Small molecule modulation of ROS release in TNF-α primed primary human neutrophils

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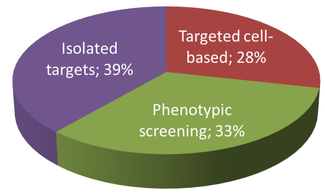
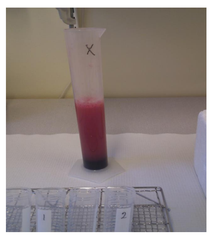
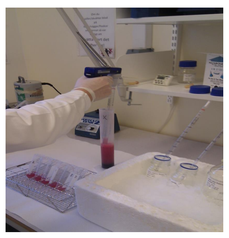
INTRODUCTION

Neutrophils are an important component of the innate immune system in the protection against invading microorganisms. Neutrophils in circulating blood are normally found in a resting state; however different inflammatory mediators transfer neutrophils into an active state by a priming procedure. Activation triggers the adhesion and penetration of neutrophils into inflamed tissue, where reactive oxygen species (ROS) are released through the activation of NADPH oxidase. Extensive ROS exposure can lead to damage also of adjacent tissue and promote inflammation. Tumor necrosis factor alpha (TNF-α) is one of the earliest cytokines produced at an inflammatory site. TNF-α itself does not activate NADPH oxidase in neutrophils to a great extent, but acts as a priming agent. Upon addition of the actin filament disrupting agent cytochalasin B to TNF-α primed neutrophils a burst of released ROS is observed, with a timing and magnitude profile resembling that observed following stimulation of pro-inflammatory GPCRs expressed on the neutrophil surface. We took advantage of this phenomenon and developed a screening assay aiming at identifying modulators of ROS release in TNF-α primed primary human neutrophils, with the hope of finding novel GPCR modulators amongst the hits. The project serves as an example of many collaborations between Chemical Biology Consortium Sweden and principal investigators in Sweden, often focused on establishing routines that allows screening of primary cells to maximize biological validity of the test system.



CBCS screening portfolio

PRIMARY CELL ISOLATIONS

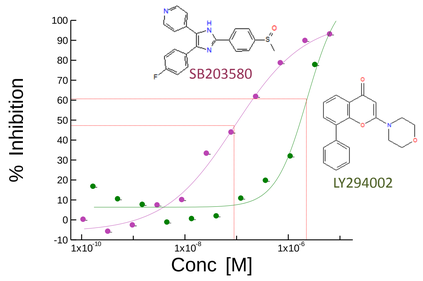




Neutrophils were prepared fresh every day from buffy coats obtained from the nearby hospital:

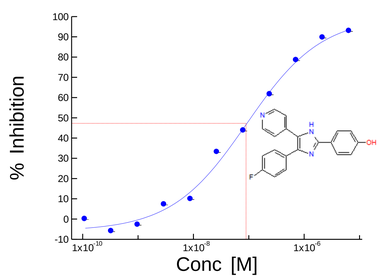
- Separation of neutrophils was achieved using a protocol that involved gradient centrifugation and osmolytic lysis of remaining erythrocytes.
- The cell identity and purity of each preparation (>90%) was validated for each neutrophil batch prior to commencing screening. The failure rate was about 10% of the donors.
- Finally a sufficient response to TNF-α priming and cytochalasin B induced ROS release was tested prior to screen start. Failure to obtain a sufficient response was about one in every fourth donor.
- Once a functional batch was identified each daily screen involved between 2 and 27 96-well plates

SCREEN SUMMARY

- A total of 7.000 compounds were tested at a compound concentration of 1.3µM (fine-tuned in pre-screening activities).
- MAPK (p38) and PI3K inhibitors are known to modulate several of the TNF-α induced effects. SB203580, a p38 inhibitor, and LY294002, a PI3K inhibitor, were used as reference compounds.
- The screen required a total of eight (8) screen days, hence using cells from eight different blood donors. Variability in compound response between donors have been tested in follow-up studies.
- 74 compounds from several different compound series were identified as inhibitors of ROS release in this system.



SB203580 IC_{50} 9 nM
LY294002 IC_{50} ~1.6 µM

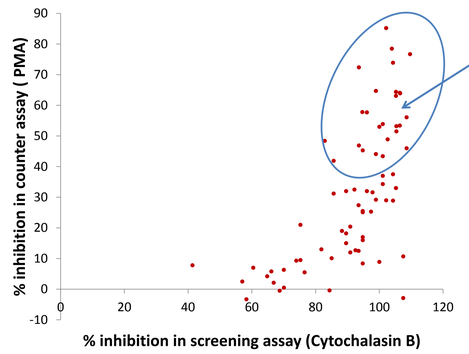


One of the most potent hits
SB202190/CBK041209 IC_{50} 7 nM

HIT VALIDATION

Several approaches have been taken to validate and rank the hits:

1. Counter-screens have been employed using various pro-inflammatory stimuli (*e.g.* PMA) to identify compounds that either interfere with the detection system or inhibit the ROS release without any relation to the TNF-α priming.
2. Variability in compound response between donors have been tested such that we focus on consistent responses in this first phase of the project.
3. Structural clustering have been employed to focus efforts on selected representatives. We have also employed kinase assays (p38-α, PI3K-δ and PI3K-ɣ) to identify obvious inhibitors of targets known to interact in the applied screening assay (identifying only the known p38 inhibitor SB202190).

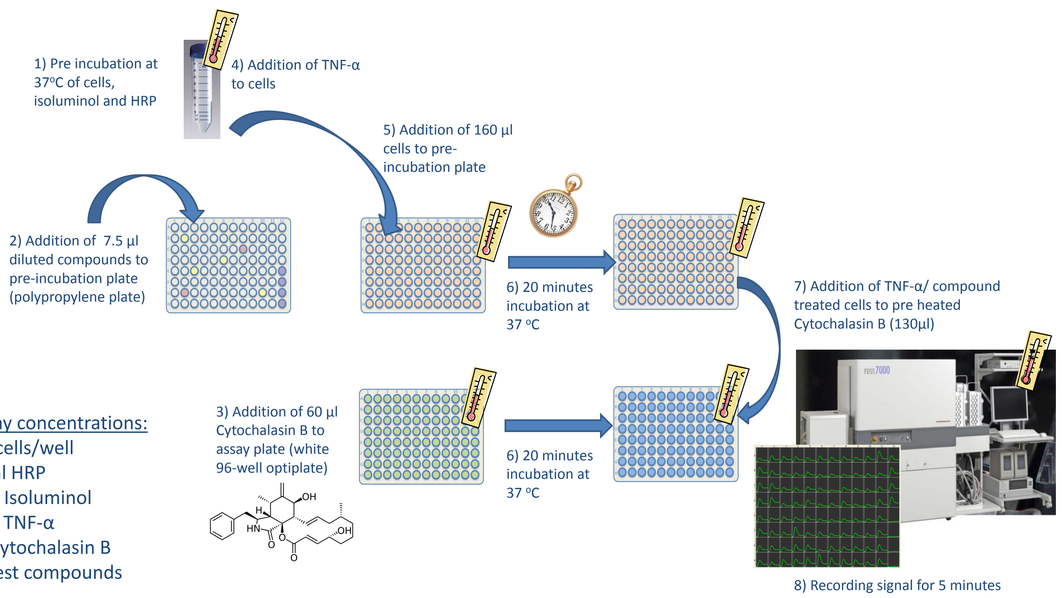


Possible interfering compounds such as: HRP inhibitors, Redox scavengers, Color quenchers, NADPH oxidase inhibitors and Broad kinase inhibitors

- The TNF-α priming step was removed.
- Cytochalasin B addition was replaced with Phorbol 12-myristate 13-acetate (PMA).

SCREENING ASSAY LOGISTICS

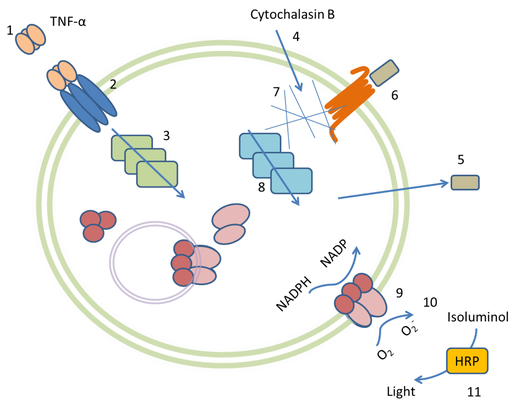
In our test system freshly isolated primary human neutrophils are first incubated with TNF-α. The cells are then triggered with cytochalasin B. The produced superoxide anions are measured by a chemiluminescence technique based on isoluminol using the single photon counting camera of the Hamamatsu FDSS 7000.



Final assay concentrations:
100 000 cells/well
4.35 U/ml HRP
11 µg/ml Isoluminol
10 ng/ml TNF-α
5µg/ml Cytochalasin B
1.3 µM test compounds

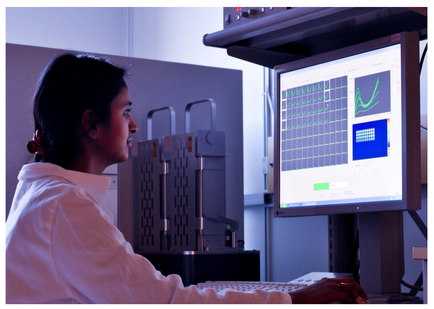
FUTURE PERSPECTIVES

A rather large number of potent nM inhibitors belonging to several compound series were identified in the primary screen. A few of the hits could be easily distinguished as known modulators of TNF-α signaling, including p38 and PDE4 inhibitors. Our present focus concerns the further characterization of compounds with hitherto unknown mechanisms and this is primarily done using two approaches. Firstly we broadly employ the same assay, but conditions are varied to include a range of different pro-inflammatory stimuli as well as cytoskeleton disrupting compounds. The latter variations serve to address the underlying question as to whether the cytoskeleton B triggering results in the reactivation of a desensitized pro-inflammatory GPCR. Secondly given the low nM potency of some of the hits we are pursuing unbiased target identification activities for compounds that belong to structural clusters and for which we have some knowledge of the structural activity relationships.



Some possible interaction points.

1. *TNF-α trimerization*
2. *TNFR1/ TNF-α interaction*
3. *TNFR1 downstream signaling*
4. *Cytochalasin B uptake and cytoskeleton interaction.*
5. *Excretion of unknown endogenous ligand*
6. *Receptor/ligand interaction*
7. *Receptor cytoskeleton interaction*
8. *Receptor downstream signaling*
9. *NADPH oxidase interaction*
10. *Redox scavenger*
11. *HRP interaction*



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