Development of methods to investigate effects of narrow spectrum kinase inhibitors on cellular phosphorylation of kinase substrates

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Introduction

- Protein kinases play a critical role in intracellular signalling by catalysing the transfer of a phosphate group to substrate proteins, leading to activation of the inflammatory signalling cascade.
- Selective kinase inhibitors have been used as therapies in the treatment of chronic inflammatory diseases, such as rheumatoid arthritis and inflammatory bowel disease (IBD), but with limited success.
- In order to improve efficacy, targeted multi-kinase inhibitors have been developed, as exemplified by narrow-spectrum kinase inhibitor (NSKI) TOP1288, which target key kinases of the inflammatory signalling cascade; mitogen-activated protein (MAP), Src-family and Syk kinases.
- TOP1288 is proposed as a potential therapy for IBD and in order to investigate activity in inflammatory cells isolated from colonic biopsies, flow cytometric methods have been developed to demonstrate inhibition of protein kinase in a clinical setting.

Methods

- Phospho-flow methods were developed *in vitro* in both a human Jurkat T cell line and primary cells derived from human whole blood (PBMC) or colonic biopsies (LPMC).
- Activation was assessed using phospho-site specific antibodies against auto-phosphorylation epitopes of two key kinases (P38α and Lck) in the pathology of IBD, and subsequent inhibition with NSKI.
- Phosphorylation induced by stimulation with hydrogen peroxide (H_2O_2) and subsequent TOP1288-mediated inhibition was assessed by intracellular flow cytometry (in morphologically intact lymphocytes).
- In all cases, cells were pre-incubated with vehicle or compound for 2hrs prior to stimulation.

Results

Jurkat T cell line

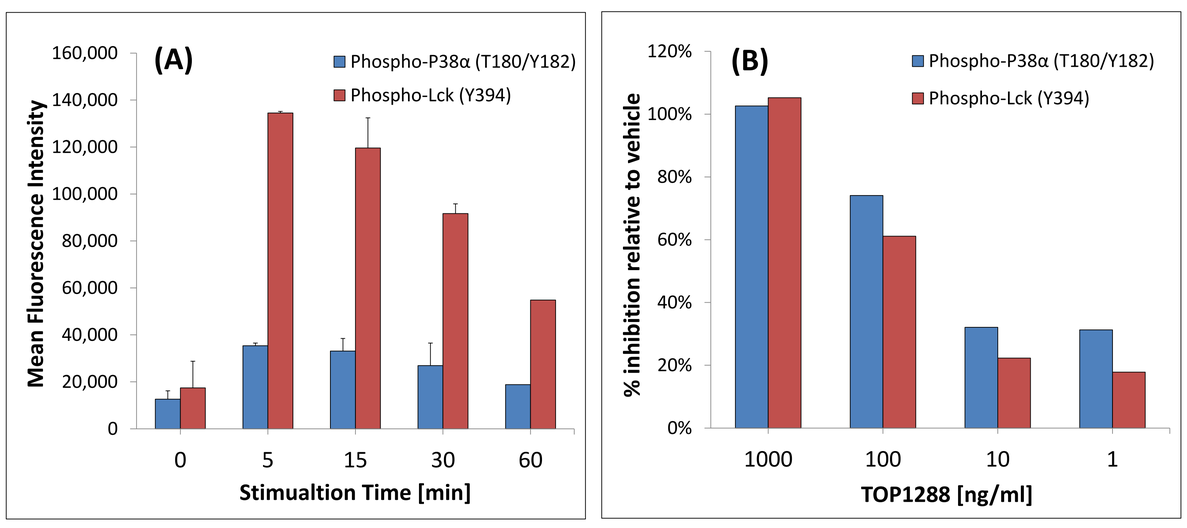


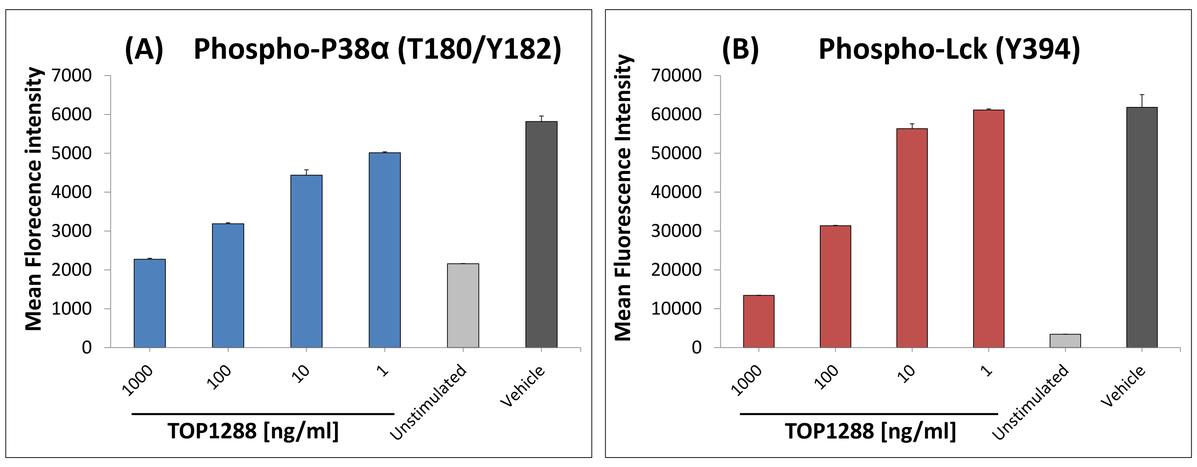
Figure 1. H_2O_2 stimulated Jurkat T cells. (A) Kinetics of P38α and Lck phosphorylation. (B) Inhibition of kinase phosphorylation in the presence of TOP1288.

- Hydrogen peroxide induces rapid transient phosphorylation of both phospho-P38α and phospho-Lck with maximum signals detectable after 5 min (Figure 1A).
- In the presence of TOP1288, activity of both phospho-P38α and phospho-Lck was inhibited with an IC_{50} of 30 & 60ng/ml, respectively. In addition, TOP1288 completely inhibited kinase phosphorylation at sub-microMolar concentrations (Figure 2B).

Results

Peripheral Blood Mononuclear Cells (PBMC)

- TOP1288 maintained similar potency and efficacy when assessed in human PBMCs isolated from healthy donors, compared to the T cell line (Figure 2).
- TOP1288 (3 µg/ml) achieved complete inhibition of the stimulated phospho-signal against both targets (Figure 2).

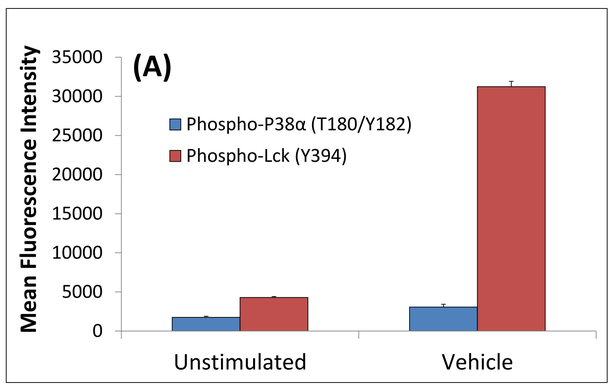


(C)

	IC_{50} (ng/ml)	Max Inh. (%)
Phospho-P38α	20	98
Phospho-Lck	100	97

Figure 2. Effect of *in vitro* TOP1288 on hydrogen peroxide stimulated human PBMCs against key inflammatory kinases; (A) phospho-P38α and (B) phospho-Lck. (C) IC_{50} values are estimated on mean fluorescence intensity.

Lamina Propria Mononuclear Cells (LPMC)



(C)

	Phospho-Lck (Y394)	
	IC_{50} (ng/ml)	Max Inh. (%)
PBMC	100	97
LPMC	90	98

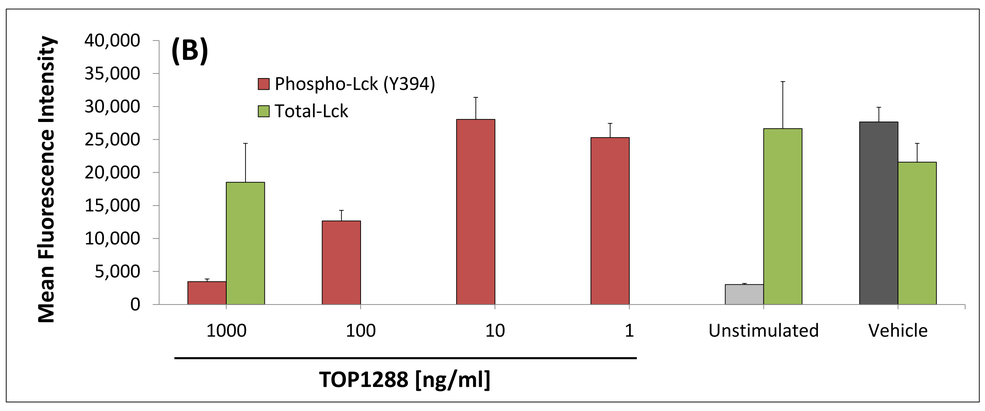


Figure 3. Hydrogen peroxide stimulated LPMCs. (A) Phosphorylation of P38α & Lck (B) Effect of *in vitro* TOP1288 against Lck (C) Comparison of TOP1288 efficacy against phospho-Lck in PBMCs vs LPMCs.

- In LPMCs, phospho-Lck stimulation was achieved in response to H_2O_2. However, only minimal induction of phospho-P38α was evident (Figure 3A).
- TOP1288 potently inhibits phospho-Lck induction with an IC_{50} of 90ng/ml (Figure 3B), achieving maximal efficacy similar to that observed in PBMCs (Figure 3C).

Conclusions

- The methods developed allow semi-quantitative measurement of target engagement in individual cells of primary origin. We have been able to demonstrate that the NSKI, TOP1288, can potently inhibit activation of kinase targets in a range of different cells. Therefore, these methods have potential for use in clinical studies for biomarker measurements in pathologically relevant cells of IBD.