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## Targeting the alternative NF- $\kappa$ B pathway in pancreatic cancer: a new direction for therapy?

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### Abstract

Pancreatic cancer, due to its late diagnosis, is difficult to treat. In addition, current therapy options are insufficient and new approaches for combination treatment are required. Recent demonstration of the importance of constitutive signaling of NF- $\kappa$ B-inducing kinase (NIK, also named MAP3K14) in maintaining the high basal activity of the alternative NF- $\kappa$ B pathway in pancreatic cancer suggests novel possibilities for therapeutic intervention. Strategies to target the alternative NF- $\kappa$ B pathway include not only the use of small molecule inhibitors for NIK and I $\kappa$ B kinase (IKK), but also broad spectrum approaches such as using proteasome inhibitors or combinatorial approaches targeting both alternative and canonical pathways. These may also act synergistically with currently used drugs.

### Keywords

alternative; MAP3K14; NF- $\kappa$ B; NIK; noncanonical; pancreatic cancer; therapy; TRAF2

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“Pancreatic cancer, due to its late diagnosis, is difficult to treat.”

### Introduction

In many cancers, NF- $\kappa$ B transcription factor complexes in malignant cells are activated via the canonical and alternative pathways. The alternative NF- $\kappa$ B pathway is induced by viral proteins and receptors of the TNF family, including B-cell activating factor belonging to TNF family receptor (BAFF-R), CD40, TNF-like weak inducer of apoptosis (TWEAK), receptor activator for NF- $\kappa$ B (RANK), TNF-R2, and lymphotoxin- $\alpha$  receptor LT- $\alpha$ -R (for a recent review on the alternative pathway) [1]. Activation of this pathway requires stabilization and activation of NIK, a serine/threonine kinase that in absence of a stimulus undergoes continuous proteasomal degradation. K48-linked polyubiquitination of NIK is induced by its association with TNF receptor-associated factors (TRAFs) TRAF3 and TRAF2, which recruit the ubiquitin ligases cellular inhibitor of apoptosis proteins (cIAPs) cIAP1 and cIAP2. In response to receptor engagement TRAFs are sequestered and, instead of NIK, TRAF3 and cIAPs, are targeted to proteasomal degradation. This results in stabilization of newly synthesized NIK and increased protein levels [2]. NIK is a basally active kinase and once stabilized phosphorylates IKK $\alpha$  at S176 in the activation loop of the kinase domain, inducing its activation [3]. Association of NIK with active IKK $\alpha$  mediates

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NF- $\kappa$ B/p100 phosphorylation, which leads to its processing to p52 and formation of the p52/RelB NF- $\kappa$ B heterodimer.

## The alternative NF- $\kappa$ B signaling pathway in pancreatic cancer

In pancreatic cancer, increased activities of the NF- $\kappa$ B pathways have been implicated in mediating proliferation, antiapoptotic signaling, metastasis and chemoresistance [4,5]. Of these, proliferation and oncogenic growth was linked to increased levels of active NIK, leading to increased activities of IKK and p52/RelB and subsequent G1- to S-phase progression [4,6–8]. Pancreatic ductal adenocarcinoma (PDAC) cell lines with increased NIK expression also displayed dramatically elevated levels of the NF- $\kappa$ B2 target genes *CCL19*, *CCL21*, *CXCL12*, *CXCL13* and *BAFF* [4]. This suggested the NIK/NF- $\kappa$ B2 pathway as a cause for rapid and aggressive growth of this cancer.

Unlike for multiple myeloma, where it is known in approximately 20% of all cases stabilization of NIK is achieved by intrinsic loss-of-function mutations for TRAF2, TRAF3 and cIAP1/2 [9], so far no genetic alterations for these molecules were described for pancreatic cancer. Recently, a permanent downregulation of TRAF2, probably due to an autocrine stimulus, has been shown to be a mechanism of how NIK stabilization is achieved [7]. Other proteins of the NIK regulatory complex, such as cIAP1/2 or TRAF3, were not significantly changed in their expression compared with normal human pancreatic ductal epithelial cells. This may be of benefit for the tumor cells since, besides their role in promoting NIK degradation, cIAPs are important regulators of tumor cell survival [10]. Therefore, loss of TRAF2 in pancreatic cancer may not only block the formation of the NIK ubiquitination complex but also prevent stimulus-dependent degradation of cIAP1/2. Utilizing this mechanism PDAC cells may gain both high capacity to survive (via cIAPs) as well as high proliferation rates (via NIK).

“NF- $\kappa$ B-inducing kinase may be an ideal target for clinical intervention.”

Due to the potential central role of the TRAF2-NIK-NF- $\kappa$ B2 signaling pathway in mediating pancreatic cancer cell proliferation, targeting key components of this pathway may be an effective approach for clinical intervention. Potential strategies are discussed below.

### Strategy I: specific inhibition of NIK

Since NIK is a kinase with a key role in the noncanonical NF- $\kappa$ B activation pathway in pancreatic cancer [6,7], inhibition of NIK would be the first choice. However, so far relatively few inhibitors have been described, and for most of them thorough analysis of their specificity is lacking or they are only effective *in vitro*. For example, pyrazoloisoquinoline derivatives such as pyrazolo[4,3-c]isoquinoline were patented for inhibition of NIK [101,102] but later were shown to inhibit the canonical NF- $\kappa$ B pathway and TGF- $\beta$ -activated kinase (TAK1). Others such as staurosporine that target the catalytic site of NIK to block ATP binding are actually pan kinase inhibitors. Another broad spectrum inhibitor, curcumin, a constituent of turmeric with anti-inflammatory activity has been shown to inhibit the NIK/IKK complex. In 2010, Mortier *et al.* identified two NIK inhibitors by performing a virtual screening. This resulted in the identification of 4H-isoquinoline-1,3-dione and 2,7-naphthydrine-1,3,6,8-tetrone [11]. However, *in vitro* kinase assays showed that their IC<sub>50</sub> to inhibit NIK kinase activity were at 51 and 90  $\mu$ M, respectively. Moreover, the kinase spectrum targeted by these molecules was not determined, and 4H-isoquinoline-1,3-dione, for example, also inhibits other kinases such as IGFR and CDK4. Recently, two groups succeeded in crystallizing and modeling of the kinase domains of human and murine NIK [12,13]. These studies now provide a foundation towards more specific inhibition of NIK, and will allow the discovery of NIK inhibitors *in*

*vitro* and *in silico* and further refinement of their structure. Additionally, the NIK kinase domain was shown to bind to and be inhibited by a 6-alkylindoline compound [103] that suppresses NIK activity with a  $K_i$  of 4 nM. Other alkynyl alcohols such as a 2-(aminothiazolyl) phenol containing compound and a des-cyano compound inhibited NIK with a  $K_i$  of 4.4 and 3.7 nM, respectively. Of note, all approaches to target NIK so far have been made by identifying compounds that target the active center in the kinase domain. However, other approaches are possible, such as targeting its interaction with binding partners. Such strategies may include targeting the TRAF or IKK binding motifs of NIK or targeting inhibitor of  $\beta$  kinase complex-associated protein (IKAP), which assembles IKKs and NIK to an active kinase complex. Additionally, heat shock protein 90 (Hsp90), which has antipancreatic tumor effects, has been shown to interact with NIK and protect it from degradation [14]. This interaction can be disrupted with geldanamycin, making this drug another potential candidate for combination therapy [15]. Another strategy would be the expression of the TRAFs and NIK-associated protein (TNAP), which has been described to inhibit NIK regulated NF- $\beta$  activation [16]. Additionally, nuclear localization of NIK may be blocked by targeting its nuclear localization signal.

### Strategy II: to increase TRAF2 stability

NIK expression and activity in most PDAC cell lines is maintained by proteasomal downregulation of TRAF2 [7]. Moreover, in 72% of grade 2 and 83% of grade 3 human tumors, a reverse correlation between NIK activity and TRAF2 expression was observed [7]. This suggests that TRAF2 levels are decreased with increased aggressiveness of tumors allowing expression of NIK. Therefore, preventing the downregulation of TRAF2 or mediating its re-expression or increasing its stability could be an alternative to targeting NIK in advanced stages of this cancer. Since TRAF2 in PDAC cell lines is downregulated through ubiquitination, the first choice would be to target proteasomal degradation processes. Reagents that may be used include bortezomib (Velcade), carfilzomib, oprozomib (ONX 0912), or MLN9708, which are already clinically used for multiple myeloma. All of these need to be further developed for pancreatic cancer. In PDAC cell lines, bortezomib, for example, has been shown to inhibit the proliferation of pancreatic cancer cells and to induce apoptosis [17]. In addition, results from a current Phase 1 clinical trial with oprozomib studying advanced refractory or recurrent solid tumors may be seminal for the use of proteasome inhibitors in pancreatic cancer. However, potential problems with proteasome inhibitors may be their off-target effects. Therefore, more specific approaches to upregulate TRAF2 or protect it from its degradation may be applied. Strategies here may include blocking the ubiquitination of TRAF2, that is, by direct inhibition of ubiquitin ligases that target TRAF2 or re-expression of TRAF2 or a dominant-active mutant that cannot be ubiquitinated. To develop such strategies for clinical use may be challenging since TRAF2 can also activate the canonical NF- $\beta$  pathway through TAK1/TAB. Also, although in most PDAC patient samples a reverse correlation between TRAF2 and NIK expression was detected, approximately 31% of samples showed high TRAF2 and NIK expression levels. In these cases the regulation of NIK may be through a different mechanism and strategies may be applied to target TRAF3 (i.e., 90% of multiple myeloma patients with decreased TRAF3 levels respond to bortezomib) [18].

### Strategy III: to block other components of the alternative NF- $\kappa$ B pathway

The list of 'NF- $\beta$ ' inhibitors is long, including the above mentioned proteasome inhibitors, as well as anti-inflammatory drugs or plant-extracted or dietary natural compounds (for overview see [19]). Natural compounds such as flavonoids and curcumin often inhibit NF- $\beta$  due to their function as antioxidants. Anti-inflammatory drugs such as aspirin, ibuprofen and sulfasalazine inhibit IKK by competing with ATP binding and thus also target the

alternative NF- $\kappa$ B pathway. Of these, aspirin has a potential preventive function for pancreatic cancer since it not only inhibits NF- $\kappa$ B but also cyclooxygenase-2, which is overexpressed in pancreatic intraepithelial neoplastic lesions [20].

“New approaches for combination treatment are required.”

The problem with all the above inhibitors is that they are not very specific and show a wide range of biological activities. A more specific approach would be the use of inhibitors for the kinases of the IKK complex, specifically for IKK $\alpha$ . IKK inhibitors include CHS-8282, MLN120B and PS-1145, but most IKK inhibitors developed so far mainly target IKK $\alpha$  and the canonical pathway, and few have entered Phase I clinical trials. To date there have been no reports of IKK $\beta$  selective compounds. Of interest for pancreatic cancer may be PS-1145, which has shown antiproliferative potential in multiple myeloma cells. Eventually, signaling through the alternative NF- $\kappa$ B pathway could also be inhibited more specifically by targeting the nuclear import of RelB or p52 with cell permeable peptides such as SN52 [21] or by inhibition of its DNA binding activity using decoy oligonucleotides (discussed in [22]).

## Expert commentary

So far, the alternative NF- $\kappa$ B pathway has mainly been linked to multiple myeloma, in which genetic mutations of TRAF2, TRAF3 and cIAPs as well as other components of this pathway were documented. In pancreatic cancer it becomes evident that both the canonical and the alternative pathways are important mediators of progression. While the canonical pathway mainly regulates antiapoptotic signaling and chemoresistance, the alternative pathway was linked to increased cell proliferation in this cancer. So far, data from cell lines and analysis of patient samples indicates that upregulation of NIK correlates with decreased TRAF2 expression, resulting in activation of the alternative pathway. Unlike for multiple myeloma, no genetic mutations have been described for key proteins in this pathway in pancreatic cancer. Moreover, NIK was shown not only to be a key regulator of the alternative NF- $\kappa$ B pathway, but when overexpressed also contributes to activation of the canonical pathway [23]. Therefore, NIK may be an ideal target for clinical intervention. Goals should be blocking the alternative NF- $\kappa$ B pathway at multiple levels or inhibiting both alternative and canonical pathways (discussed in [24]). For this the most effective strategy may be combination therapy using NIK inhibitors in combination with pan IKK inhibitors or proteasome inhibitors such as bortezomib. These combination therapies then may be combined with apoptosis-inducing drugs such as gemcitabine or 5-FU.

## Five-year view

Given the importance of NIK not only in the alternative, but when highly expressed, also canonical NF- $\kappa$ B signaling pathways, we predict that it is an ideal drug target for this cancer. So far, development of NIK inhibitors was marginal, due to lack of stability of recombinant protein as well as the lack of a structural model for the kinase domain. Both recently have been solved [12,13], opening the field of drug discovery for this kinase. We also predict that in addition to developing NIK as a drug target in pancreatic cancer, analysis of patient samples for mutations in the genes involved in the alternative pathway will lead to a better understanding of the signaling mechanisms involved in this cancer. Moreover, the analysis of patient biopsies for specific markers of both NF- $\kappa$ B pathways will allow tailored treatment of patients by targeted combination therapies.

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