

# Identifikacija malih molekula s inhibirajućim djelovanjem na onkogeni mutant tirozinsko kinaznog receptora FGFR4 metodom MaMTH-DS

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University of Zagreb  
Faculty of Science  
Department of Biology

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**Identification of small molecule inhibitors of oncogenic FGFR4 tyrosine  
kinase mutant using MaMTH-DS method**

Master thesis

Zagreb, 2019.

This thesis was performed in Stagljär Lab, Department of Biochemistry and Department of Molecular Genetics, Faculty of Medicine, University of Toronto, Toronto, Canada under supervision of Dr. Igor Štagljär, Prof. Thesis is submitted on evaluation to Department of Biology, Faculty of Science, University of Zagreb, Zagreb, Croatia in order to acquire academic title of Master of Molecular Biology.

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## BASIC DOCUMENTATION CARD

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University of Zagreb  
Faculty of Science  
Division of Biology

Master Thesis

### **Identification of small molecule inhibitors of oncogenic FGFR4 tyrosine kinase mutant using MaMTH-DS method**

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Cells receive signals from beyond the cell membrane which are detected by specific receptors and that renders a cellular response. One of the systems that enable such signalling is through receptor tyrosine kinases (RTKs). RTKs are membrane proteins which have been proven as crucial regulators of pivotal cellular processes including proliferation, differentiation, cell survival, cell cycle control and metabolism. Consequently, constitutive and aberrant activation of RTKs and downstream pathways is shown to lead to tumorigenesis through disruption of these processes. Fibroblast growth factor receptor 4 (FGFR4) is an RTK whose aberrant regulation is associated with various cancers and the ability to metastasize. Its mutation V550E is considered activating in rhabdomyosarcoma, a childhood tumor with inadequate therapy for later stages. As knowledge of molecular basis of cancer expands it enables development of novel, targeted therapies. High-throughput screening approach to drug discovery involves biochemical and cell-based assays to screen collections of compounds in order to find specific hits on a target. Aim of this study was to identify small molecule inhibitors specific to the FGFR4 V550E using MaMTH assay, a recently developed technology for mapping interactions of membrane proteins, modified into a high-throughput drug screening method, MaMTH-DS. Collection of 5570 compounds was tested and five were identified as mutant specific inhibitory hits.

(43 pages, 11 figures, 4 tables, 33 references, original in English)

Key words: FGFR4 V550E, MaMTH, mutant specific hits, inhibitory hits

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Diplomski rad

### **Identifikacija malih molekula s inhibirajućim djelovanjem na onkogeni mutant tirozinsko kinaznog receptora FGFR4 metodom MaMTH-DS**

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Stanice primaju signale iz (mikro)okoliša putem stanične membrane. Te signale prepoznaju specifični receptori što se prevodi u stanični odgovor. Jedan od sustava koji omogućava takvu signalizaciju jest putem tirozinskih kinaznih receptora (RTK). RTK su membranski proteini koji su regulatori središnjih staničnih procesa uključujući proliferaciju, diferencijaciju, stanično preživljenje, kontrolu staničnog ciklusa i metabolizam. Posljedično, za konstitutivna i aberantna aktivacija RTK-a i nizvodnih signalnih putova sudjeluju u tumorigenezi zbog remećenja navedenih procesa. Receptor fibroblastnog faktora rasta 4 (FGFR4) je RTK, čija je aberantna regulacija povezana s raznim karcinomima i sposobnošću metastaziranja. Njegova mutacija V550E se smatra aktivirajućom kod rabdomiosarkoma, tumora dječje dobi s neodgovarajućom terapijom u kasnijim stadijima. Kako znanje o molekularnoj osnovi karcinoma raste, omogućava razvoj novih, ciljanih terapija. Probir visoke protočnosti pristup je otkrivanju lijekova koji uključuje biokemijske testove i testove koji se temelje na korištenju stanica za probir zbirki spojeva u svrhu nalaženja specifičnih ciljanih pogodaka. Cilj ovog istraživanja bio je identificirati male molekule s inhibirajućim djelovanjem specifičnim za FGFR4 V550E korištenjem testa MaMTH, nedavno razvijene tehnologije za istraživanje interakcija membranskih proteina, modificirane u visokoprotočnu varijantu, MaMTH-DS. Testirana je zbirka od 5570 spojeva te je za pet malih molekula identificirano inhibirajuće djelovanje specifično za mutirani receptor FGFR4.

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## **1. Introduction**

### **1.1. Cancer as a result of aberrant cell signalling**

All cells can receive and act on signals from beyond the cell membrane and that ability is fundamental to life. These signals represent information which is detected by specific receptors and renders a cellular response. There are several cell signalling systems in eukaryotes one of which is signaling through receptor tyrosine kinases (RTKs)<sup>1</sup>. Molecular architecture of RTKs is similar among them, prevalently consisting of a ligand-binding extracellular domain, a single transmembrane helix and a cytoplasmic region with the tyrosine kinase domain. Upon binding of a specific ligand, predominantly growth factors, leads to homo or hetero dimerization of the receptor which promotes phosphorylation and thus activation of intracellular signaling proteins<sup>2,3,4</sup>. Over decades of studying RTKs, many members of this family have emerged as crucial regulators of pivotal cellular processes, such as proliferation, differentiation, cell survival, cell-cycle control and cell metabolism<sup>2</sup>. Moreover, mutations in RTKs and abnormalities in their intracellular signaling pathways are thought to cause various diseases, among them various types of cancer, since they lead to cell transformation, observed in wide range of malignancies<sup>2,4</sup>. Constitutive and aberrant activation of RTKs and downstream pathways leads to increased cell proliferation, survival, invasion and metastasis<sup>4</sup>.

#### **1.1.1. Roles of FGFR4 tyrosine kinase in oncogenesis**

Fibroblast growth factor receptors (FGFRs) are one of the RTK subfamilies, comprised of four family members FGFR1-4. They are activated by binding of fibroblast growth factors and are associated with multiple cellular cascades and responses including cell growth, proliferation, differentiation and survival, playing an important role in fundamental embryonic development patterns as well as adult life<sup>2,6,7</sup>. As such, FGFRs are shown to have oncogenic roles in many cancers which is supported by several types of genetic evidence including activating mutations, single nucleotide polymorphisms, gene amplifications and chromosomal translocations<sup>6,7</sup>. FGFR4 is essential to myogenesis and repair of skeletal muscle. Aberrant regulation of its activity has been observed in breast, prostate and hepatocellular cancer as well as rhabdomyosarcoma (RMS) and it has been linked to later stages of tumorigenesis<sup>6,8,9</sup>. Activating mutations, specific to FGFR4, at amino acids 535 and 550 of the kinase domain

were identified in 7.5% of RMS tumors and are thought to promote tumor growth and metastasis by constitutive activation <sup>6,9,10</sup>.

## **1.2. Targeted cancer therapy**

As knowledge and understanding of molecular origin of cancer expands it enables development of novel therapies. These targeted agents specifically interfere with key molecular events responsible for tumor development, unlike conventional chemotherapy. As a result of that property, widening of the therapeutic window is expected along with providing opportunities for combining therapies with other anticancer drugs without overlapping toxicity <sup>11</sup>. The goal of targeted cancer therapy or precision medicine is to describe molecular architecture of tumor cells at different stages of tumorigenesis and detect features amenable to therapeutic hindering <sup>12</sup>. As signaling pathways through RTKs regulate cell proliferation, survival and differentiation, processes behaving abnormally in tumor cells, they make for a promising target, and indeed many of to date developed targeted therapies which affect and interfere with interactions between signaling proteins and pathways <sup>12</sup>.

### **1.2.1. Drug discovery**

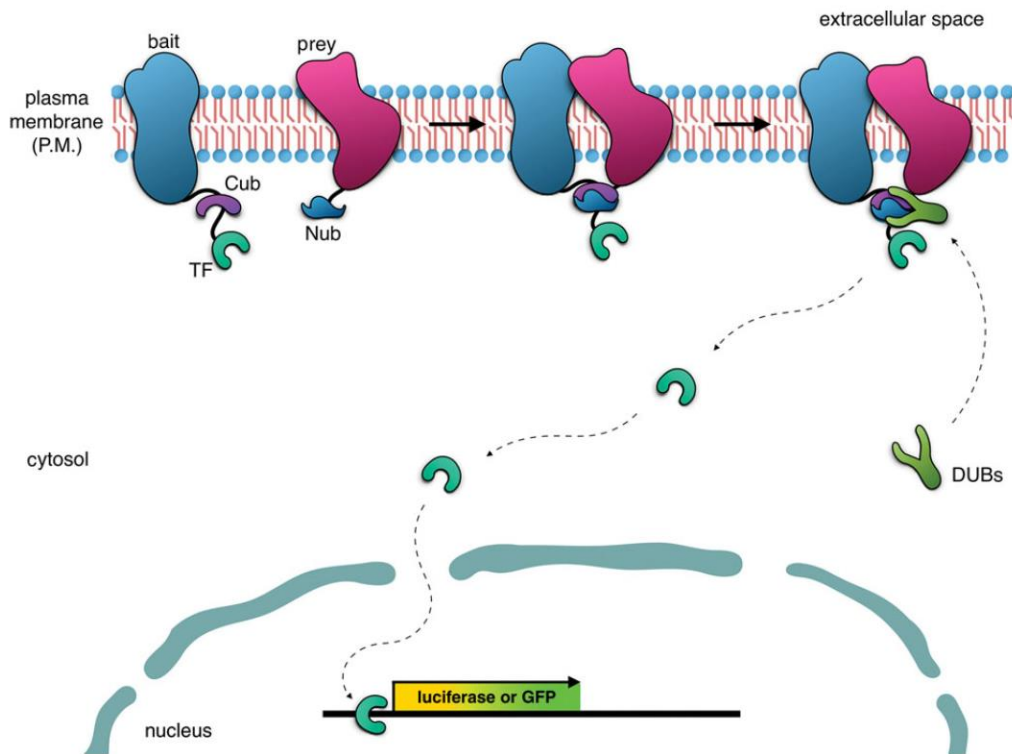
Drug discovery is an exhaustive process which allows for finding new therapies and medication candidates. With success of first wave of molecular therapeutics that specifically attack oncogenic pathways, and new findings in genomic profiling of cancers, this field has grown and evolved. It involves several steps and is usually a lengthy process. To accelerate development of new therapies, high-throughput technologies have arisen as an effective strategy. High-throughput screening (HTS) is an extremely eminent approach to identifying small molecule hits on a novel target. It involves using biochemical and cell-based assays to screen collections of compounds. When structures of targets are known and can be modelled, virtual libraries containing “drug-like” compounds can be screened *in silico* using computer algorithms. Fragment based screening is an approach that relies on X-ray crystallography or nuclear magnetic resonance to search for hits. Following identification of screening hits, their quality is evaluated through investigating their physicochemical properties <sup>12,13</sup>. Novel methods to investigate molecular targets such as protein-protein interactions have emerged in recent years and are rapidly modified and expanded into large scale platforms.

### 1.3. MaMTH assay

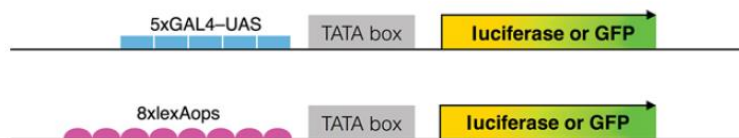
The Mammalian Membrane Two-Hybrid (MaMTH) assay was developed by Dr. Stagljar's group and first published by Julia Petschnigg *et al.* It is a technology emerged from Membrane Yeast Two-Hybrid (MYTH) assay<sup>14</sup> which was based on earlier split ubiquitin approaches<sup>15</sup>. MaMTH enables studying protein-protein interactions (PPIs) of full-length integral membrane proteins or membrane associated proteins in their natural context in live mammalian cells<sup>16,17</sup>.

A membrane protein of interest that is assigned as “bait” is tagged with C-terminal portion of ubiquitin (Cub) and a chimeric transcription factor (TF). Cytosolic or membrane-bound “prey” protein is tagged with the N-terminal portion of ubiquitin (Nub). Upon interaction of “bait” and “prey” proteins, Cub and Nub associate thus forming “pseudo-ubiquitin” which is recognized by cytosolic deubiquitinating enzymes (DUBs). Recruited DUBs then cleave the “pseudo-ubiquitin” construct consequently releasing TF which was fused to Cub. After entering the nucleus, TF binds to TF binding sites located upstream of the reporter system and, by doing so, activates it (Figure 1). Stably integrated reporter system could be either luciferase or Green Fluorescent Protein (GFP) with respective TFs used to activate it (Figure 2), while the reporter construct can consist of either five repeats of *GAL4* upstream activating sequence or eight *lexA* operator repeats followed by the reporter gene which make up the TF binding region<sup>18, 19</sup>. When it comes to luciferase reporter system, two options are possible: using the Firefly luciferase which is intracellular and requires cell lysis prior to measuring luciferase activity in presence of substrate or using *Gaussia princeps* luciferase which is secreted into the media the cells grow in and measurement is done without lysis. Expression of “bait” and “prey” proteins can be achieved by transiently transfecting with both proteins, generating reporter cells that are also stably expressing either “bait” or “prey” followed by transient transfection of the other or by generating reporter cells stably expressing both proteins.

The assay is a robust tool for detecting binary PPIs, mapping the interactome of membrane proteins and characterizing how these interactions are affected by different conditions. MaMTH can be used to detect changes in interaction following ligand binding, starvation, posttranslational modifications and is suitable for observing interaction patterns of wild type and mutant proteins or drug-inhibited interactions. As MaMTH can identify changes in PPIs acquired by aberrant signalling pathways, it can contribute to clarifying disease mechanisms and defining new drug targets<sup>16, 18, 19</sup>.



**Figure 1. Schematic outline of MaMTH.** The assay allows for identification of PPIs through detection of transcriptional activation of a reporter gene. That detection is dependent upon interaction of “bait” and “prey” protein which are fused each to a segment of split ubiquitin molecule. In an event of interaction, the segments reunite into “pseudo-ubiquitin” which can consequently recruit deubiquitinating enzymes (DUBs). DUBs cleave the “pseudo-ubiquitin” hence releasing the transcription factor (TF) fused to its C-terminus. TF then enters the nucleus and activates the reporter system located downstream of multiple TF binding sites <sup>17</sup> (Saraon et al, 2017.)



**Figure 2. Reporter constructs stably expressed in MaMTH reporter cells.** Two reporter system that could be used in MaMTH assays. (Saraon et al, 2017.)

### 1.3.1. MaMTH-DS method

Recently, MaMTH had been adjusted to MaMTH-Drug screening platform (MaMTH-DS), a high-throughput method developed to carry out screenings of small molecules in order

to detect compounds specifically targeting functional interactions of RTKs. The modifications required for expanding the assay in such manner included generating reporter cells stably expressing “bait” and “prey” proteins, have increased adherence to tissue culture plastic and use the *Gaussia princeps* reporter system. All these modifications minimized variability and noise, allowed for automated handling and more sensitive detection of change in activity <sup>20</sup>.

#### **1.4. Hypothesis and aim of the study**

The hypothesis is that the interaction of FGFR4 wild type receptor variant with the downstream signalling protein Shc1 and the interaction of oncogenic mutant variant of FGFR4 with the same downstream protein will be unequally susceptible to the effect of small inhibitory molecules.

Aims of the study were to investigate impact of 5570 small molecules on the interaction of FGFR4 V550E with protein Shc1 and determine which compounds significantly inhibit said interaction. Subsequently examining effect of identified inhibitors on the interaction of wild type FGFR4 and Shc1 and with that find small molecules that specifically inhibit the mutant variant of FGFR4.

## 2. Materials and methods

### 2.1. Cell culture

During development of the MaMTH assay in the lab of Dr. Igor Stagljar, various HEK 293 reporter cell lines were created. Preceding this work, cell lines with stable expression of FGFR4 wild type and FGFR4 V550E baits were generated by Dr. Punit Saraon, using the FLP-In TREx technique on a reporter cell line HEK 293 which has stably integrated *Gaussia princeps* luciferase under control of the Gal4/UAS system. Upon expression, luciferase is secreted into the media. Also, these MaMTH reporter cells have increased adherence to tissue culture plates due to stable integration of macrophage scavenger receptor (MSR1) surface protein.

Cell culture work was performed in CLASS II Type A2 (*Microzone Corporation*) biological safety cabinet. Cells were grown and maintained at 37 °C, 5% CO<sub>2</sub> in the cell culture incubator Hera cell 150i (*Thermo Fisher Scientific*), in 10 cm tissue culture dishes containing Dulbecco's Modified Eagle Medium (DMEM) (*Multicell, Wiesent Bioproducts*) with 10% Fetal Bovine Serum (FBS) (*Multicell, Wiesent Bioproducts*) and 1% Penicillin/Streptomycin (P/S) (10000 U/ml / 10000 µg/ml, *Gibco*). When full confluency was reached, cells were passaged.

### 2.2. Cell passaging

After confirming cells were ready to be split by observing under a microscope (*Vista Vision, VWR*), old media was aspirated followed by washing of the cells with 5 ml of Dulbecco's Phosphate Buffered Saline (PBS) (*Sigma Aldrich*) at room temperature to remove leftover media and detached cells. PBS was then also aspirated and 1 ml of TrypLE Express (*Gibco*) added to dissociate the viable, attached cells. Incubation was at 37 °C for 10 minutes after which 4 ml of pre-warmed media was added to inactivate TrypLE Express. When maintaining cultures, 1 ml of cell suspension was added to a new culture dish and diluted with 9 ml of fresh media. When cells were supposed to be seeded for an experiment, 50 µl of cell suspension was diluted with 450 µl of media and cells were counted with Scepter™ 2.0 Cell Counter (*Millipore*).

## **2.3. Generation of HEK 293 FGFR4 WT “bait” with Shc1 “prey” and HEK 293 FGFR4 V550E “bait” with Shc1 “prey” double stable cell lines**

### **2.3.1. Thawing of FGFR4 WT and FGFR4 V550E frozen stable cell lines**

Both cell lines were stored at -80 °C in freezing media (DMEM with 10% DMSO). The vials were held in hand to thaw the cell suspension. When liquid, the entire volume (1 ml) was added to 9 ml of fresh media, resuspended and then centrifuged for 3 minutes at 1000 rpm at room temperature in the centrifuge 5810R (*Eppendorf*) to wash and separate the cells from the freezing media. Supernatant was removed, 10 ml of fresh media added to the cell pellet and resuspended. The diluted cell suspension was then transferred to a 10 cm cell culture dish and placed in the incubator.

### **2.3.2. Single sorting of thawed cells**

Single sorting was done by Fluorescence-activated cell sorting (FACS). The thawed FGFR4 WT and FGFR4 V550E single stable cell lines were grown to confluency. After aspirating media, cells were washed with PBS and dissociated using TrypLE Express. To stop the reaction 4 ml of FACS sorting buffer was used. Sorting buffer contained 1x PBS, 1 mM Ethylenediaminetetraacetic acid (EDTA), 25 mM HEPES (pH 7.0) and 1% FBS. This was followed by collection of the cells into a 15 ml tube and spinning down for 3 minutes at 1000 rpm using the centrifuge 5810R (*Eppendorf*). Supernatant was discarded and cells were resuspended in fresh sorting buffer. Cells were counted and diluted to 1 million cells per ml. Prepared samples had to be filtered to eliminate large aggregates which was done by passing the samples through nylon mesh with a pore size of 40 µM using BD Falcon 5 ml tubes with 40 µM filter top caps. Individual cells were then sorted into 96-well plates using FACS Aria II Flow Cytometer (*BD Biosciences*).

### **2.3.3. Expansion and validation of single sorted cells**

Monoclonal populations were gradually expanded and individually tested with MaMTH for reporter activity and Western blot for “bait” expression. Cell line of each “bait” variant that showed strong activity was chosen to be used for generating double stable cells by stably integrating “prey” protein.

### 2.3.4. Integrating Shc1 “prey” into FGFR4 WT and FGFR4 V550E using CRISPR/Cas9 genome editing

Expanded single sorted FGFR4 WT and FGFR4 V550E cells were grown in a 6-well format, seeded 100 000 cells per well to around 70% confluency. Transfection mixture was prepared as stated in Table 1. and was added to cells in dropwise fashion after which the plate had been gently swirled to ensure even distribution of complexes. The plate was then left at room temperature for 20 minutes, incubated for 5 hours at 37 °C, 5% CO<sub>2</sub> followed, after which transfection media was replaced with 2 ml of fresh media.

**Table 1.** Transfection mixture for integrating Shc1 “prey”.

Serum free OptiMEM		97 µl	
Xtreme Gene 9		3 µl	
Plasmid DNA	iCRISPR gRNA Temp2 px330	333 ng	1 µg
	AAV gRNA px330	333 ng	
	N-prey (Shc1 or Pex7), Zeocin resistance	333 ng	
<i>Plasmids were added to OptiMEM first, then Xtreme Gene 9. The mixture was pipetted up and down 30 times in order to mix the complexes well and left to incubate at room temperature for 15 minutes.</i>			

### 2.3.5. Expansion and validation of generated double stable cell lines

Transfected cells were left to grow for 48 hours and were then split into new plates with media containing 0.5 µg/ml zeocin for selection and grown until individual foci appeared. Foci were expanded and resulted in cell populations which were then tested with MaMTH for reporter activity and Western blot for stably integrated “bait” and “prey” expression.



### **2.3.6. Freezing of generated double stable cells**

After confirming activation and “bait” and “prey” expression, cell lines were grown and frozen. After detaching, cells were centrifuged for 3 minutes at 1000 rpm using 5810R (*Eppendorf*) and resuspended in freezing media (90 % DMEM/10% FBS/ 1% P/S + 10 % DMSO). Aliquots of 1 ml of so prepared cell suspension were frozen and kept in liquid nitrogen.

## **2.4. Western blot**

### **2.4.1. Cell lysis and sample preparation**

In preparation of the experiment cells were seeded in a 6-well plate, 100 000 cells per well after which they were grown until confluent. Following steps were done with the plate on ice. Media was removed and the cells were washed with 500 µl of ice-cold PBS. Upon removal of PBS, 120 µl of cell lysis buffer (1x dilution of the Cell Lysis Buffer 10x, *Cell Signalling Technology*) supplemented with protease inhibitors was added. Plate was then left to shake for 10 minutes at 4 °C. Afterwards, cells were scraped off and vortexed at 4 °C. The lysate was then centrifuged for 20 minutes at 4 °C at 16000 x g (*Microfuge 18, Beckman-Coulter*). Supernatant was collected, mixed with 4x Sample buffer and heated for 5 minutes at 95 °C. Sample buffer was prepared using 40% glycerol, 250 mM Tris-Cl (pH 6.8), 8% sodium dodecyl sulphate (SDS), 0.04% bromophenol blue and 5% 2-mercaptoethanol. The samples were then stored at -20 °C.

### **2.4.2. Total Protein Assay**

The concentration of proteins in a sample was determined using Pierce BCA Protein Assay Kit (*Thermo Fisher Scientific*). The standard curve was calibrated using a series of albumin dilutions: 0, 31, 62.5, 125, 250, 500, 1000, 2000 ng/µl. Volume of 10 µl of each dilution was added to a 96-well plate in triplicates, while 2 µl of sample, before mixing with 4x Sample buffer, was added in duplicates. The working reagent was prepared as stated in the manufacturer’s protocol of which 100 µl was added to each well. The plate was incubated for 30 minutes at 37 °C and subsequently absorption was measured at 562 nm using CLARIOstar® *Plus* microplate reader (*BMG LABTECH*).

### 2.4.3. Electrophoresis and Western blotting

The proteins were separated by SDS- PAGE electrophoresis on 10% resolving gels containing per 15 ml: 5.9 ml of ddH<sub>2</sub>O, 5.0 ml of 30% Acrylamide/Bis Solution, 3.8 ml of Tris, pH 8.8, 0.075 ml of 20% SDS, 0.15 ml of 10% Ammonium peroxydisulfate (APS) and 0.006 ml of Tetramethylethylenediamine (TEMED). For 4 ml of stacking gel was used: 2.7 ml ddH<sub>2</sub>O, 0.67 ml of 30% Acrylamide/Bis Solution, 0.5 ml of Tris, pH 8.8, 0.02 ml of 20% SDS, 0.04 ml of APS and 0.004 ml of TEMED. Running buffer 10x was prepared by mixing 250 mM Tris, 960 mM glycine and 1% SDS with 1 L ddH<sub>2</sub>O and diluted to 1x prior to use. To perform electrophoresis Mini-PROTEAN Tetra Cell (*BioRad*) apparatus was used. Samples were loaded in volumes that corresponded to equal amounts of proteins, and PageRuler™ Plus Prestained Protein Ladder (*Thermo Scientific™*) was used as a molecular weight marker. Voltage of 150 V was applied for 55 to 60 minutes. Thereafter, proteins were transferred from the gel onto a nitrocellulose membrane by wet transfer in 1x Transfer buffer. 10x transfer buffer was prepared by mixing 150 mM Tris base and 1.2 M glycine with 1 L of ddH<sub>2</sub>O and diluted to 1x prior to use. The transfer was done at 300 mA lasting for 90 minutes using Criterion™ Blotter (*BioRad*) apparatus. To verify transfer quality, membranes were then stained with Ponceau S (*Sigma Aldrich*) for 30 seconds and washed with 1% acetic acid (*BioShop*). Tris-buffered saline/Tween 20 (TBST) prepared by dissolving 20 mM Tris, 150 mM NaCl and 0.1 % of Tween 20 in 1 L of ddH<sub>2</sub>O, pH adjusted to 7.6, and diluted to 1x prior to use. Washing with TBST fully destained the membranes which were then blocked for 2 hours on a rocking platform with 2% Bovine Serum Albumin (*BioShop*) in TBST blocking solution. This was followed by overnight incubation of membranes in primary antibodies diluted in 1x TBST to 1:10000 ratio, at 4 °C on a rocking platform. Washing of the membranes with 1x TBST was done in three rounds of 15 minutes, prior to incubation with secondary antibodies diluted also to 1:10000 ratio, on a rocking platform at room temperature, lasting for one hour. Next was three more rounds of 1x TBST washing for 15 minutes and imaging. That was done by soaking the membranes in SuperSignal™ West Pico PLUS Chemiluminescent Substrate (*Thermo Scientific™*) prepared according to the instructions of the manufacturer and developed using SRX-101A (*Konica Minolta*) developer.

## **2.5. MaMTH assay**

### **2.5.1. Cell seeding**

MaMTH reporter cells were grown as described in chapter 2.1. and detached as described in chapter 2.2. After cell counting, cell suspension was diluted with fresh media to a concentration which allowed seeding 10 000 cells per well of a 96-well plate in a volume of 95  $\mu$ l per well. The plate was then placed into the incubator and left to grow for 18 hours or to reach confluency of 60-70%.

### **2.5.2. Transfection and induction**

The following day the mixture for transfection was prepared by diluting 0.4  $\mu$ l of MaMTH “prey” plasmid in 2  $\mu$ l of PBS as mix A and 0.12  $\mu$ l Polyethylenimine (PEI) (*Sigma-Aldrich*) again in 2  $\mu$ l of PBS as mix B, amounts stated per replicate. The concentration of the MaMTH “prey” plasmid was 100 ng/ $\mu$ l. Mix A and B were combined, mixed by pipetting up and down 10 to 15 times and left to incubate at room temperature for 10 minutes. Then, 4  $\mu$ l of transfection mix was added to each well, the plate was stirred gently and placed back in the incubator. All transfections were done in triplicates. Five hours later, 5  $\mu$ l of media with tetracycline (*BioShop*) was added so that the final concentration of Tetracycline per well was 0.5  $\mu$ g/ml. Plates were incubated at 37 °C/5% CO<sub>2</sub> over the next 18 hours.

### **2.5.3. Luciferase reading**

The substrate solution was prepared the following way. Coelenterazine (CZ) (*NanoLight Technology*) was diluted in PBS to a concentration of 20 mM and left in dark for 30 to 45 minutes. In the meantime, 5  $\mu$ l of the media from the 96- well plates were aliquoted into a 96-well plate with white bottom and walls and diluted with PBS to a volume of 100  $\mu$ l. The plate was then incubated for 10 to 15 minutes at room temperature on a rocking platform. Luminescence was then measured using CLARIOstar® *Plus* (*BMG LABTECH*).

## **2.6. Assessment of positive controls**

Compounds Ponatinib and BLU 9931, which were previously reported to have an inhibitory effect on FGFR4, were tested using MaMTH assay to confirm they could be used as positive controls for the drug screen. FGFR4 V550E cells were seeded as reported previously. A day later they were transfected with Shc1 “prey” as described and 5 hours later, along with inducing with tetracycline, cells were treated with the Ponatinib and BLU 9931. Luciferase reading was done as described.

## **2.7. FGFR4 V550E-Shc1 drug screen using MaMTH-DS**

### **2.7.1. Cell preparation and seeding**

A week prior to seeding for the screen, FGFR4 V550E-ShcI cells were thawed as described in chapter 2.3.1. They were left to recover and grow until confluent which took 3 days. They were split and passaged once in order to grow required number of cells for the screen. On the day of seeding they were detached as described in chapter 2.2., counted and diluted in fresh media. The rest of the work was done in collaboration with the lab of Dr. Alessandro Datti of S.M.A.R.T. Facility, Center for High-Throughput Screening, of Lunenfeld Tanenbaum Research Institute at Mount Sinai Hospital, Toronto. Cells were seeded in 384-well plates with clear bottom and white walls, 5000 cells per well, using Multidrop<sup>TM</sup> Combi Reagent dispenser (*Thermo Scientific*) and placed in an incubator MCO-19AIC (*Sanyo*) at 37 °C, 5% CO<sub>2</sub> to grow for 18 hours.

### **2.7.2. Induction and treatment**

Next day, media with Tetracycline was prepared, as well as a 384-well plate with Ponatinib and BLU 9931 as positive and DMSO as negative controls. Using the automated system at S.M.A.R.T. compounds from libraries (Table 2) and Multimek<sup>TM</sup> (*Beckman Coulter*) automated pipettor, controls were added to the cells to a final concentration of 4 µM and media with tetracycline (final concentration 0.5 µg/ml). Cells were again left to incubate for 18 hours.

**Table 2.** List of libraries of small molecules used for the drug screen.

<b>Library</b>	<b>Number of compounds</b>
OICR Kinase inhibitors	560
Lopac	1263
NIH	446
Prestwick	1120
Selleck	885
Tocris	1296

### 2.7.3. Luciferase reading

Substrate solution was prepared in the same manner as before. Luminescence measuring was done by injecting substrate directly into the plates with cells using PHERAstar® FSX (BMG LABTECH).

### 2.7.4 Data analysis

Readings were analysed with an in house made software (developed by Dr. Jamie Snider) using R programming language<sup>20, 21</sup>. Raw data was transformed using Box-Cox transformation<sup>22</sup> for the purpose of improving symmetry and normality of data distribution. Values of  $Z'$ <sup>23</sup> were calculated on a per plate basis with FGFR4 V550E-Shc1 treated with BLU 9931 as a positive control and FGFR4 V550E-Shc1 with added DMSO as negative control.  $Z'$  is a method of verifying suitability of an assay for use on a high-throughput scale. If a single most extreme value of separate control datasets was classified as an outlier relying on a cut-off of 1.5 times the interquartile range, it was excluded from the  $Z'$  value calculation. In order to normalize data, Normalized Percent Inhibition (NPI) and BScore were used. NPI is based upon controls and calculated as  $(\text{Negative Control Signal} - \text{Sample Signal}) / (\text{Negative Control Signal} - \text{Positive Control Signal}) * 100$ , whereas BScore was calculated using cellHTS2 package. A combined cut-off of 70% NPI and a BScore of -3 or less was used for rating inhibitory hits.

## **2.8. Retesting of inhibitory hits against FGFR4 V550E-Shc1 and FGFR4 WT-Shc1**

### **2.8.1. Cell preparation, seeding and induction**

Analysis of results from the FGFR4 V550E-Shc1 screen against compounds from the aforementioned libraries resulted in a number of inhibitory hits. Those were then singled out and used in the second round of screening which included retesting on the FGFR4 V550E-Shc1 cells as well as testing their effect on the FGFR4 WT-Shc1 in order to investigate specificity towards the mutated variant. This round of experiment was done in duplicates.

Cells of both cell lines were thawed and left to recover and grow in the same manner as for the first round of the screen. On the day of seeding they were detached as described in chapter 2.2., diluted to desired concentration with fresh media and tetracycline was added to the suspension immediately (final concentration of tetracycline 0.5 µg/ml). Prepared cell suspension was taken to S.M.A.R.T. Facility and 5000 cells were seeded per well in 384 well plates with white walls and clear bottom using Biomek® FX automated liquid handler (*Beckman Coulter*).

### **2.8.2. Treatment**

Cells were kept in the incubator at 37 °C, 5% CO<sub>2</sub> for 3 hours and subsequently treated with controls, Ponatinib and BLU 9931 as positive and DMSO as negative, along with the preselected compounds. The addition was done with Multimek™ 96 automated pipettor (*Beckman Coulter*). The plates were then returned to the incubator at 37 °C, 5% CO<sub>2</sub>.

### **2.8.3. Luciferase reading**

Luminescence was measured 48 hours after treatment. It was performed the same way as in the first round of the screen.

### **2.8.4. Data analysis**

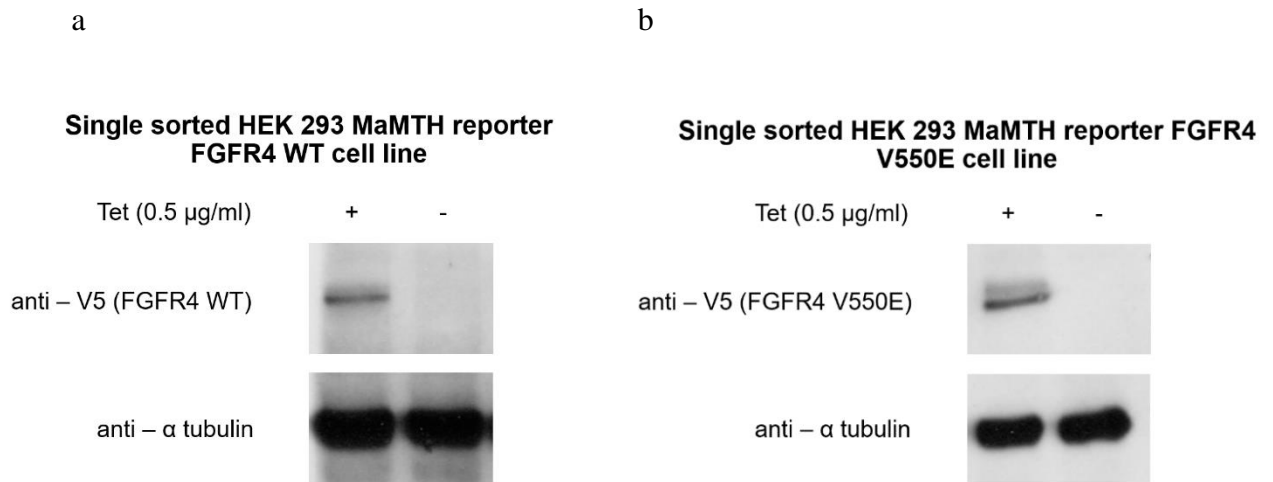
Percent of activity was calculated for each sample relative to negative control on a per plate basis and averaged between the duplicates of each cell line tested. This was followed by identifying compounds that have decreased activity of FGFR4 V550E-ShcI by more than 50% while keeping percent of activity of FGFR4 WT-ShcI above 50%.

### **3. Results**

#### **3.1. Generation of HEK 293 FGFR4 WT “bait” with Shc1 “prey” and HEK 293 FGFR4 V550E “bait” with Shc1 “prey” double stable cell lines**

##### **3.1.1. Validation of “bait” expression in single sorted FGFR4 WT and FGFR4 V550E stable cell lines**

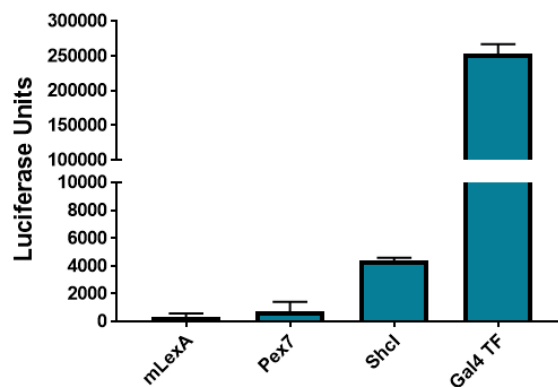
MaMTH reporter cell lines with stably integrated “bait” protein FGFR4 in two variants, wild type and oncogenic V550E mutation of the kinase domain, were thawed upon start of the study. After recovery and preliminary MaMTH assays they were single sorted in the interest of getting monoclonal cell populations for further use. Following expansion, selected populations were tested by Western blotting to confirm bait expression (Figure 3). For that purpose, anti-V5 tag antibody (*Cell Signaling Technology*) was used since FGFR4 protein was tagged with V5. As a loading control, expression of  $\alpha$ -tubulin was checked using anti-  $\alpha$  tubulin (*Santa Cruz Biotechnology*). MaMTH assay was used to assess activity of reporter system and to show interaction of FGFR4 variants with downstream adaptor protein Shc1 (Figure 4). Cells were transfected with mLexA as a background control since used cells are stably expressing reporter system with five Gal4 upstream activating sequence repeats, Pex7, a protein expected not to interact, as negative control and Gal4 TF as transfection and reporter control.



**Figure 3. Expression of FGFR4 WT and FGFR4 V550E “baits” in sorted MaMTH reporter cell lines.** Single sorted HEK 293 cell lines were cultured with and without 0.5 µg/ml of tetracycline for 18 hours and then lysed. “Bait” expression was checked using anti-V5 antibody, while anti-α tubulin was used to assess loading control. a) shows expression of FGFR4 WT. b) shows expression of FGFR4 V550E in reporter cells. Thereby “bait” expression after inducing with tetracycline was confirmed.

a

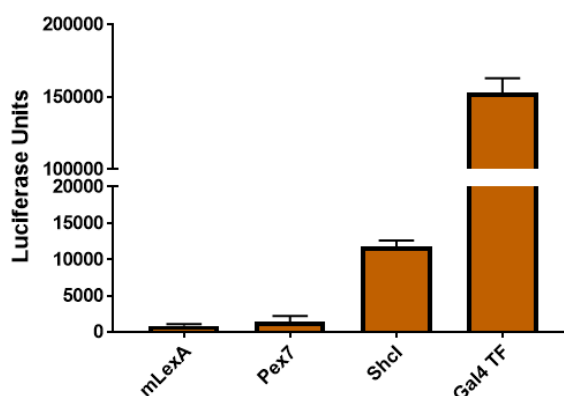
Single sorted HEK 293 MaMTH reporter FGFR4 WT cell line





b

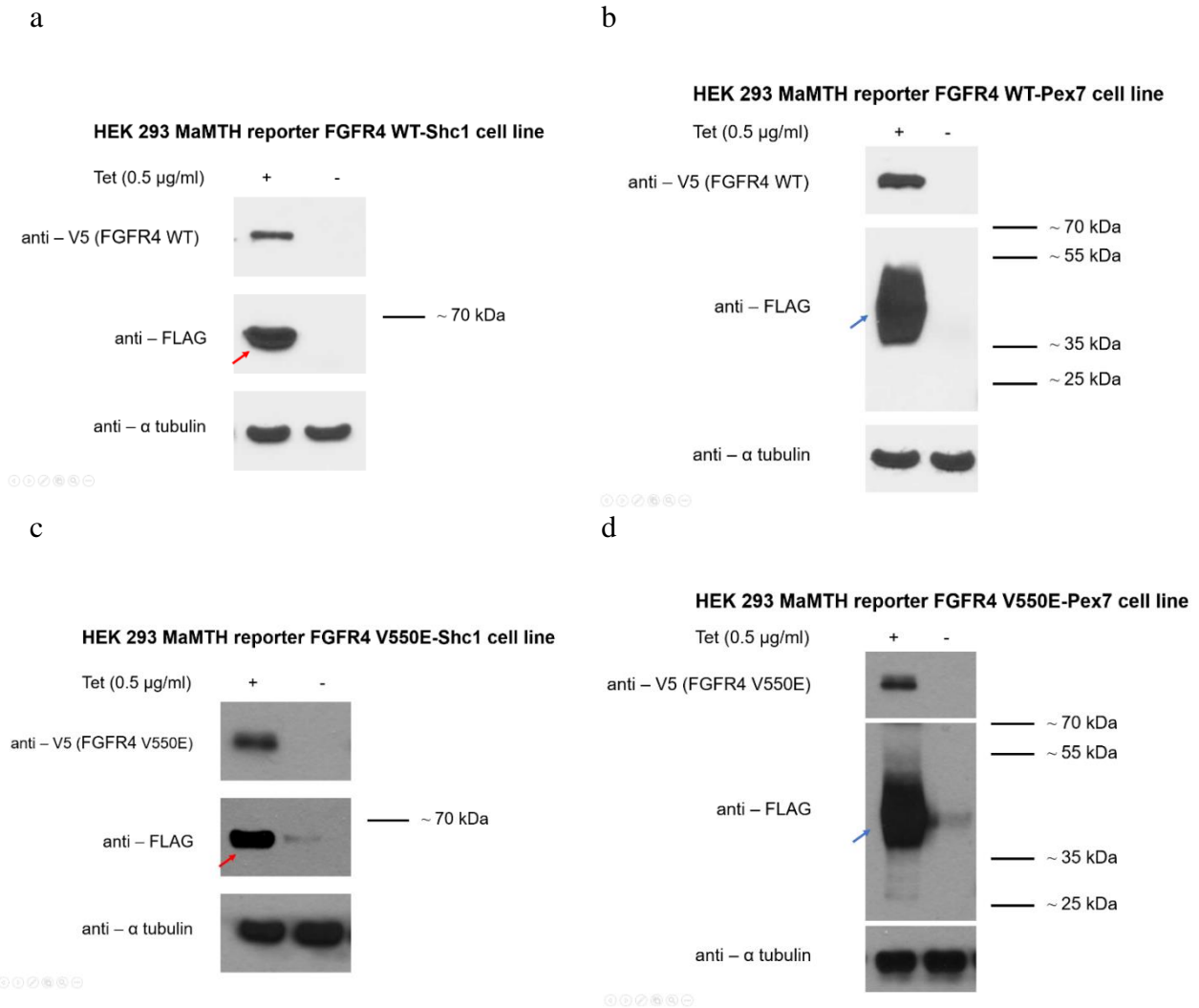
**Single sorted HEK 293 MaMTH reporter FGFR4 V550E cell line**



**Figure 4. MaMTH assay validation of sorted stable MaMTH reporter cell lines.** MaMTH assay was performed using sorted cell lines and confirmed activity of reporter system and interaction with Shc1. a) shows results from assay performed on FGFR4 WT which report very low background activity (mLexA), no interaction of negative control (Pex7), successful transfection (Gal4 TF) and interaction with adaptor protein Shc1. b) displays results of FGFR4 V550E which also indicate low background, successful transfection but more interaction with Shc1.

### 3.1.2 Validation of both “bait” and “prey” expression in FGFR4 WT-Shc1 and FGFR4 V550E-Shc1 double stable cell lines

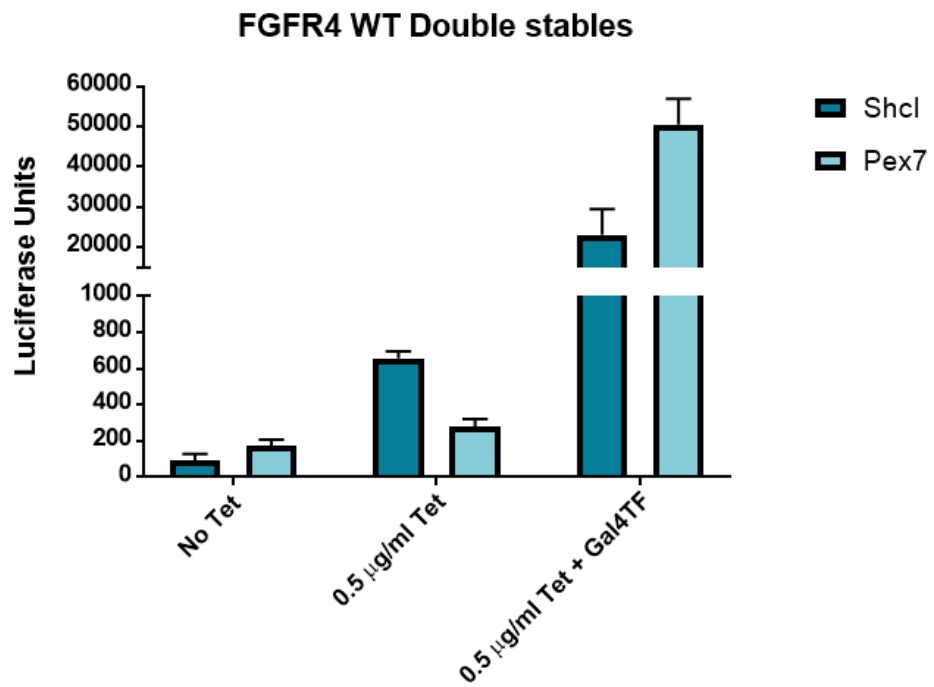
After selection and expanding of MaMTH reporter cells with stably integrated both “baits”, FGFR4 WT or FGFR4 V550E, and “prey”, Shc1, proper functioning of the system had to be verified. When generating double stable cell lines, a negative control cell line with stably integrated Pex7 was made as well, for both of “bait” variants. All four cell lines were analysed using Western blot to confirm expression of baits and preys (Figure 5). Antibodies used for detecting V5 tagged bait was anti-V5 antibody (*Cell Signaling Technology*), for detecting FLAG tagged preys, anti-FLAG antibody (*Sigma Aldrich*) and for loading control anti- $\alpha$  tubulin (*Santa Cruz Biotechnology*). All four cell lines were tested with MaMTH as well (Figure 6).



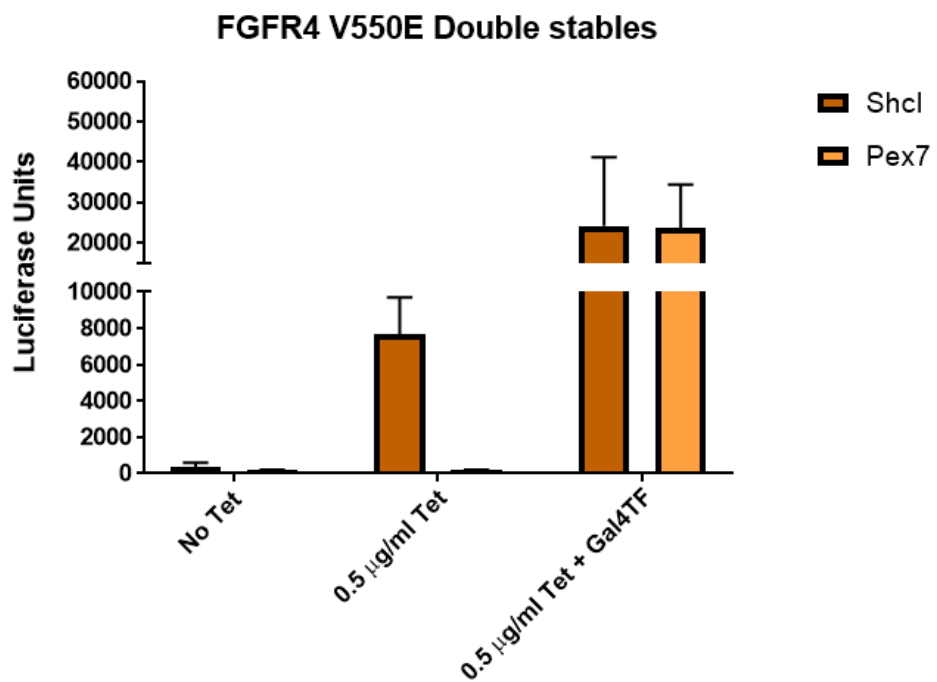
**Figure 5. Expression of stably integrated baits and preys into MaMTH reporter cell lines.**

Double stable cells were cultured with and without 0.5 μg/ml of tetracycline overnight. Anti-V5 tag antibody was used for bait detection, anti-FLAG tag antibody for prey detection and anti-α tubulin was used to assess loading control. a) and b) show expression of wild type “bait” and “prey” proteins Shc1 and Pex7 stably integrated into MaMTH reporter cell lines. c) and d) show expression of mutant “bait” and “prey” proteins Shc1 and Pex7. Red arrow on a) and c) marks FLAG tagged Shc1 prey determined by comparing theoretical size (~ 66 kDa) to position of band on the blot and molecular weight marker. Blue arrow in b) and d) points to FLAG tagged Pex7 prey (~ 44 kDa) determined in the same way.

a



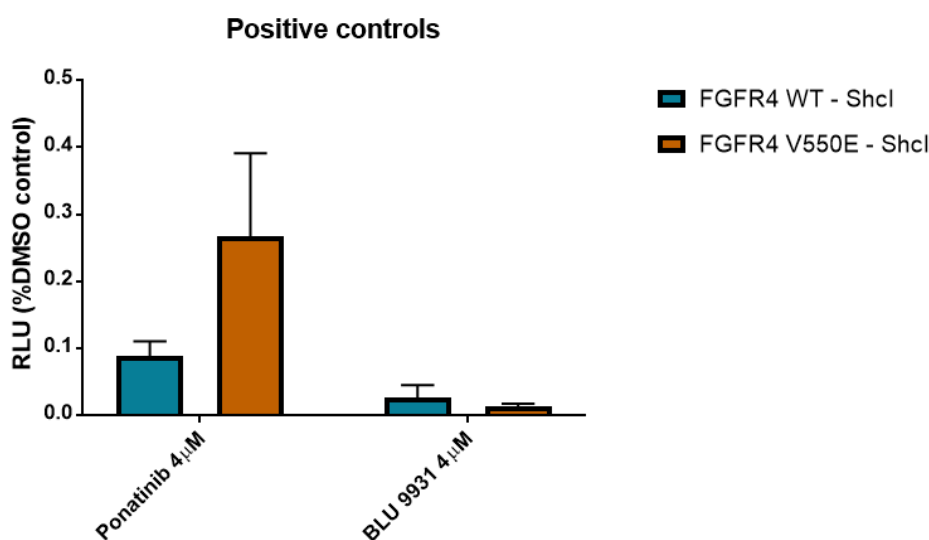
b



**Figure 6. MaMTH assay validation of double stable MaMTH reporter cell lines.** a) FGFR4 WT-ShcI in comparison to negative control FGFR4-Pex7 shows interaction of stably integrated bait and prey upon tetracycline induction, despite lower activity of reporter system than expected. This was addressed and resolved later in preparation for the screen. b) FGFR4 V550E-ShcI shows interaction in presence of tetracycline.

### 3.2. Positive controls for the drug screen

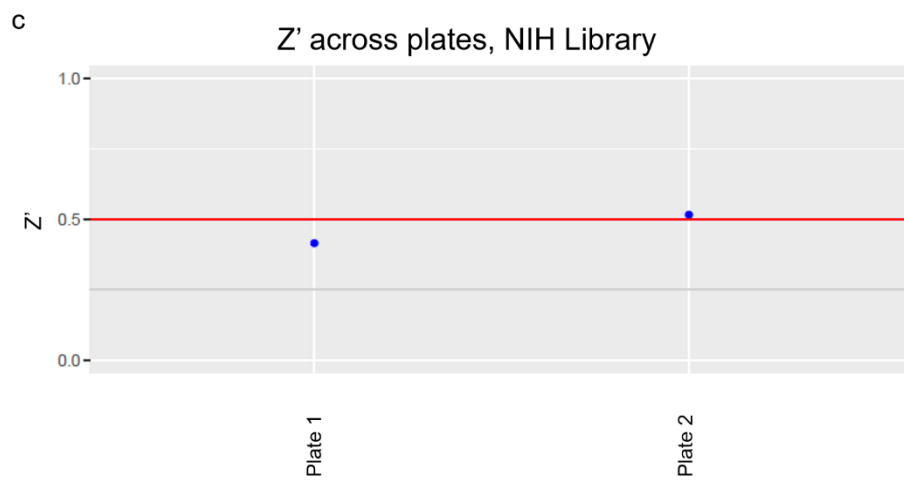
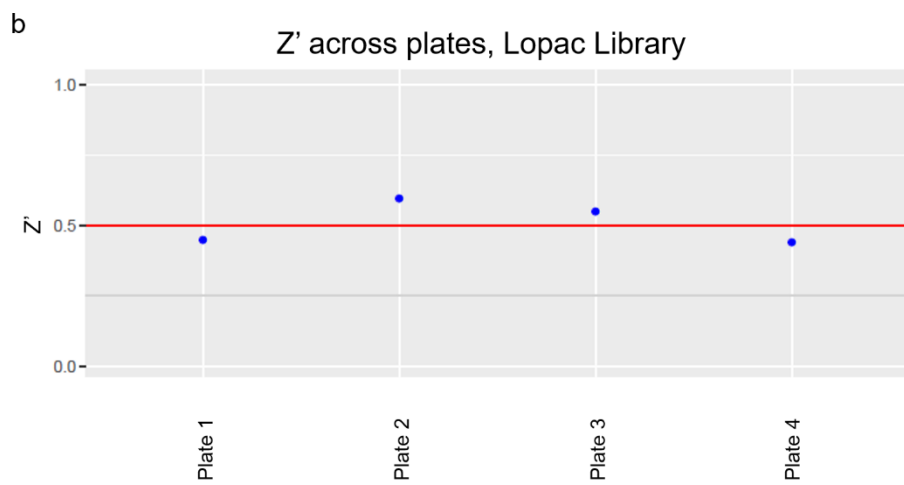
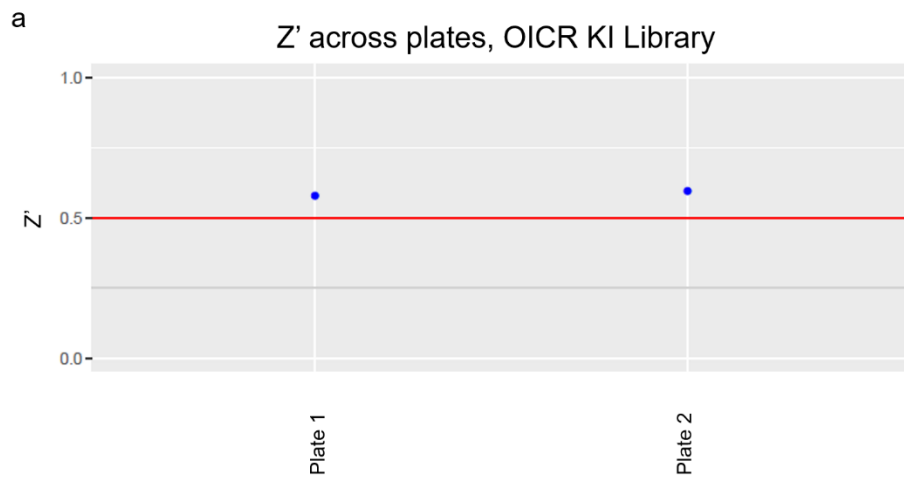
For the purpose of finding appropriate positive controls for the MaMTH-DS, compounds Ponatinib and BLU 9931 were tested against both FGFR4 WT and FGFR4 V550E (Figure 7). Both of these compounds were previously reported as FGFR4 inhibitors<sup>9, 24</sup>. The used concentration of 4  $\mu$ M was chosen to match the final concentration of compounds screened using MaMTH-DS. Assay showed substantial decrease of signal for both FGFR4 variants compared to DMSO control and hence inhibition, although BLU 9931 arose as more potent and almost equally so between FGFR4 WT and FGFR4 V550E. This showed they could indeed both be used as positive controls for drug screening, preferably BLU 9931, leaving a sizeable span of difference in activity that could be observed.

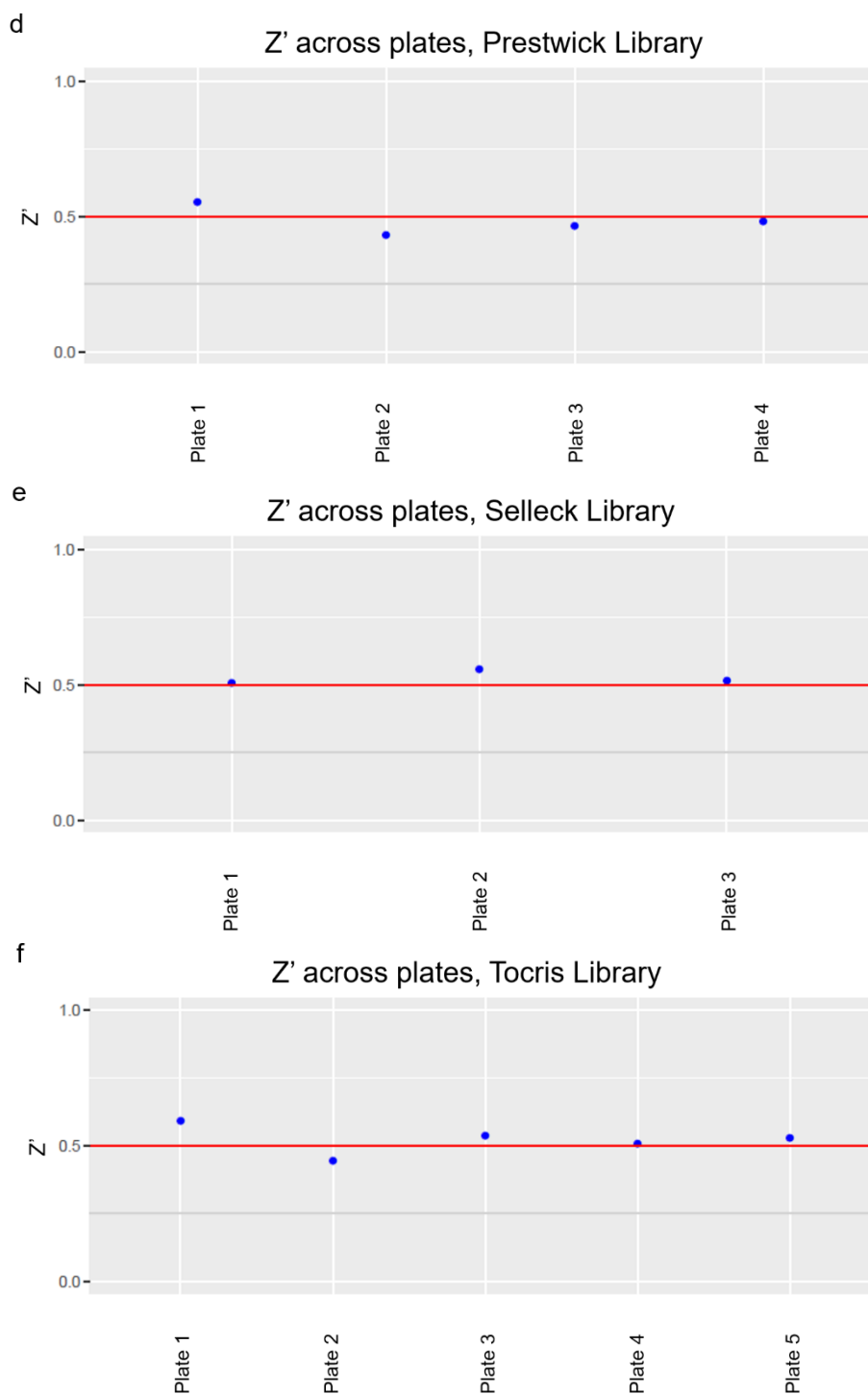


**Figure 7. MaMTH assay on FGFR4 WT-ShcI and FGFR4 V550E-ShcI after treating with Ponatinib and BLU 9931.** Both compounds showed strong inhibition at 4  $\mu$ M concentration, albeit BLU 9931 came up as more potent and with less variability.

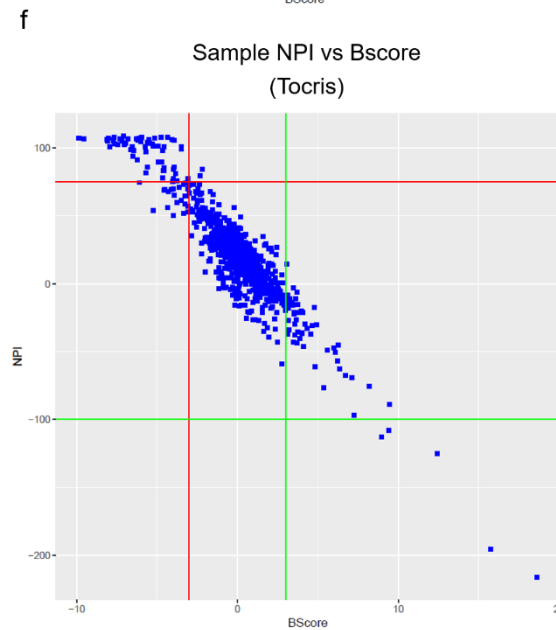
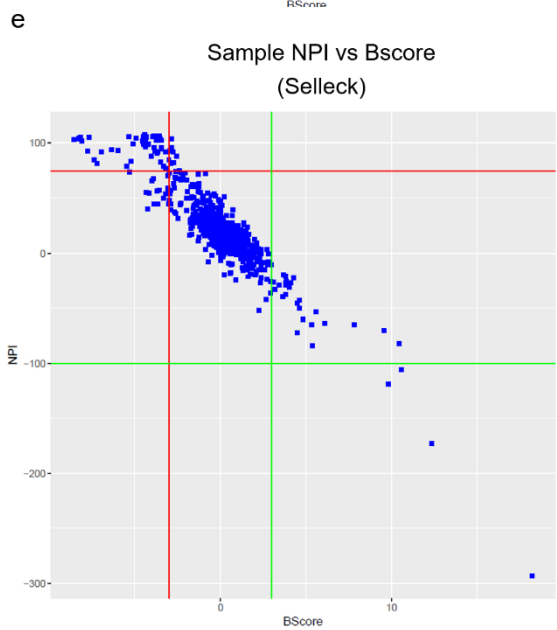
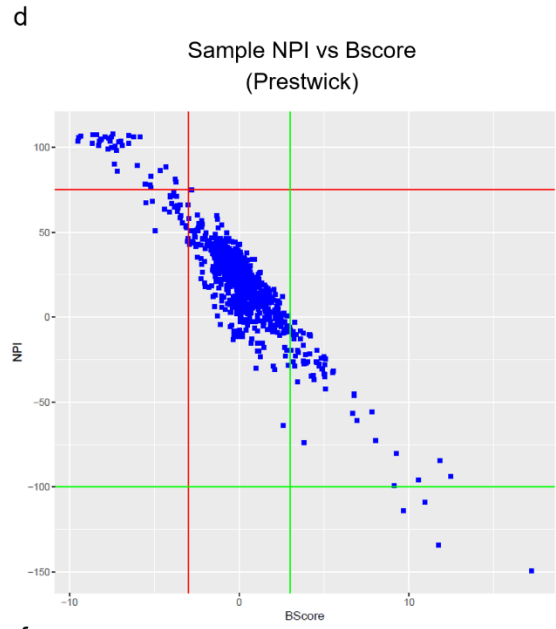
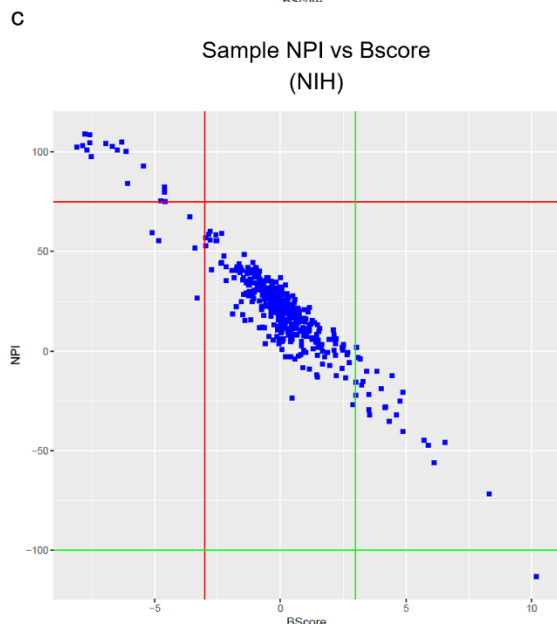
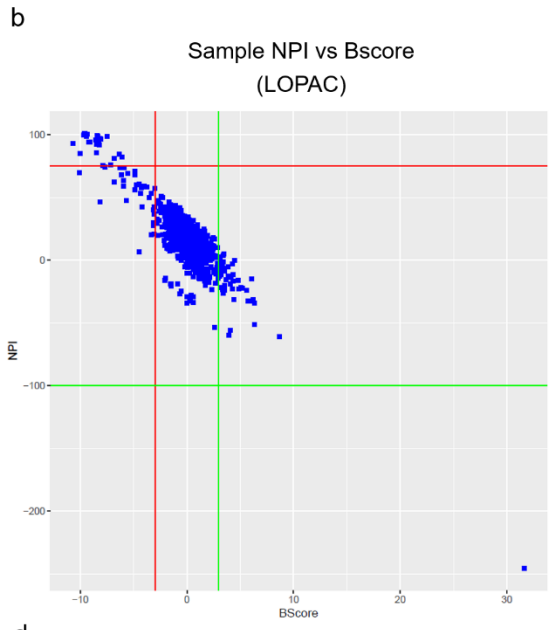
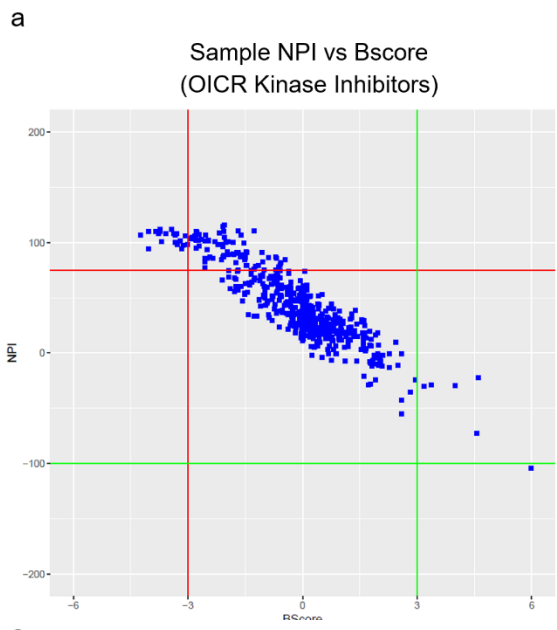
### **3.3. FGFR4 V550E-Shc1 inhibitory hits**

The first round of screening was performed on FGFR4 V550E-Shc1 double stable cells, at the S.M.A.R.T. Facility in Lunenfeld Tanenbaum Research Institute at Mount Sinai Hospital, Toronto. Six libraries of small molecules, 5570 compounds in total, were screened in a semi-automated high-throughput fashion using MaMTH-DS in a 384-well plate format. Raw data was subjected to Box-Cox power transformation before analysis for the purpose of improving symmetry and normality of sample data distribution. Calculated  $Z'$  values across all 20 plates were above 0.41 with an overall average of 0.51 which suggests the assay worked properly as  $Z'$  values that fall into the interval of 0.5 to 1.0 indicate an excellent assay. (Figure 8). To correct for plate variation and positional effects two approaches of data normalization were used, NPI, which is control based, and BScore which is sample based. Inhibitory hits were scored using a combined cut-off of higher than 70% NPI and a BScore of -3 or below (Figure 9). These conditions yielded 165 FGFR4 V550E-Shc1 inhibitory hits (Table 3).





**Figure 8. Z' values across all 20 plates.** All exceeding 0.41 and averaging 0.51. a) Z' values across two plates treated with compounds from OICR Kinase inhibitors library b) Z' values across four Lopac plates. c) Z' values across two NIH plates in. d) Z' values across four Prestwick plates. e) Z' values across three Selleck plates. f) Z' values across five Tocris plates.





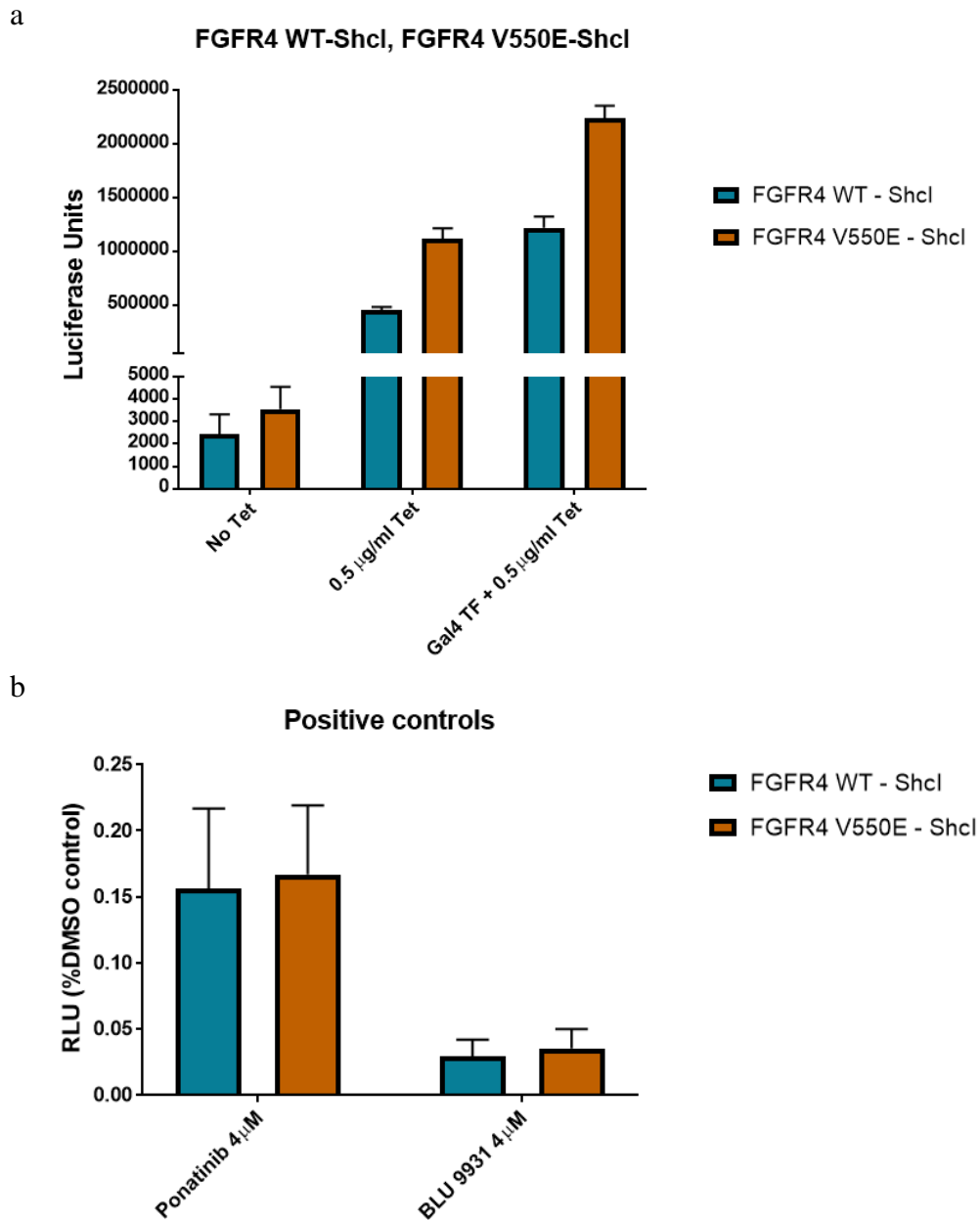
**Figure 9. NPI and BScore scatterplots.** Horizontal lines represent NPI values of 70% and -100%, red line indicating 70%, while vertical lines represent BScore values of 3 and -3, red line indicating -3. Values that fall in the upper left quadrant, enclosed with red lines, were designated as hits. Scatterplot of NPI and BScore values a) across the OICR Kinase inhibitors library, b) across Lopac library, c) across NIH library, d) across Prestwick library, e) across Selleck library, f) across Tocris library.

**Table 3.** Number of hits per library and total number of hits identified in the first round of screening.

Library	Number of compounds	Number of hits
OICR Kinase inhibitors	560	9
Lopac	1263	24
NIH	446	15
Prestwick	1120	34
Selleck	885	33
Tocris	1296	50
<b>Total</b>	<b>5570</b>	<b>165</b>

### 3.4. FGFR4 V550E-Shc1 specific inhibitory hits

For the second round of screening, hits identified from the first screen were singled out and only those compounds were used. Both FGFR4 V550E-Shc1 and FGFR4 WT-Shc1 cells were used and assay was performed in duplicates. Due to low signal of FGFR4 WT-Shc1 observed during validation of generated cell line, protocol was modified so the signal would increase and therefore enable more stringent analysis. It included seeding cells in media with added tetracycline and treating with controls and compounds after 2-3 hours. MaMTH assay data from optimization experiment is shown in Figure 10. Raw data from the second round of screening was analysed in the following way: after normalizing recorded sample activity to averaged activity of negative control BLU 9931 per plate, calculated value of sample activity was averaged between respective samples on two duplicate plates. Afterwards, values of samples exceeding 50% activity for FGFR4 WT-Shc1 were compared to values of corresponding samples for FGFR4 V550E-Shc1. Of those, ones with activity lower than 50% and at least twofold less active were scored as hits (Table 4). There were five compounds that fulfilled all criteria and were considered mutant specific hits (Figure 11).



**Figure 10. MaMTH reporter activity after optimization of protocol in preparation for the second round of screening.** a) Both cell lines were seeded in media (DMEM/10% FBS/ 1% P/S) with tetracycline and thus induced immediately. Luciferase was measured 48 hours later. b) Two hours after seeding Ponatinib and BLU 9931 were added to check if their effect could still be observed. As shown, both were very potent and the effect on each of the cell lines was more similar.

**Table 4.** Mutant specific inhibitory hits after analysis of second round of MaMTH-DS.

FGFR4 WT-ShcI	FGFR4 V550E-ShcI	Fold change	Compound
74.42	4.07	18.28	6-Azauridine
92.97	6.63	14.03	Antimycin A
207.67	34.20	6.07	Malonoben
52.51	11.96	4.39	Pralatrexate
89.41	38.51	2.32	Trifluridine



**Figure 11. Overview of MaMTH-DS workflow done with the aim of identifying oncogenic FGFR4 mutant specific hits.** First round of screening included testing effects of compounds from six libraries in a 384-well plate format and resulted in 165 inhibitory hits. Those were retested in the second round of screening including both mutant and wild type cell lines. Finally, five compounds were characterized as mutant specific hits.

#### 4. Discussion

Results of validation experiments showed generation of HEK 293 FGFR4 WT-Shc1 and HEK 293 FGFR4 V550E-Shc1 double stable cell lines was done successfully. By single sorting and expanding cells stably expressing “bait” protein of interest, monoclonal cell populations were obtained. Thereby noise was reduced and better chances of subsequently making cells stably expressing both “bait” and “prey” proteins secured. Also, it was shown that the reporter system is active and that both FGFR4 WT and FGFR4 V550E exhibited interaction with Shc1 “prey” protein. Those improvements in precision were expected from preliminary experiments using unsorted populations of cells in which same results were reported but with larger variability. As presented, following integration of Shc1 “prey” protein, double stable cells were indeed procured. Using of a high efficiency transfection reagent, adapted CRISPR/Cas 9 genome editing method and stringent selection process was chosen due to preliminary transfection experiments and this protocol resulted in highly successful integration. Both of the FGFR4 variants stably expressing Shc1 displayed reporter activity upon induction with tetracycline whereas both variants stably expressing Pex7 did not. This reconfirmed interaction of both “bait” variants with Shc1 as Pex7 is a protein unexpected to interact with neither FGFR4 WT nor FGFR4 V550E hence used as negative control. However, overall activity of FGFR4 WT-Shc1 was much lower than of the FGFR4 V550E-Shc1 which required optimization of the protocol used in further research. This could be due to V550E being an activating mutation<sup>10</sup> which would reasonably result in higher reporter activity in presence of “prey”. As the aim of the subsequent drug screening was to identify compounds specifically inhibiting the mutant variant as accurately as possible, it was necessary to improve readability of the high throughput version of the assay by increasing reporter activity and with that resolution of effective and ineffective compounds. The optimization was introduced in the second round of screening, for both FGFR4 WT-Shc1 and FGFR4 V550E-Shc1 cell lines, after testing out and confirming in small scale assays. New protocol included immediate tetracycline induction upon cell seeding and afterwards treatment as soon as the cells adhered, not only allowed for needed increase in reporter activity but also decreased variability which was observed among controls in the first round of screening, when only FGFR4 V550E-Shc1 cell line was used.

Usability of positive controls, Ponatinib and BLU 9931, previously reported as FGFR4 inhibitors<sup>9,24</sup> was confirmed. Ponatinib is an inhibitor of multiple tyrosine kinases<sup>9</sup> while BLU

9931 is a compound developed as a specific FGFR4 inhibitor <sup>24</sup> and obtained results show higher potency of BLU 9931 on both wild type and mutant variant. Consequently, all analysis was performed using results of treating with BLU 9931 which was proved as a powerful positive control.

Results obtained from the first round of drug screening, where only FGFR4 V550E-Shc1 cell line was tested against 5570 compounds, show inhibitory effect of 165 small molecules. By putting raw data through Box Cox power transformation to improve distribution and normality of sample data and then verifying quality of the assay by calculating Z', the conditions of performing valid analysis of results were satisfied. Then, using two approaches for normalization, one controls-based (NPI) one sample-based (BScore), addressed the problem of positional effects on each plate as well as variation between plates which was necessary in order to minimize potential bias in further analysis. NPI and BScore correlated really well and using a combined cut-off ensured more stringent filtering of hits than relying on only one as can be seen on scatterplots. As the aim was to find potentially powerful mutant specific inhibitors, performing a counter screen on wild type FGFR4 was most reasonable using only very potent inhibitors of FGFR4 V550E. Hit rates across libraries varied which was expected since they contain groups of related molecules or molecules used for similar purposes.

Performed research identified five compounds that specifically inhibit mutant activity. With this, the stated hypothesis was confirmed and aims achieved.

Compound Antimycin A showed almost no effect on FGFR4 WT (92.97 % activity detected) while exhibiting inhibition of FGFR4 V550E by a fold change of 14.03 compared to WT. Antimycin A is an antibiotic substance produced by *Streptomyces* and has been reported to inhibit mitochondrial respiration and may be depleting cell of ATP. It is used commercially as fish poison and has antifungal properties. No studies on effect on humans have been conducted but there are no reports on observed adverse effects upon accidental exposure <sup>25</sup>. Compound 6-Azaauridine decreased activity of FGFR4 WT to 74.42% but showed 18.28-fold change in inhibiting FGFR4 V550E. It is an antimetabolite, a triazine nucleoside analogue of uridine, which interferes with pyrimidine synthesis thereby impeding production of nucleic acids. It was previously used to treat psoriasis and mycosis fungoides but exhibited potential neurotoxicity <sup>26</sup>. Trifluridine, also a nucleoside analog antimetabolite, inhibiting nucleic acid synthesis by incorporating into DNA and inhibiting thymidylate synthase, showed a 2.32-fold change in activity between FGFR4 WT and FGFR4 V550E, keeping WT active at 89.41% and

inhibiting mutant activity to 38.51 %. It is used as an antiviral agent in treatment of primary keratoconjunctivitis and recurrent epithelial keratitis due to *Herpes simplex* virus and has been considered for antineoplastic treatment <sup>27</sup>. Compound pralatrexate decreased activity of WT down to 52.51% but still kept a 4.39-fold more activity compared to the mutant. Pralatrexate is a more cytotoxic and better internalized analog of methotrexate, inhibitor of dihydrofolate reductase thereby inhibitor of DNA, RNA and protein synthesis <sup>28</sup>. In 2011 a study on therapeutic effect of pralatrexate on peripheral T-cell lymphoma in patients with relapsed or refractory disease was successfully conducted. Subsequently pralatrexate became the first drug approved for this disease with poor prognosis and no preceding accepted standard care <sup>29</sup>. A very interesting find is Malonoben also known as tyrphostin A9, which increased activity of FGFR4 WT to 207.67% while inhibiting FGFR4 V550E to 34.20% activity. It has been noted as a platelet-derived growth factor receptor (PDGFR) inhibitor <sup>30</sup>.

Four more molecules fulfilled the criteria of keeping wild type activity above 50% while decreasing mutant activity to less than 50% but were discarded due to fold change of activity less than 2. For all four compounds fold change was between 1.63 and 1.69.

As a follow up validation after screening, dose response MaMTH experiments should be done, as well as cell viability assays on rhabdomyosarcoma cells with the V550E mutation (such as RMS772 models). Western blot analysis of signalling pathways in presence of detected hits at various doses should also be done to check for potential changes.

This study was conducted in order to identify small molecules that specifically target FGFR4 V550E mutant associated with a pediatric disease RMS. Said mutation was previously reported to be a gain-of-function mutation, present in one of two RMS subtypes, as well as breast cancer <sup>6, 10</sup>, and is regarded as promoting tumor growth and metastasis by constitutively activating FGFR4 <sup>9</sup>. Conventional chemotherapeutics, combined with surgery and radiation, are an effective treatment against localized disease with the survival rate of 70-75 %. On the other hand, patients with relapsed or metastatic forms of disease have a poor prognosis at overall survival rates of 20-30 % <sup>9, 33</sup>. Since FGFR4 V550E is considered to have an important role in tumorigenesis in such advanced stages it imposes itself as a target with potentially major therapeutic value.

In this version of the drug screen FGFR4 WT and FGFR4 V550E interaction with Shc1 was measured in presence of numerous small molecules. Further screenings should be performed using other FGFR4 interactors such as phospholipase C $\gamma$ , v-crk sarcoma virus CT10

oncogene homolog (avian) (Crk), docking protein FGFR substrate 2 (FRS2) and signal transducers and activators of transcription (STAT)<sup>6,32</sup>. In addition to screening in presence of alternative interacting partners, it would be interesting to conduct screening with other identified FGFR4 mutations. The other mutation of the kinase domain that is considered activating in RMS is N535K<sup>9,10,32</sup> and by screening both of the activating mutations it could give more insight in signaling pathways in RMS and specificity. Another possible target with a lot of potential is a single nucleotide polymorphism G388R of FGFR4. It is identified in many different cancers such as prostate, hepatocellular, breast as well as RMS and is associated with more aggressive and metastatic forms and poor prognosis<sup>6,33</sup>.

## **5. Conclusions**

Wild type and oncogenic mutant variants of FGFR4 showed different response to being treated with the same molecules as observed with MaMTH-DS method.

Out of 5570 small molecules tested for effect on FGFR4 V550E-Shc1 interaction, five showed significant decrease of reporter activity hence inhibition of interaction in said oncogenic mutant while not causing cell death or affecting interaction of the FGFR4 WT-Shc1.



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