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The pseudokinases, MLKL, and IRAK3 are promising drug targets.

What is a pseudokinase?

Pseudokinases have the same overall kinase domain fold as catalytically active kinases, but lack one or more of the key conserved catalytic residues, therefore most of them are incapable of catalyzing phosphotransfer. The key conserved catalytic residues in the active site of a kinase and their significance are as follows[1)2)3)4)]:

• **The three glycine residues within GxGxxG motif (Glycine-rich loop)** :
The small side-chain of a single hydrogen in these glycine residues allows for close contact of the glycine-rich loop with the adenosine ring of ATP, which enables ATP binding and proper positioning of ATP for catalysis.

• **The lysine residue within VAIK motif (β3 strand)** :
This lysine residue forms a salt bridge between a conserved glutamate residue in helix αC to correctly position the β3 lysine to coordinate the α- and β- phosphates of ATP.

• **The aspartate residue within HRD motif (Catalytic loop)** :
This aspartate residue's negatively charged side-chain serves as a catalytic base, abstracting a proton from the substrate's hydroxyl group, promoting nucleophilic attack of the ATP γ-phosphate.

• **The aspartate residue within DFG motif (Activation loop)** :
This aspartate residue is involved in binding key divalent cations (Mg^{2+} or Mn^{2+}) that stabilize the bound ATP.

On the basis of the changes in their nucleotide and cation binding properties, pseudokinases are categorized into four classes:

(Class 1) devoid of detectable nucleotide or cation binding.
(Class 2) able to bind nucleotide but not cation.
(Class 3) able to bind cation but not nucleotide.
(Class 4) able to bind nucleotide and cation.

Among the pseudokinases classified as Class 2 and Class 4, which are capable of binding nucleotides, several have been reported to interact with ATP-competitive small-molecule compounds[2)3)].

Within the human kinome, approximately 50 protein kinases, accounting for roughly 10% of the total, have been classified as pseudokinases. Pseudokinases regulate biological processes through diverse mechanisms of non-catalytic kinase activity: as allosteric modulators; protein-based switches that regulate signal transduction by exploiting conformational changes in proteins; scaffolds for complex assembly; and as competitive inhibitors in signaling pathways (Fig.1)[5)]. Mutations in pseudokinases and dysregulation of their expression have been linked to several developmental and morphological disorders, as well as a wide range of diseases, such as cancer, neurological disorders, metabolic disorders and autoimmune diseases[3)]. Recent advancements in targeted protein degradation technologies have made drug development targeting pseudokinases - previously considered difficult to regulate with conventional therapeutics – a more realistic and promising approach.

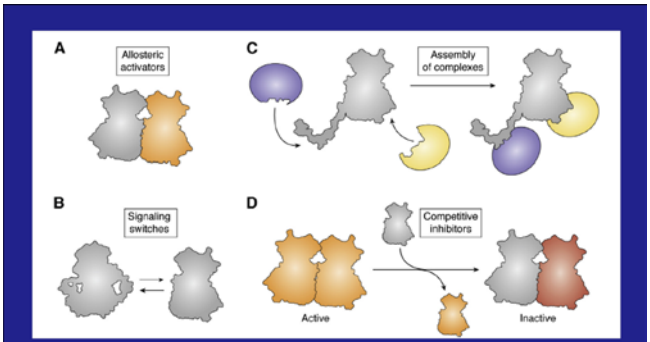

Fig.1. Modes of noncatalytic kinase activities[5)].
Cartoon depiction of the four modes of noncatalytic kinase function: (A) allosteric activators, (B) signaling switches, (C) assembly of complexes, and (D) competitive inhibitors. In each case, kinase domains that play noncatalytic roles are depicted in gray.

MLKL is a key mediator in initiating necroptosis.

Mixed lineage domain-like protein (MLKL) is a pseudokinase classified as Class 2 and plays a role as the terminal effector of necroptosis. Necroptosis is a proinflammatory, regulated form of cell death characterized by rupture of the cell membrane and release of Damage Associated Molecular Patterns (DAMPs). Necroptosis pathway has garnered significant interest as a potential therapeutic target for a broad range of pathologies including oncogenic, neuro-degenerative, hepatic, autoimmune, and inflammatory conditions[6)].

Prior to stimulation, RIPK3 is associated with MLKL and maintains it in an inactive conformation. Upon necroptotic stimulation including cytokines such as TNF-α, double-stranded RNA and lipopolysaccharides, and IFN-γ signaling, RIPK3 phosphorylates MLKL to induce a conformational change, which disengages MLKL from RIPK3. Once released, MLKL oligomerizes and integrates into the cell membrane where it forms pores that cause cell

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lysis and the release of DAMPs. The absence of MLKL has been shown to be protective in models of autoimmune hepatitis and psoriasis, acute pancreatitis, nonalcoholic fatty liver disease, and acute and chronic kidney disease[6].

MLKL consists of two domains, the N-terminal 4-Helix Bundle (4HB) executioner domain and the C-terminal pseudokinase domain. To date, small-molecule compounds have been reported to bind to MLKL at three distinct sites: Cys-86 on the 4HB domain, another site at the N-terminus of the 4HB domain, and the ATP binding pocket in the pseudokinase domain[6][7][8]. PROTACs that bind to Cys-86 on the 4HB domain and to the ATP binding pocket in the pseudokinase domain of MLKL have been developed. These compounds have been shown to degrade intracellular MLKL and protect cells against necroptosis[6][7].

IRAK3 is a promising drug target with synergistic effects with immune checkpoint blockade therapy.

Interleukin-1 receptor associated kinase 3 (IRAK3) is a Class 1 pseudokinase which negatively regulates IL-1/TLR innate immune signaling. In the RNA-seq analysis of the IMvigor210 clinical trial, in which patients with locally advanced or metastatic urothelial carcinoma treated with atezolizumab (an anti-PD-L1 antibody) participated, it has been shown that patients with low IRAK3 mRNA expression levels, at pretherapy, exhibit prolonged survival compared to those with high expression levels. Additionally, IRAK3-high tumors coincided with enriched TGFβ signaling. The TGFβ pathway has been identified to be a resistant mechanism to immune checkpoint blockade (ICB) therapy, providing an insight that IRAK3 serves as a key regulator for response to ICB therapy[9].

In syngeneic murine tumor model, IRAK3 deficiency delayed tumor growth. Importantly, IRAK3 deficient mice showed enhanced response to PD-1 blockade in an ICB-refractory MYCN-driven neuroblastoma tumor model. In addition, a concurrent increase of the TCF1$^+$PD-1$^+$stem-like CD8$^+$T (T_{SL}) cells and highly activated myeloid cells in the IRAK3-KO mice treated with the PD-1 blockade was identified[9]. T_{SL} cells are a subset of immune cells with superior persistence and antitumor immunity. They are important for the expansion of tumor specific CD8$^+$T cells in response to ICB therapy[10]. Therefore, it is possible that IRAK3 deficiency caused-activation status of the innate immune cell types sustains the function of CD8$^+$T cell subsets. These findings highlight IRAK3 as a promising therapeutic target with potential synergistic effects in combination with ICB therapy.

To date, FIM-001 and PROTAC 23 have been identified as IRAK3-binding degraders, and both have demonstrated IRAK3 degradation in the THP-1 cell line. Additionally, FIM-001 exhibited anti-tumor efficacy upon subcutaneous administration in the LLC syngeneic mouse model, which is known to be poorly responsive to ICB therapy[11][12].

With the emergence of targeted protein degradation strategies, pseudokinases – once deemed undruggable – are now gaining recognition as promising drug targets, paving the way for future advancements in novel therapeutic development.

Pseudokinase-related products provided by Carna Biosciences

Carna has launched biotinylated pseudokinase protein products and plans to expand the product lineup in the future. The five pseudokinase proteins released this time are as follows:

- **MLKL**
- **IRAK3**
- **ERBB3 (HER3)**
- **TYK2 JH2 domain**
- **JAK2 JH2 domain**

For more information about each product, please click the following links.
https://www.carnabio.com/english/product/protein-pseudokinase.html

If you have any questions about our products, including pseudokinase protein products, or if you would like to discuss custom requests, please feel welcome to contact us at info@sb.carnabio.com.

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