

# **A Small-Molecule Inhibitor to the Cytokine Interleukin-4**

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## **Supplementary Information**

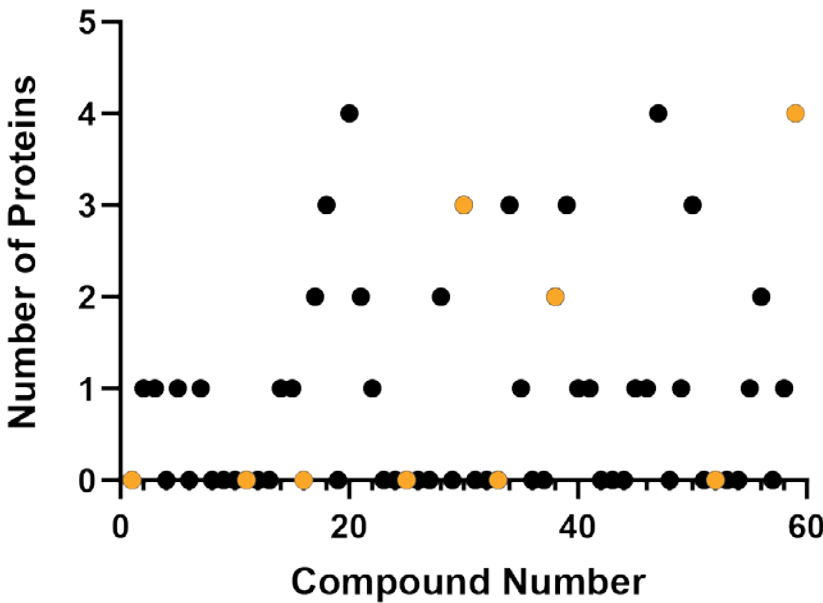
## Supplemental Tables and Figures

**Supplemental Table 1.** List of proteins used to generate SMM binding signatures in Figure S1

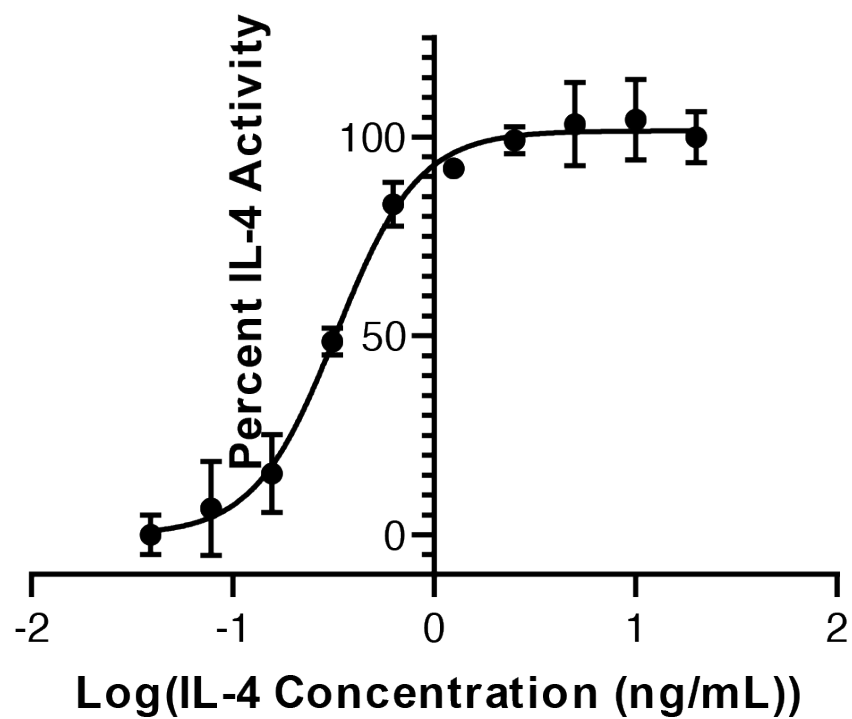
Protein Name	Protein Class
Androgen Receptor Variant 7 (ARv7)	Transcription regulator
BCL11AXL	Transcription regulator
Bromodomain Containing Protein 4 (BRD4)	Transcription regulator
Carbonic Anhydrase II (CAII)	Enzyme
EWS/FLI	Transcription regulator
Forkhead Box A1 (FOXA1)	Transcription regulator
Forkhead Box P3 (FOXP3)	Transcription regulator
High Mobility Group Nucleosome Binding Domain 1 (HMGN1)	Transcription regulator
Heat Shock Factor 1 (HSF1_HA)	Transcription regulator
Heat Shock Factor 1 (HSF1_His)	Transcription regulator
Interleukin 4 (IL-4)	Cytokine
$\beta$ -ketoacyl ACP synthase I (KasA-His)	Enzyme
MYB fragment	Transcription regulator
MYB_MYBNFIB IVT	Transcription regulator
Prion Protein (PrP)	Surface receptor
Rev1	DNA Polymerase
SOX2	Transcription regulator
YAPD1	Transcription regulator
Yes-Associated Protein (YAP-HA)	Transcription regulator
B-Cell Lymphoma/Leukemia 11A (BCL11A)	Transcription regulator
Succinyl-CoA ligase [GDP-forming] subunit beta (SucI2)	Enzyme
MYC-Associated Protein X (Max)	Transcription regulator
Chromodomain Helicase DNA Binding Protein 4 (CHD4)	Enzyme
Glutathione S-transferases (GST)	Enzyme
3C-Like Protease (3CLpro)	Protease
mp97	Transcription regulator
Sialic Acid-Binding Immunoglobulin-Like Lectin F (Siglec-F)	Glycan receptor
Immunoglobulin G (IgG)	Antibody
Siglec	Glycan receptor

**Supplemental Table 2.** Control data for HEK-Blue IL-4/IL-13 inhibition experiments

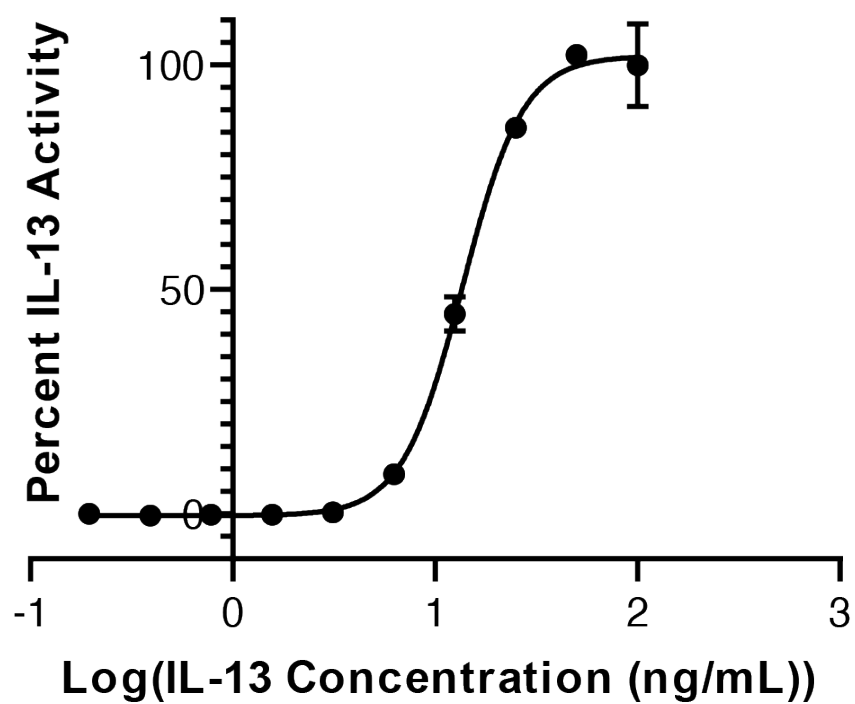
Condition	Abs650
Cells Alone	0.0961 ± 0.0113
Cells with Vehicle	0.0923 ± 0.0079
Cells with IL-4 and Vehicle	1.391 ± 0.0650
Cells with 100 µM Compound 1	0.0955 ± 0.0072
Cells with 100 µM Compound 25	0.0648 ± 0.0012
Cells with 100 µM Compound 52	0.0588 ± 0.0175



**Supplemental Figure 1.** SMM binding signatures for compounds 1-59 derived from SMM assays with >28 unrelated proteins. The number of proteins that these compounds were measured as binding positives is used as an indication of binding selectivity. The 10 compounds advanced into functional cell-based assays (**Figure 2**) are highlighted in orange.



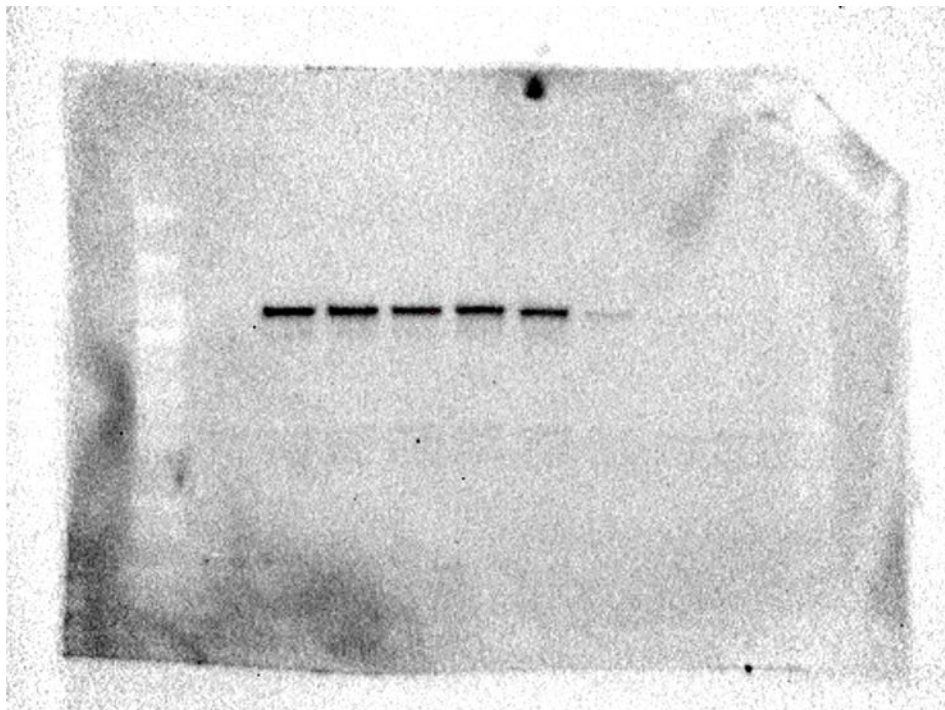
**Supplemental Figure 2.** Activity of IL-4 with 0.2% DMSO in HEK-Blue IL-4/IL-13 cells



**Supplemental Figure 3.** Activity of IL-13 with 0.2% DMSO in HEK-Blue IL-4/IL-13 cells



**Supplemental Figure 4.** Full STAT-6 Western Blot



**Supplemental Figure 5.** Full p-STAT-6 Western Blot

## **Materials and Methods**

### **Reagents**

His-tagged mammalian-expressed IL-4 for SMM and DSF was purchased from Abcam (ab185869). Tag-free HEK-293 expressed IL-4 for HEK-Blue IL-4/IL-13 and THP-1 inhibition assays was purchased from Acros Biosystems (IL4-H4218). Avitag IL-4 for surface plasmon resonance was purchased from Acros Biosystems (IL4-H82E0). Blasticidin, Zeocin, Normocin, Quanti-Blue was purchased from Invivogen. Commercial small molecules used in SMM were purchased from Chembridge. STAT-6 and p-STAT-6 antibodies were purchased from Cell Signaling Technology. All other chemicals and reagents were purchased from Fisher Scientific.

### **SMM Screening of Recombinant IL-4**

Each SMM slide contained approximately 5000 printed features in duplicate and was prepared as described previously.<sup>1</sup> In total 50,000 compounds were screened. The collection contained computationally selected commercially available compounds as well as products of diversity-oriented synthesis and known bioactive compounds. Each sample was screened against two replicate SMMs. Each slide was incubated with 3 mL of a solution of 0.1 µg/ml recombinant IL4-His protein in PBS-T buffer for 0.5 hour at room temperature. The slides were then incubated with a 3ml solution of anti-His mouse monoclonal antibody conjugated to AlexaFluor 647 (Qiagen) at a concentration of 1:1000. Each incubation step was followed by three washes in PBS-T. Finally the slides were briefly rinsed in distilled water and spin-dried. The slides were immediately scanned using a GenePix 4000B fluorescence scanner (Molecular Devices). The image was analyzed using GenePix Pro software (Axon Instruments) and data analyzed as described below.

### **SMM Statistical Analysis.**

Raw data was analyzed based on the signal-to-noise ratio and reproducibility. For each feature, the signal to noise ratio (SNR) was defined as the median fluorescence intensity of the feature divided by the median fluorescence intensity of the surrounding slide area, defined as a radius 3 times the radius of the spot, excluding pixels within a certain overlap threshold of neighboring features. Then, for each feature, a robust z-score ( $z_i$ ), which is less influenced by outliers compared with the mean-based z statistic, was calculated for each feature ( $i$ ) by the following equation:

$$z_i = \frac{SNR_i - Mdn(SNR)}{MAD(SNR) * 1.486}$$

Where  $SNR_i$  is the SNR value for a given feature,  $Mdn(SNR)$  is the median of the SNR values for all features in the subarray, and the  $MAD(SNR)$  is the maximum absolute deviation of the SNR values for all features in the subarray. Assay positives with an average robust Z score greater than 1.645 ( $\alpha = 0.05$ ) on duplicate slides were compared to both a counter screen for the detection antibody and a database representing > 25 unique targets screened by SMM against the same chemical library to filter nonspecific binders.

### **DSF**

Top hits from SMM were made into 1 mM stock solutions in pure DMSO and dispersed in 384-well plates. 1  $\mu$ g of protein in PBS was added to each well, then 8X Spiro-Orange was added, followed by PBS, DMSO, or small molecules. Small molecules were screened

at either 20  $\mu$ M or 2  $\mu$ M in 0.2% DMSO. DSF was performed on a BioRad CFX384. Change in melting temperature was calculated by comparing the nadir of the melting curve of protein with vehicle to the nadir of the melting curve of protein with small molecule. Plots were made in Graphad Prism 8.

### **HEK-Blue IL-4/IL-13 Inhibition Assays**

HEK-Blue IL-4/IL-13 cells were purchased from Invivogen and maintained in DMEM complete with 10% fetal bovine serum, 10  $\mu$ g/ml of blasticidin and 100  $\mu$ g/ml of Zeocin, 100  $\mu$ g/mL Normocin, and 100U/mL-100  $\mu$ g/mL Pen-Strep. Cells used for assays were between passages 12 and 18. Prior to screening small molecules, the SEAP production induced by IL-4 alone was tested in the cells (**Figure S2**) and determined to be 0.3 ng/mL. The EC25 for the IL-4 induced SEAP production (0.1 ng/mL final well concentration) was chosen for running small molecule inhibition assays.

For screening small molecules, 25  $\mu$ L of each molecule (**Figure 2**) in DMEM with 4% DMSO was added to 25  $\mu$ L of 1 ng/mL IL-4 in DMEM and incubated for 2 hours at 37°C. HEK-Blue IL-4/IL-13 cells were trypsinized, spun down, and resuspended in DMEM with 10% heat-inactivated FBS, and 100U/mL-100  $\mu$ g/mL Pen-Strep at a concentration of  $3.125 \times 10^5$ /mL. 160  $\mu$ L of the cell suspension was added to each well of a 96 well plate, avoiding the edges of the plate and equilibrated in an incubator for 1 hour. After 2 hours, 40  $\mu$ L of the small molecule/IL-4 solution was added to each well. Each measurement was performed in triplicate. Cells were incubated for 20 hours at 37°C/5% CO<sub>2</sub>. Quanti-Blue was prepared according to manufacture and 160  $\mu$ L added to each well of a 96 well plate, avoiding edges. 40  $\mu$ L of cell supernatant was added to each well and then incubated for 3 hours at 37°C. The optical density was then read at 650 nm with a



Molecular Devices SpectraMax M5 Microplate Reader. Data was plotted and analyzed in GraphPad Prism 8.

Inhibition assays with IL-13 were performed similarly. First, the activity of IL-13 in HEK Blue IL-4/IL-13 cells was tested at concentrations from 0.2 - 100  $\mu\text{g/mL}$  (final well concentrations) with 0.2% DMSO (**Figure S3**). 8 ng/mL was then incubated with increasing concentrations of D9 in 2% DMSO for 2 hours at 37°C before being added to cells. The supernatant was assayed for SEAP with Quanti-Blue and then absorbance taken at 650 nm. Data was again plotted and analyzed in GraphPad Prism 8.

#### **THP-1 STAT-6/pSTAT-6 assay**

THP-1 cells were received as a gift from the laboratory of Dr. Mark Grinstaff and maintained in RPMI-1640 with 10% heat-inactivated fetal bovine serum and 50  $\mu\text{M}$  beta-mercaptoethanol. Cells used for assays were between passages 6 and 10. 10 ng/mL IL-4 was preincubated with experimental sample (vehicle or small molecules) for 2 hours at 37°C. 1 mL of each solution was then added to a pellet of 2E6 THP-1 cells and pipetted up and down. THP-1 cells were incubated for 30 minutes in incubator at 37°C 5% CO<sub>2</sub>. Cells were then spun down, lysed with 200  $\mu\text{L}$  ice-cold RIPA buffer complete with phosphatase and protease inhibitors. Cell lysate was incubated on ice for 30 minutes and then stored at -20°C until western blot analysis. Undiluted cell-lysate was run SDS-PAGE followed by transfer to membrane using Biorad equipment. Membranes were incubated with 1:1000 primary antibody overnight at 4°C. Membranes then incubated with 1:50,000 dilution secondary for 1 hour at RT. Membranes were then exposed to Femto ECL for 5 minutes and imaged using a Bio-Rad Gel Doc. The brightness/contrast was adjusted in FIJI (**Figures S4 and S5**) and the area under the curve of each lane was quantified. The

ratio of phosphorylated STAT6 to STAT6 was calculated and normalized to the amount of total STAT6. The data was plotted and analyzed using Graphpad Prism 8.

### **Immunofluorescence for pSTAT-6**

THP-1 cells were plated at a density of  $1.5 \times 10^6$ /mL into a MatTak dish coated with Cell Tak adhesive. Cells were allowed to attach for 1 hour. IL-4 and small molecule or vehicle (DMEM with 2% DMSO) was pre-incubated for 2 hour at 37°C. Media was aspirated and IL-4 or IL-4/small molecule were added to cells and incubated for 30 minutes at 37°C 5% CO<sub>2</sub>. Cells were rinsed twice with PBS and fixed with 2% formaldehyde for 30 minutes at room temperature. Cells were rinsed twice and then permeabilized with 0.1% Triton-X for 10 minutes. Cells were blocked for 1 hour in superbloc with 0.05% Tween-20, rinsed twice, and then incubated with anti-pSTAT6 1:100 diluted in SuperBlock 0.05% Tween-20 overnight at 4°C. Cells were rinsed three times with PBS 0.05% Tween-20 and then incubated with Goat anti-rabbit IgG (H+L) Secondary Antibody - Alexa Fluor 647 mixed with a 1:250 dilution of Hoechst 33342. Cells were rinsed twice and left in 1 mL of PBS. Confocal imaging was done using an Olympus FV10i and images were processed using FIJI.

### **Surface plasmon resonance (SPR)**

Surface plasmon resonance was performed on a Biacore T200. SA sensor chips were purchased from GE Healthcare. The running buffer was 1X PBS pH 7.40 with 2% DMSO and 0.1% Tween-20 and the flow rate was kept constant at 50  $\mu$ L/min unless otherwise noted. The SA chip was activated with three 1 minute injections of 1M NaCl in 50 mM NaOH followed by a wash of 50% isopropanol in 1M NaCl and 50 mM NaOH. 50  $\mu$ L of a 5 mg/mL solution of Avitag-IL-4 in DMSO free buffer was injected to immobilize 3000 RU

on the chip surface using the software wizard. Two manual 50  $\mu$ L injections of 50 mM biocytin dissolved in running buffer were then performed to block all biotin sites on the sensor chip. The system was then primed with running buffer for 10 minutes. Compound 8 was diluted in running buffer and injected for 300 second cycles in triplicate for each concentration, starting from low concentration. The dissociation phase was 600 seconds long. Sensogram figures were created by exporting the raw data and plotting in Graphpad Prism 8.

## References

- (1) Bradner, J. E.; McPherson, O. M.; Koehler, A. N. A Method for the Covalent Capture and Screening of Diverse Small Molecules in a Microarray Format. *Nat. Protoc.* **2006**, 1 (5), 2344–2352. <https://doi.org/10.1038/nprot.2006.282>.