic agent for treating pancreatic cancer. We previously reported that down-regulation of IKK β expression directly reduced the high level of NF- κ B constitutively activated by mutant Kras in PDAC cells, and knockout of IKK β in pancreas suppressed mutant Kras-driven PDAC development, suggesting that IKK β is required for mutant Kras-induced PDAC and potential therapeutic target [9]. As depicted in Fig. 9, in the current study, we demonstrate that NBDP directly inhibited the NF- κ B pathway by competitively blocking the assembly of IKK α /IKK β with IKK γ as shown in the immune precipitations (Figure 2A). NBDP also inhibited levels of downstream target genes in PDAC cells, as

shown by decreased phosphorylated I κ B α and phosphorylated p65 (Figure 6B–F). These results show that NBDP works well as an inhibitor of NF- κ B pathway and we found that NBDP suppresses the tumorigenesis of PDAC in vivo (Figures 7 and 8). However, it is unclear how gemcitabine-enhanced mutant Kras-induced NF- κ B activation and leads to increased resistance. One of the possible explanations is that ROS generated from gemcitabine-damaged DNA increase NF- κ B activity. ROS is in turn neutralized by anti-oxidant enzymes in a Nrf2-regulated feedback pathway to limit the duration of increased level of ROS and NF- κ B activation [20]. The anti-apoptotic molecules induced by activated NF- κ B may have longer effect in the cells to give the cells gemcitabine resistance.

To determine whether NBDP cooperated with gemcitabine at clinically relevant doses to inhibit the growth of tumors, we performed the experiments using the orthotopic mouse model. Our study showed that NBDP promotes chemo-sensitization of PDAC to gemcitabine. By directly targeting the NF- κ B pathway, NBDP decreased the proliferation and increased apoptosis of PDAC cells, and these effects were magnified when NBDP was combined with gemcitabine. We also found there were significantly increased expression of cleaved PARP and cleaved caspase 3 in the apoptosis pathway in response to NBDP combined with gemcitabine (Figures 4–6). These in vitro results suggest that NBDP as an adjuvant therapy could sensitize pancreatic cancer to gemcitabine.

In conclusion, NBDP blocks the constitutively activated NF- κ B pathway in PDAC cells by acting as a competitive antagonist of IKK γ and could potentially be used as an adjuvant to increase the chemo-sensitivity of PDAC to gemcitabine in clinical therapy. More preclinical research and clinical trials are needed to explore the efficacy and safety of this use of NBDP.

MATERIALS AND METHODS

Cell lines and reagents

In a previous study, we built the HPNE/Kras^{G12V}/P16sh cell line for studying Kras-induced NF- κ B activation function. The human PDAC cell line MiaPaca2 was purchased from the American Type Culture Collection. The Colo357 cell line was obtained from the laboratory of Dr. Isaiah J. Fidler (M. D. Anderson Cancer Center. All cell lines were cultured in the medium of Dulbecco Modified Eagle Medium (Caisson Laboratories, Inc.) that contained L-glutamine and 15 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and were supplemented with 10% heat-inactivated fetal bovine serum and penicillin (100 IU/mL) and streptomycin (100 μ g/mL) in an atmosphere of 5% carbon dioxide at 37°C. NBDP was manufactured by Peptide 2.0, Inc., and its sequence was designed according to a previous study [16]. The molecular weight of NBDP was 3693.3 g/mol, and its purity (per high-performance liquid chromatography) was 95%. Gemcitabine hydrochloride was purchased from Sigma-Aldrich. TNF- α factor was purchased from R&D Systems, Inc.

Cell proliferation assays

For the MTT assay, 3000 PDAC cells per well were seeded in 96-well plates. After 12 h, attached cells were treated with various doses of NBDP or gemcitabine. The cells were treated for 1, 2, 3, 4, and 5 days. MTT solution at 5 mg/mL was used to crystallize the cells

for 4 h, and 100 μ L of DMSO was added to solute crystalloid cells, which were tested at 490 nm by an immunosorbent instrument. For the colony formation assay, 500 PDAC cells cultured in each well of six-well dishes and then were treated with various doses of NBDP and/or gemcitabine for 2 weeks. Then, the colonies were fixed with formalin (Sigma-Aldrich) within 30 min and stained with crystal violet (Sigma-Aldrich) within 1 h. For the wound-healing assay, PDAC cells were cultured to be confluent in six-well plates. The wound was scratched horizontally by a 1-mL pipette tip on monolayers of PDAC cells and then treated with different doses of NBDP and gemcitabine for 12 h. The healing area was observed and photographed by a phase-contrast inverted microscope in each well.

Cell apoptosis assays

For the flow cytometry assay for apoptosis, cells were washed twice with cold phosphate-buffered saline (PBS) and then resuspended in $1 \times$ binding buffer at a dose of 1×10^6 cells/mL. Then, the cells were stained by 5 μ L of APC Annexin V (BD Pharmingen, Inc.) and 5 μ L of propidium iodide staining solution (BD Pharmingen, Inc.) with 100 μ L solution for 15 min incubation at 25°C in the dark. After the incubation, 400 μ L of $1 \times$ binding buffer was added; the tubes were analyzed by flow cytometry within 1 h. For the TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay, slices of pancreatic tumor tissue were stained by the Situ cell death detection kit and fluorescein (Roche Diagnostics) to evaluate apoptosis induction. The number of TUNEL-positive cells was counted and photographed.

Western blot assay

The cell lysates from HPNE/Kras^{G12V}/P16sh, MiaPaca2, and Colo357 cell lines were lysed in immunoprecipitation assay protein lysis buffer. A total of 30 μ g of protein extract was loaded and run on the gel and then transferred to nylon membranes (Immobilon-P; Millipore) to detect phosphorylated NF- κ B, NF- κ B, Lamin A/C, phosphorylated IKK β , cleaved caspase 3, PARP, and cyclin D1 (Cell Signaling Technology) and IKK β , IKK γ , phosphorylated-I κ B α , I κ B α , phosphorylated ERK, ERK, and caspase 3 (Santa Cruz Biotechnology). Gel-Pro Analyzer 4.0 software (Media Cybernetics) was used to determine the integrated optical density values of the Western blots, and Graph Pad Prism 5.0 software was used to tabulate the values.

Electrophoretic mobility shift assay

The wild-type double-stranded oligonucleotides containing the NF- κ B site were purchased from Santa Cruz Biotechnology and labeled with ³²P to be used as probes. The method of Andrews and Faller was used to collect nuclear extract from HPNE/Kras^{G12V}/P16sh cancer cells. A total of 10 μ g of nuclear extract was loaded on 4% polyacrylamide gels containing $0.25 \times$ Tris/Borate/EDTA (ethylenediaminetetraacetic acid) buffer to perform DNA-binding assays for NF- κ B protein. OCT1 is one member of the OCT transcription factor family, and it is detected in a wide range of mammalian cells. The POU domain includes the POU - box and Homeo domain. We chose OCT1 homologous polynucleotide primer as an internal reference.

Immunoprecipitation Assay

Cell lysates were prepared with lysis buffer and added to 20 μ L of protein G Sepharose beads on a rotating wheel at 4°C for 1 hour. After reaction, spin the tube and transfer supernatants. Sepharose beads for each sample were incubated with IKK β antibody (Santa Cruz Biotechnology) together with 20 of protein G and rotated at 4°C for overnight. The beads were then washed with TT buffer (Tris 20mM, NaCl150mM, EDTA 1mM, NP40 0.5mM, and Triton X-100) six times following by 25 μ L 2 $\times$ sample buffer addition and boiled at 100°C for 4 min before loading sample to sodium dodecyl sulfate polyacrylamide gel electrophoresis. Western blotting was performed with IKK γ antibodies (Santa Cruz Biotechnology).

Histologic and immunohistochemical assay

The PDAC tumors were separated from the pancreatic tissues of the orthotopic xenograft model and fixed in formalin for 1 day. Paraffin-embedded tissue sections were subjected to immunostaining using the streptavidin-peroxidase technique with diaminobenzidine as a chromogen. Hematoxylin and eosin and immunohistochemical analyses were conducted according to standard procedures. Sections were incubated at 4°C overnight with the primary mouse anti-human monoclonal antibody anti-Ki67 (1:200 dilution), the primary mouse anti-human monoclonal antibody of anti-phosphorylated ERK (1:300 dilution; Santa Cruz Biotechnology), and anti-cyclin D1 and anti-NF- κ B phosphorylated P65 (1:300 dilution; Cell Signaling Technology). Slides were washed in Tris-buffered saline buffer and incubated for 30 minutes with the appropriate horseradish peroxidase-conjugated secondary antibody before being counterstained with Mayer's hematoxylin (Peroxidase Detection System; Leica Microsystems, Inc.). The expression levels of Ki67, cyclin D1, phosphorylated ERK, and activated P65 were detected as nuclear brown staining of varying intensities in neoplastic cells. The slides were evaluated independently using light microscopy (Olympus) and analysis by ImageJ software (National Institutes of Health).

PDAC orthotopic xenograft model

Twenty female athymic nude mice (NCI-nu), which were 4–6 weeks old and weighed approximately 24.9–33.0 g, were purchased from the Animal Production Area of the National Cancer Institute Frederick Cancer Research Facility. All mice were housed and treated in accordance with the guidelines of The University of Texas MD Anderson Cancer Center's Animal Care and Use Committee and were maintained in specific pathogen-free conditions. The facilities were approved by the Association for Assessment and Accreditation of Laboratory Animal Care; they meet all current regulations and standards of the U.S. Departments of Agriculture and Health and Human Services and the National Institutes of Health.

HPNE/Kras^{G12V}/P16sh cells were harvested from cell culture dishes in PBS with 20% Matrigel (Fisher Scientific) for the orthotopic xenograft nude mouse model. In each mouse, 1.0×10^6 cells per 50 μ L tumor cells were injected subcapsularly into the pancreatic tissue by 1-mL syringes and 30-gauge needles (Hamilton Company). The abdominal incisions were closed by wound clips (Braintree Scientific, Inc.), which were removed 10 days later, after the incisions had healed. For the in vivo studies, 20 nude mice were orthotopically injected

with HPNE/Kras^{G12V}/P16sh cells and randomly assigned into four groups (n=5 per group). Fourteen days after tumor cell injection, the mice were subjected to drug treatments via peritoneal injection. The therapeutic regimen of gemcitabine was 25 mg/kg twice per week for a total of 4 weeks. NBDP was used every other day for the 4-week period at a dose of 2 mg/kg, in accordance with the phase I clinical trial with NBDP [21]. All mice were weighed weekly and observed for tumor growth during the 4-week treatment.

Statistical analysis

We used the SPSS 19.0 software to conduct all statistical analyses. A one-tailed independent-sample t-test was used to determine the significance of differences between groups. The error bars in all experiments were determined by GraphPad Prism 5 software. All statistical tests considered $P < 0.05$ to be statistically significant.

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Highlights

- The NEMO-binding domain peptide (NBDP) of IKK was found to inhibit constitutive NF- κ B activation and promote apoptosis in cultured pancreatic cancer cells.
- The preclinical and therapeutic experimental evidence supports direct targeting of IKK activation in therapy pancreatic cancer.
- NBDP combined with gemcitabine significantly decreased levels of NF- κ B activity and inhibited the growth of PDAC in vivo in an orthotopic xenograft mouse model.
- Mechanistic investigations showed that NBDP effectively competed with NEMO/IKK γ for binding to IKKs and thus inhibited IKK and NF- κ B activation,

Figure 1: Kras-activated NF- κ B was inhibited by NBDP in PDAC cells

A. Kras-induced constitutive NF- κ B expression was measured by Western blot analysis in HPNE, HPNE/Vec, HPNE/Kras^{G12V}, HPNE/Kras^{G12V}/P16sh, MiaPaca2, and Colo357 cell lines; **B.** NF- κ B expression levels in HPNE/Kras^{G12V}, HPNE/Kras^{G12V}/P16sh, MiaPaca2, and Colo357 cells were significantly higher than in HPNE and HPNE/Vec cells; **C.** Western blot analysis shows phosphorylated NF- κ B expression in nuclear extract of HPNE/Kras^{G12V}/P16sh cells treated with 50, 100, or 200 μ M NBDP; **D.** electrophoretic mobility shift assay shows phosphorylated NF- κ B expression in HPNE/Kras^{G12V}/P16sh cells treated with 50, 100, or 200 μ M NBDP; **E.** Western blot analysis shows phosphorylated NF- κ B expression in total cell lysates of HPNE/Kras^{G12V}/P16sh cells treated with 50, 100, or 200 μ M NBDP; **F** and **G.** Western blot analysis shows phosphorylated NF- κ B expression in total cell lysates of MiaPaca2 and Colo357 cells treated with 50, 100, or 200 μ M NBDP. IOD: integrated optical density

Figure 2: NBDP inhibits gemcitabine-activated NF- κ B.

A. Immunoprecipitation analysis showed that TNF- α (10 ng/mL) factor stimulated and promoted IKK γ to combine with IKK β in HPNE/Kras^{G12V}/P16sh cells; NBDP (100 μ M) was competitive with IKK β , decreasing the IKK γ combination under TNF- α (10 ng/mL) factor stimulation; **B.** Western blot analysis showed the change of NF- κ B pathway in HPNE/Kras^{G12V}/P16sh cells treated with NBDP (100 μ M); **C.** Western blot analysis measured phosphorylated NF- κ B expression in HPNE/Kras^{G12V}/P16sh cells treated with gemcitabine (10 μ M) in 0 h, 1 h, 2 h, 4 h, 6 h, 12 h; **D.** phosphorylated NF- κ B expression was significantly increased in HPNE/Kras^{G12V} and HPNE/Kras^{G12V}/P16sh cells treated with gemcitabine at 2 h and 4 h (2 h vs 0 h, *P=0.0118; 4 h vs 0 h, **P=0.0029); **E.** Western blot analysis measured phosphorylated NF- κ B expression in HPNE/Kras^{G12V}/P16sh, MiaPaca2, and Colo357 cells treated with gemcitabine (10 μ M) combined with NBDP (100 μ M); **F.** NBDP decreased gemcitabine chemo-resistance due to increased phosphorylated NF- κ B

expressions in HPNE/Kras^{G12V}/P16sh (gemcitabine vs control, *P=0.0001; combination vs gemcitabine, **P<0.0001), MiaPaca2 (gemcitabine vs control, *P<0.0001; combination vs gemcitabine, **P=0.0002), and Colo357 (gemcitabine vs control, *P=0.0014; combination vs gemcitabine, **P=0.0005) cell lines. Gem: gemcitabine; IOD: integrated optical density.

Figure 3: The growth of NBDP expressing PDAC cells in response to gemcitabine.

A. MTT analysis showed that proliferation of HPNE/Kras^{G12V}/P16sh, MiaPaca2, and Colo357 cells was inhibited by NBDP at doses of 5, 10, 25, 50, 100, 200, and 400 μM within 5 days; **B.** MTT analysis showed that proliferation of HPNE/Kras^{G12V}/P16sh, MiaPaca2, and Colo357 cells was inhibited by gemcitabine at doses of 250 and 500 nM and 1, 5, 10, and 20 μM within 5 days; **C.** IC₅₀ values of NBDP were detected by MTT analysis in HPNE/Kras^{G12V}/P16sh, MiaPaca2, and Colo357 cells; **D.** MTT analysis showed that NBDP combined with gemcitabine inhibited proliferation in HPNE/Kras^{G12V}/P16sh, MiaPaca2, and Colo357 cells. Ctrl: control; Gem: gemcitabine; IOD: integrated optical density.

Figure 4: Inhibition of NF- κ B activity by NBDP reduced colony formation and cell migration in PDAC cells treated with gemcitabine.

A. Colony formation analysis showed that tumor cell proliferation was inhibited by NBDP at doses of 50, 100, and 200 μ M in HPNE/Kras^{G12V}/P16sh (50 μ M NBDP vs control, * P =0.0004), MiaPaca2 (50 μ M NBDP vs control, * P =0.0085), and Colo357 (50 μ M NBDP vs control, * P =0.0218) cells; **B.** Colony formation analysis showed that NBDP treatment combined with gemcitabine inhibited proliferation of HPNE/Kras^{G12V}/P16sh (NBDP vs control, * P =0.0123; combination vs gemcitabine, ** P =0.0165), MiaPaca2 (NBDP vs control, * P =0.0114; combination vs gemcitabine, ** P =0.0008), and Colo357 (NBDP vs control, * P =0.0031; combination vs gemcitabine, ** P =0.0026) cells; **C.** Wound-healing analysis showed that NBDP decreased invasion of HPNE/Kras^{G12V}/P16sh, MiaPaca2, and Colo357 cells at doses of 50, 100, and 200 μ M. Ctrl: control; Gem: gemcitabine

Figure 5: Inhibition of NF- κ B pathway by NBDP increased apoptosis in PDAC cells treated with gemcitabine:

A. Flow cytometry analysis of the Annexin V staining assay showed the apoptosis of HPNE/Kras^{G12V}/P16sh cells treated with NBDP at 50, 100, and 200 μ M; **B.** the ratio of apoptosis increased with increasing NBDP doses in HPNE/Kras^{G12V}/P16sh cells (50 μ M NBDP vs control, *P=0.0004); **C.** Flow cytometry analysis showed that 100 μ M NBDP combined with 5 μ M gemcitabine increased apoptosis in HPNE/Kras^{G12V}/P16sh, MiaPaca2, and Colo357 cells; **D.** The ratio of apoptosis increased with NBDP combined with gemcitabine in HPNE/Kras^{G12V}/P16sh (NBDP vs control, *P=0.0870; combination vs gemcitabine, **P=0.0061), MiaPaca2 (NBDP vs control, *P=0.0055; combination vs gemcitabine, **P=0.0407), and Colo357 (NBDP vs control, *P=0.0659; combination vs gemcitabine, **P=0.0522) cells. Ctrl: control; Gem: gemcitabine

Figure 6: Cleavage of Parp and caspase 3 in PDAC cells treated with NBDP and gemcitabine.

A Western blot analysis showed the expression of Parp in HPNE/Kras^{G12V}/P16sh cells treated with NBDP (50, 100, and 200 μM); **B**. The expression of Parp increased after NBDP treatment in HPNE/Kras^{G12V}/P16sh cells (50 μM NBDP vs control, *P=0.0007); **C**. Western blot analysis showed the expression of cleaved-Parp and cleaved-caspase 3 in HPNE/Kras^{G12V}/P16sh cells treated with NBDP (100 μM) combined with gemcitabine (10 μM); **D–E**. cleaved-Parp expression and cleaved-caspase 3 expression were both increased under the combination treatment with NBDP (100 μM) and gemcitabine (10 μM) in HPNE/Kras^{G12V}/P16sh cells (C-Parp: combination vs gemcitabine, **P=0.0235; C-caspase 3: combination vs gemcitabine, **P=0.0075); **F–G**. MiaPaca2 and Colo357 cells were measured for expression of cleaved-Parp and cleaved-caspase 3 by Western blot analysis after the combination treatment with NBDP (100 μM) and gemcitabine (10 μM); **H–K**. cleaved-Parp expression and cleaved caspase 3 expression were both increased under the

combination treatment with NBDP (100 μ M) and gemcitabine (10 μ M) in MiaPaca2 (C-Parp: combination vs gemcitabine, **P=0.0017; C-caspase 3: combination vs gemcitabine, **P=0.1033) and Colo357 (C-Parp: combination vs gemcitabine, **P=0.0585; C-caspase 3: gemcitabine vs gemcitabine, **P=0.0032) cells. Gem: gemcitabine; C: cleaved

Figure 7: NBDP and gemcitabine inhibited tumorigenesis of PDAC cells in an orthotopic xenograft model *in vivo*.

A. Twenty athymic mice bearing orthotopic HPNE/Kras^{G12V}/P16sh tumors (n=5 per group) were euthanized by carbon dioxide inhalation after being treated for 4 weeks. The therapeutic regimen of gemcitabine was 25 mg/kg twice per week for a total of 4 weeks. NBDP was used every other day for the 4-week period at a dose of 2 mg/kg. The tumors were excised from the abdominal cavity; **B.** The tumors were separated from pancreatic tissue and measured at the maximal diameter in each group (gemcitabine vs control, *P=0.368; combination vs control, **P=0.001); **C.** Tumor weights analyzed by an independent-sample t-test showed the inhibition of HPNE/Kras^{G12V}/P16sh tumor growth after treatment with NBDP(5 mg/Kg) combined with gemcitabine (25 mg/Kg) (combination vs gemcitabine, *P=0.0367); **D.** Representative micrographs show the histopathologic features of HPNE/Kras^{G12V}/P16sh tumor (a, b), margin (c), and normal pancreatic tissue (d)

by hematoxylin and eosin staining; **E**. There was no significant weight loss in 20 athymic mice; (**F**), there was a significant body weight increase in the combination treatment group compared with the control group (* $P=0.0437$); **G-H**. TUNEL apoptosis analysis was performed by staining HPNE/Kras^{G12V}/P16sh tumors; there were more apoptotic cells in tumor tissue treated with NBDP combined with gemcitabine compared with gemcitabine alone (* $P=0.0122$). Ctrl: control; Gem: gemcitabine

Figure 8: A. Inhibition of NF-κB downstream target genes by NBDP and gemcitabine. Immunohistochemical analysis of HPNE/Kras^{G12V}/P16sh tumors and normal pancreatic tissues stained with antibodies to Ki67, cyclin D1, phosphorylated ERK, and the active P65 subunit of NF-κB; **B, C.** Levels of Ki67 and cyclin D1 expression on immunohistochemical analysis were decreased in tumor tissue treated with NBDP combined with gemcitabine compared with the control, NBDP, and gemcitabine (Ki67: combination vs gemcitabine, *P=0.0345; cyclin D1: combination vs gemcitabine, *P=0.0118); **D, E.** Levels of phosphorylated Erk and phosphorylated P65 expression in tumor tissue treated with gemcitabine on immunohistochemical analysis were increased compared with the control

but were decreased in tumor tissue treated with NBDP combined with gemcitabine compared with the control and gemcitabine alone (P-Erk: gemcitabine vs control, *P=0.0386, combination vs gemcitabine, **P=0.0172; P-P65: gemcitabine vs control, *P=0.0263, combination vs gemcitabine, **P=0.0080); **F.** Western blot analysis showed the expression of phosphorylated P65 and P65 in a total cell lysate of HPNE/Kras^{G12V}/P16sh tumor tissue in each group; **G.** Phosphorylated P65 expression of HPNE/Kras^{G12V}/P16sh tumor tissue treated with NBDP combined with gemcitabine was significantly down-regulated compared with that in the control and gemcitabine groups (gemcitabine vs control, *P=0.0479, combination vs gemcitabine, **P=0.0043). Ctrl: control; Gem: gemcitabine.

Figure 9. Target IKK γ to inhibit IKK/I κ B/NF- κ B pathway in PDAC.

The NEMO-binding domain peptide (NBDP) of IKK was found to inhibit NF- κ B activation in cancer cells. Mechanistic investigations showed that NBDP effectively competed with IKK γ for binding to IKKs and thus inhibited IKK/I κ B/NF- κ B pathway activation in PDAC.