

## Abstract

**Background:** NF- $\kappa$ B signaling plays a critical role in MCL as evidenced by the high response rate observed in refractory/relapsed MCL patients treated with BTK inhibitor ibrutinib. However, the majority of these patients relapse with a dismal prognosis. Thus, identifying additional targeted agents that affect the eventual activation of NF- $\kappa$ B mediated through the B-cell receptor (BCR) or other signaling pathways is needed to address primary or relapsed ibrutinib-resistance. As an essential component in the IL1R/toll-like receptor (TLR) mediated NF- $\kappa$ B signaling pathway, IRAK4 is one such target. For these studies, we tested the novel oral IRAK4 kinase inhibitor CA-4948, which is currently in a Phase I trial for R/R non-Hodgkin lymphoma (clinicaltrials.gov NCT03328078).

**Experimental procedures:** Six MCL cell lines (JeKo-1, MAVER-1, Mino, GRANTA-519, REC-1, and Z-138) were exposed to escalating doses of CA-4948 either alone or in combination with other targeted agents, and changes in viability were evaluated after 24-96 hr. These same six cell lines were also treated with CA-4948 to assess its effect on TLR- agonist-induced NF- $\kappa$ B signaling by evaluating cytokine production and western blot analysis of intracellular signaling pathway components. Finally, the *in vivo* efficacy of CA-4948 was evaluated in mouse xenograft subcutaneous tumor models of these six cell lines.

**Results:** Similar to previously observed results in CA-4948 treated DLBCL cell lines, blocking IRAK4 kinase function was neither cytostatic nor cytotoxic in these six MCL lines under standard *in vitro* growth conditions (EC50 > 10  $\mu$ M). In contrast, CA-4948 blocked TLR- agonist-induced pro-inflammatory cytokine production and TLR pathway activation markers (e.g. p-IKK) in MCL lines, including Mino and REC-1. Interestingly, GRANTA-519 and Z-138 cells exhibited constitutive production of a subset of cytokines in the absence of TLR stimulation, consistent with reports that these lines have deregulated alternative NF- $\kappa$ B signaling. *In vivo*, CA-4948 exhibited anti-tumor activity in the Mino and REC-1 xenograft models. Consistent with constitutive NF- $\kappa$ B activation independent of BCR and TLR signaling, CA-4948 demonstrated no activity against the GRANTA-519 and Z-138 xenograft tumor models.

**Conclusion:** Our findings reveal a requirement for IRAK4 kinase function in TLR- agonist-induced NF- $\kappa$ B signaling and cytokine production in MCL cell lines. Oral administration of CA-4948 demonstrated an essential *in vivo* role for IRAK4 function in certain MCL cells grown as xenograft tumors. These results provide the rationale for continued testing of CA-4948 in combination with canonical and alternative NF- $\kappa$ B pathway-targeted agents.

## In vitro CA-4948 Viability Assays in MCL Cell Lines

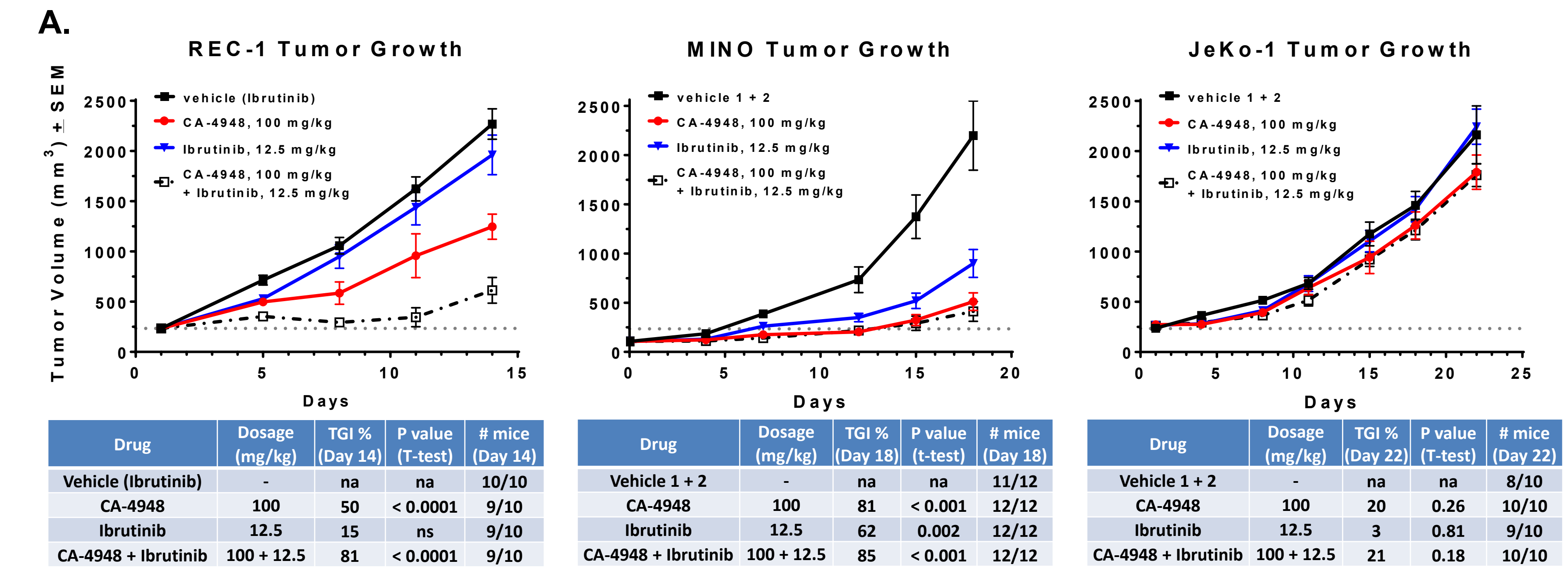
Cell Line	CA-4948 EC50 ( $\mu$ M)*		
	3 days	5 days	7 days
REC-1	>10	4.7	2.9
Mino	>10	>10	>10
JeKo-1	>10	>10	>10
GRANTA-519	>10	>10	>10
Z-138	>10	>10	>10
MAVER-1	>10	>10	>10

\*: average of 2 or more CellTiter Glo viability assays

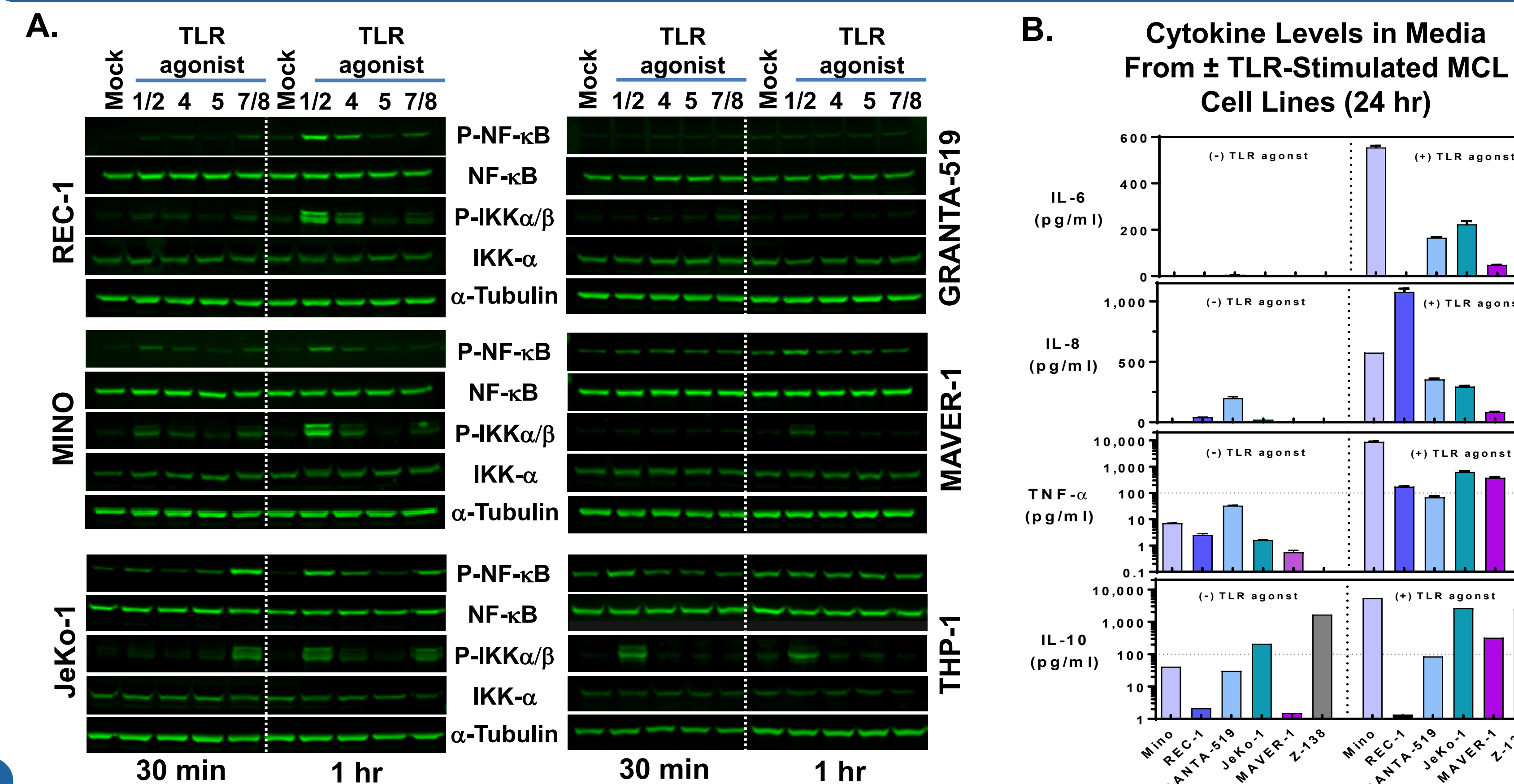
Cell Line	CA-4948 Combination*			
	Palbociclib (CDK4/6i)	Ibrutinib (BTKi)	Venetoclax (BCL2i)	Bortezomib (Proteasome)
REC-1	no synergy	mild synergy	mild synergy	mild synergy
Mino	no synergy	no synergy	no synergy	no synergy
JeKo-1	no synergy	no synergy	no synergy	mild synergy
GRANTA-519	no synergy	no synergy	no synergy	no synergy
Z-138	no synergy	no synergy	no synergy	mild synergy
MAVER-1	no synergy	no synergy	no synergy	no synergy

\*: summary results of CellTiter Glo viability assays after 24, 48, 72 and 96 hr treatments

## CA-4948 Exhibits Anti-Tumor Efficacy in Xenograft MCL Models with Canonical NF- $\kappa$ B Activation

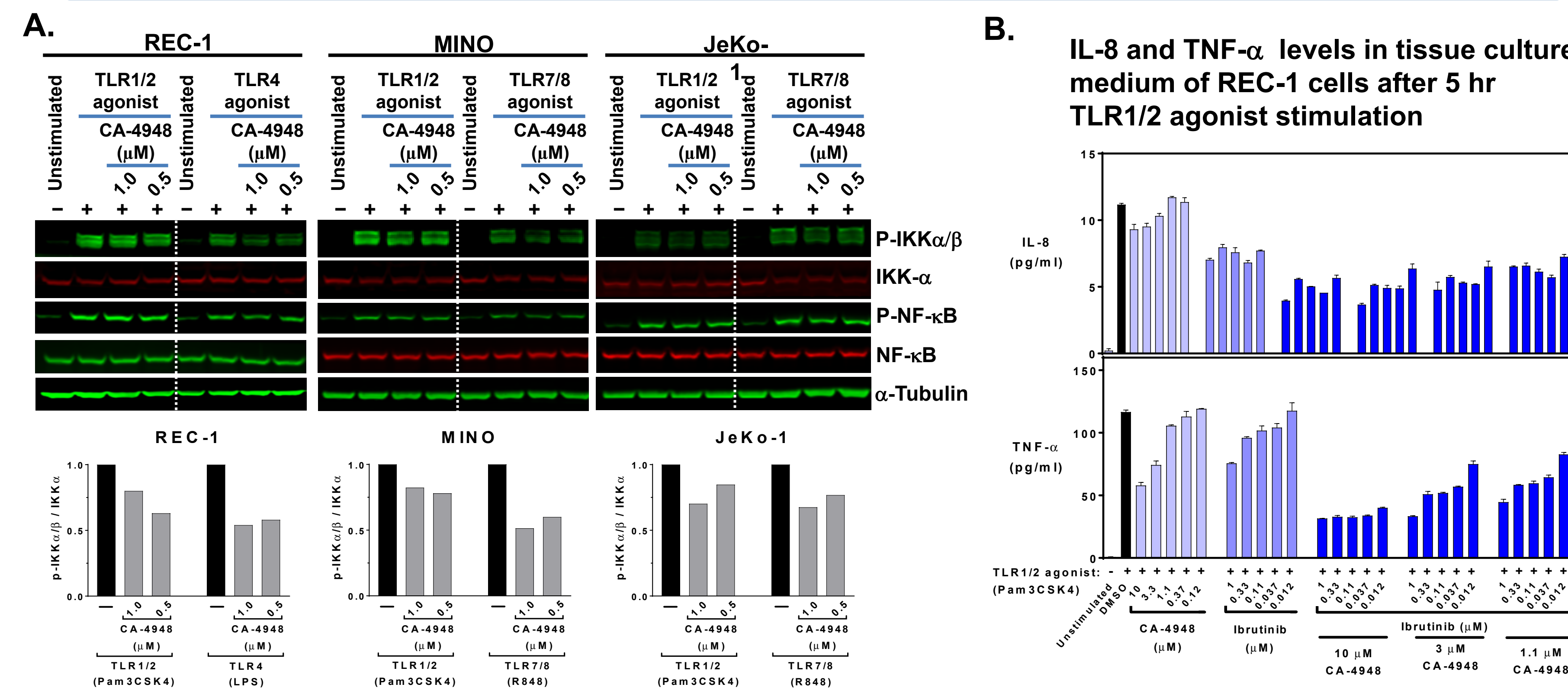


## Activation of NF- $\kappa$ B pathway and Cytokine Production in Response to TLR Agonists in MCL Cell Lines



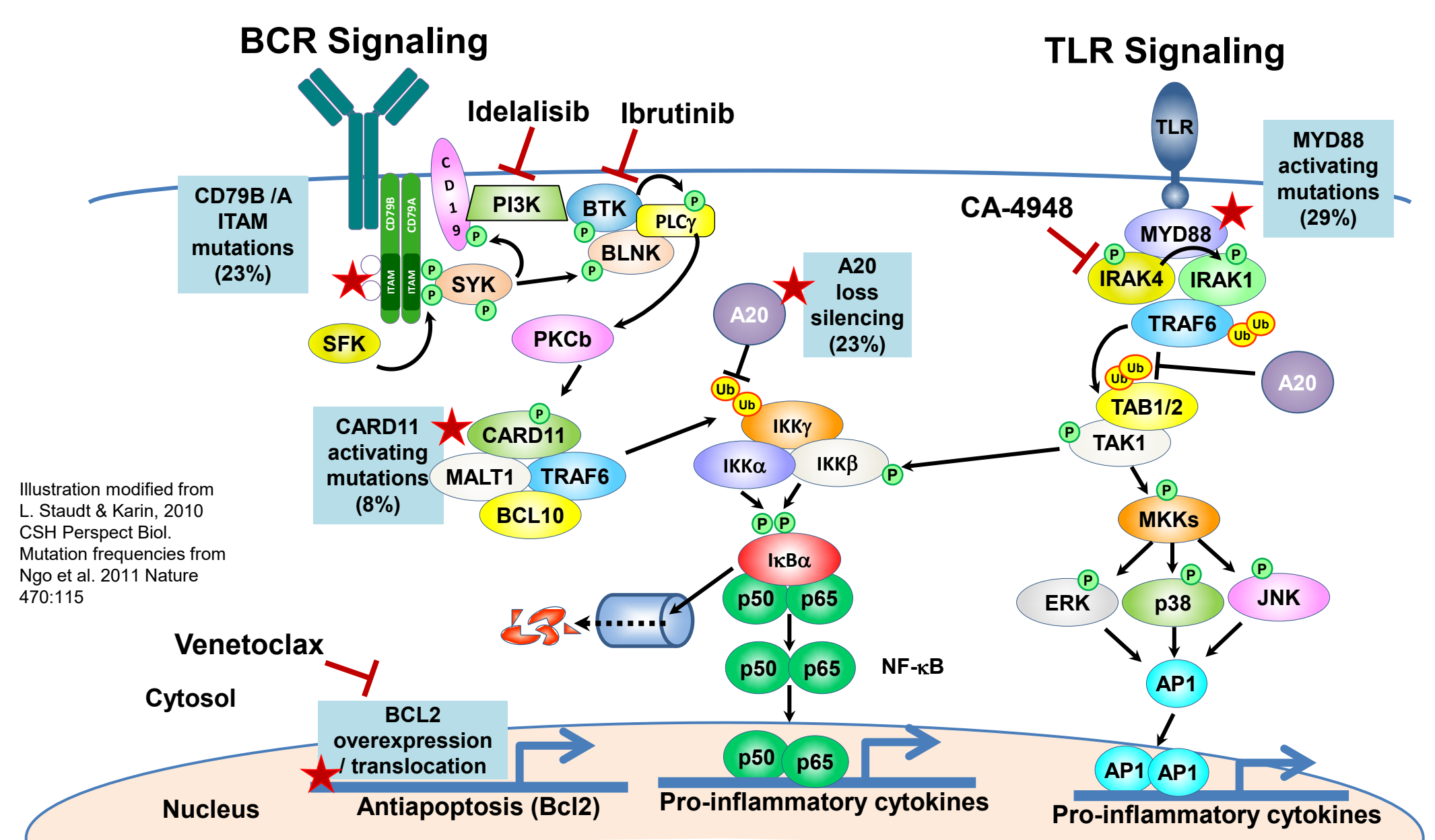
A. MCL cell lines were stimulated with agonists for TLR1/2 (Pam3CSK4), TLR4 (LPS), TLR5 (FLA-ST), or TLR7/8 (R878). NF- $\kappa$ B p65 blots are shown  
B. MCL cells were stimulated with TLR cocktail (Pam3CSK4, LPS, FLA-ST, and R848)

## CA-4948 $\pm$ Ibrutinib Inhibition of NF- $\kappa$ B p65 Signaling and Cytokine Production

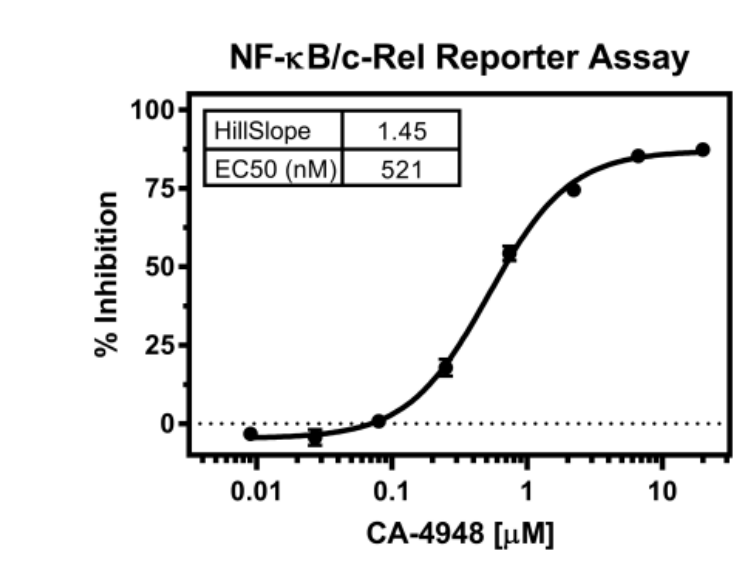


A. CA-4948 inhibition of TLR signaling pathway components after 1 hr stimulation  
B. Combination effect of CA-4948 + ibrutinib on TLR-induced cytokine production

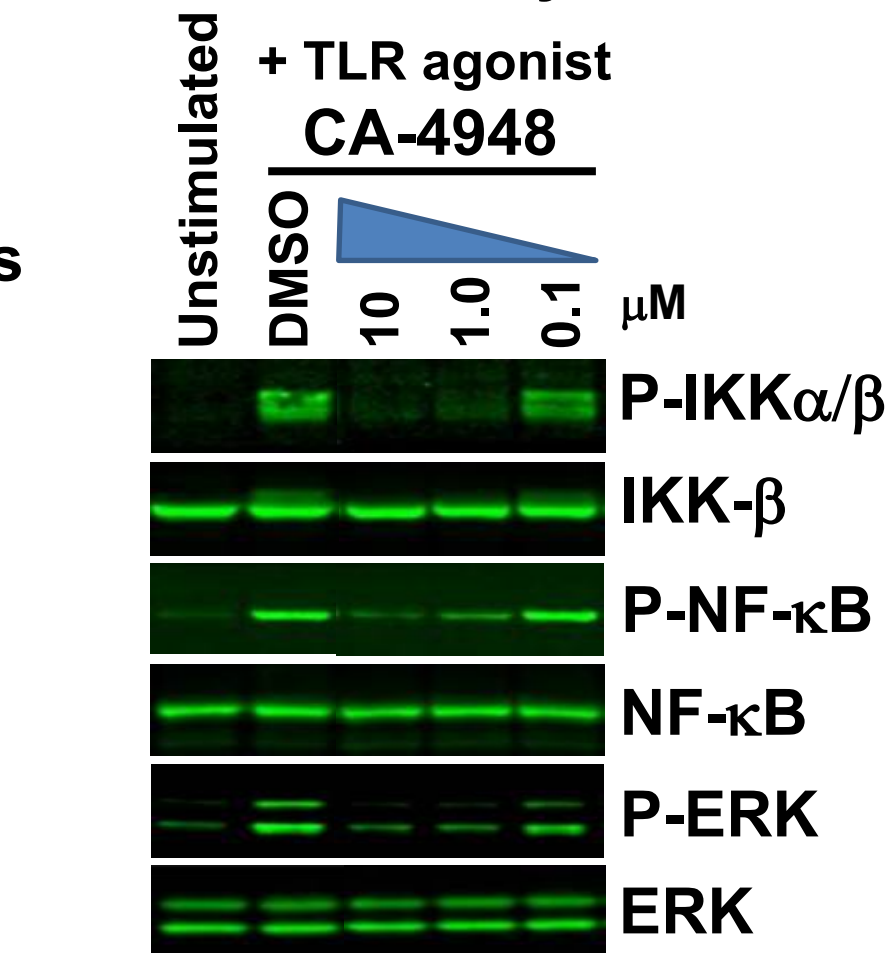
## CA-4948 Blocks the TLR/IL-1R Induced Canonical NF- $\kappa$ B signaling Pathway



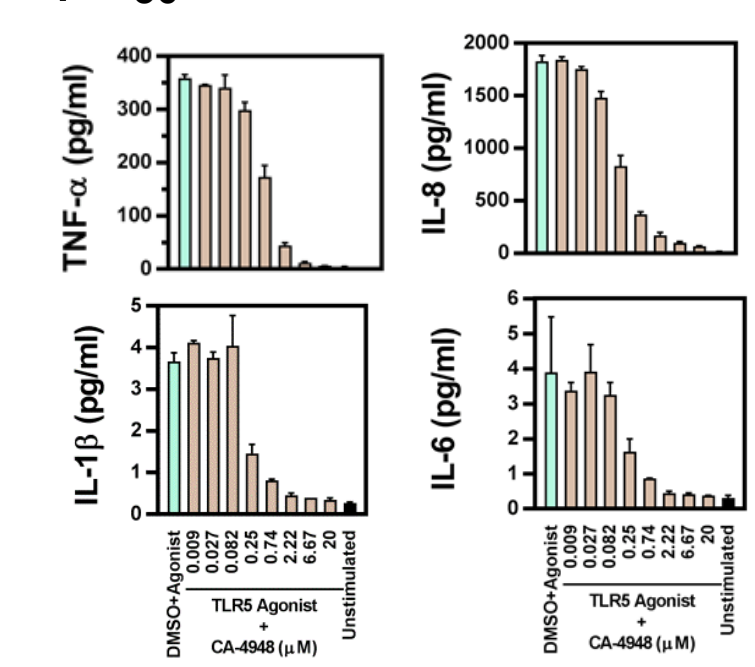
TLR-induced NF- $\kappa$ B reporter assays (IC<sub>50</sub> = 520 nM, THP1)



In vitro THP1 monocytic cell assays + TLR agonist CA-4948



TLR-induced cytokine release assays (IC<sub>50</sub> = 150-220 nM, THP1)



- CA-4948:**
- > Small molecule inhibitor
  - > ATP-competitive, reversible
  - > Oral bioavailable

Kinase	Kd (nM)
IRAK4	23
IRAK1	12,000

**CA-4948 Binding Affinity Activity**

CA-4948 inhibition of NF- $\kappa$ B reporter, secreted cytokine levels, and phospho-signals in THP1 monocytic cells

**In vivo efficacy studies of xenograft subcutaneous MCL tumor models. MCL cell lines with chronic activation of (A) BCR-driven classical and (B) alternative NF- $\kappa$ B pathways (Rahal R. et al., (2014) Nature Medicine 20(1) 87-94)**

## Summary

- ❖ CA-4948 is a potent, oral IRAK4 Ser/Thr kinase inhibitor with >500-fold against IRAK1
- ❖ CA-4948 treatment in culture did not result in antiproliferative/cytotoxicity activity in the 6 MCL cell lines tested (>10  $\mu$ M at 3 days)
- ❖ CA-4948 inhibited TLR-induced signaling and cytokine production in MCL cell lines with an intact BCR-driven canonical NF- $\kappa$ B pathway
- ❖ CA-4948 resulted in potent *in vivo* anti-tumor activity in MCL models with intact canonical NF- $\kappa$ B signaling, which was enhanced in combination with ibrutinib treatment
- ❖ CA-4948 exhibited weakest *in vivo* activity in models with alternative NF- $\kappa$ B signaling
- ❖ These results underscore the therapeutic potential of targeted IRAK4 kinase inhibition by CA-4948 in combination with BTK inhibitors for the treatment of MCL

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