MSc Jacek Plichta,
Medical University of Łódź
E-mail: jacek.plichta@umed.lodz.pl

MEDICAL UNIVERSITY OF LODZ

# Evaluation of MAP kinase activation levels after TGF-β receptor inhibition through quantitative measurements of JNK1/2, p44 and SMAD1/5 protein phosphorylation

## Introduction

The primary aim of this study is to define the effects of TGF-β receptor inhibition on the levels of MAP kinase (MAPK) pathway activation. Within the study, the level of MAPK pathway activation is defined by the levels of JNK1/2, p44 and SMAD1/5 protein phosphorylation. The inhibition of the TGF-β receptor is assumed to lead to a reduction in MAPK pathway activation.

Transforming growth factor β (TGF-β) is an important mediator protein involved in the proinflammatory reaction and pathological tissue transformation in the asthmatic lung. Pathways related to inflammation regulate these processes, and TGF-β plays key roles in many of these pathways.

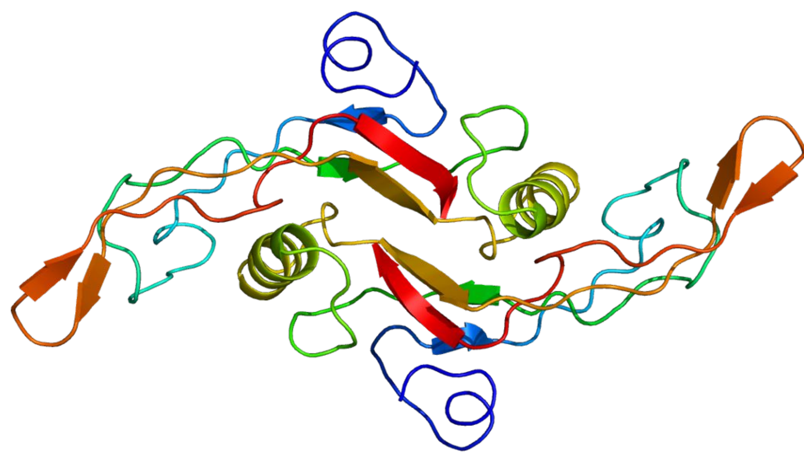

*Figure 1. Crystal structure of human TGF-β 1. Source: RCSB Protein Data bank.*

Upon activation, TGF-β receptors stimulate a cascade of endoplasmic protein intermediaries named Smad, which in turn are transcription factors which regulate the expression of effector protein genes. Smad3 signalisation is required for the process of allergen-induced airway remodelling and myofibroblast accumulation in asthma. An intensification of TGF-β signalisation is found in bronchial biopsies from asthmatic patients. TGF-β also activates Smad-independent pathways such as MAPK pathways. Measuring phosphorylation levels of SMAD and MAPK proteins can be used to measure TGF-β activity in examined cells.

In the context of asthma, TGF-β induces both proapoptotic and antiapoptotic effects in airway epithelial cells. Antiapoptotic effects are induced in the absence of physical or chemical stressors through the activity of the Smad2/3 pathway. Chronic allergen exposure allows TGF-β to induce apoptosis through the activation of MAPK signalling. Epithelial damage intensified through the dysregulation of cellular repair processes lead to the detachment of cells from the epithelium and epithelial-mesenchymal transition (EMT).

| Features of asthmatic respiratory tract remodeling |
|---|
| subepithelial fibrosis |
| extracellular matrix deposition |
| goblet cell proliferation |
| hypertrophy of smooth muscles and mucous glands |
| epithelial damage |

*Table 1. Features of asthmatic airway remodelling*

As TGF-β is a protein which plays a central role in asthma pathogenesis, the effective inhibition of TGF-β receptors may allow the development of novel therapeutics. In the clinical environment, said therapeutics may limit occurrence of airway constriction episodes, pathological airway remodelling and other asthma symptoms associated with chronic inflammation.

## Materials and methods

**Cell line:**
The human embryonic kidney cell line HEK293T was modified for use as a reporter cell line for the measuring of TGF-β signaling. The presence of CAGA12-Luc reporter activity allows HEK293T cells to be used to measure TGF-β signaling activity using luciferase assays.

**TGF-β receptor inhibitors:**
Commercially available small molecule TGF-β receptor inhibitors SD-208 and SB-431542 were used as reference samples. 14 novel peptide inhibitors of the TGF-β receptor designed by Dr. Michał Karbownik and synthesised by GenScript Biotech Corporation were used in the assays.

**Flow cytometry:**
HEK293T cells are seeded in 12-well plates and incubated in empty culture medium, or with addition of commercially available, small molecule, or novel peptide TGF-β inhibitors. After the incubation period, cells are washed in PBS, and suspended in binding buffer at a concentration of 1 million cells/mL. 100 000 cells from each population are then stained with propidium iodide and FITC-Annexin V. The stained cells are then used to carry out flow cytometry and measure the degree of apoptosis and necrosis in cell populations.

**Luciferase luminescence assay:**
The Promega Dual-Luciferase® Reporter Assay System kit is used for testing on the HEK293T line. Cells are seeded in 96-well plates coated with poly-d-lysine (PDL) in DMEM 10% FBS medium. The cells are then incubated with TGF-β and either commercially available or novel TGF-β inhibitors. Before measurement, cells are lysed using a passive lysis buffer. The lysate from each well is added to the appropriate wells on a new 96-well plate containing a reagent which induces reporter protein luminescence. Luminescence is read using a plate reader with a luminescence measurement function.

## Results - Flow cytometry

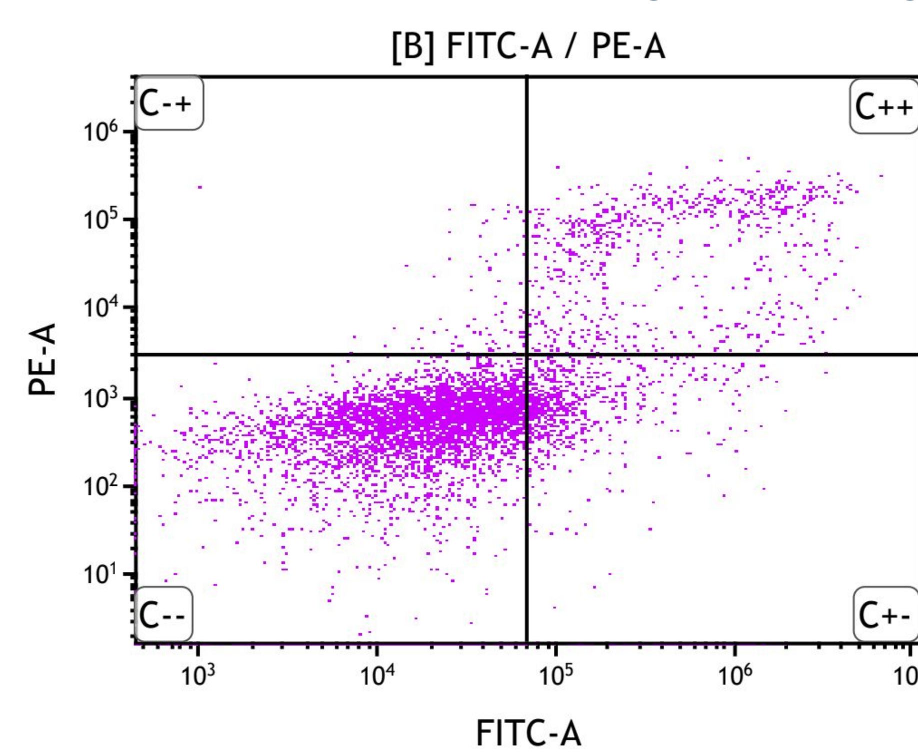


*Figure 2. Cell cytometry readout for a population of cells incubated with peptide inhibitor 2_6 for 24 hours. Cells stained with propidium iodide and FITC-Annexin V.*

| Cell type | Proportion of total population |
|---|---|
| Viable (C--) | 73% |
| Necrotic/late apoptotic (C++) | 12,5% |
| Early apoptotic (C+-) | 13% |

*Table 2. Proportion of each cell type present in the readout presented above.*

A series of measurements of the impact of the tested peptide inhibitors and commercially available small-molecule inhibitors on the survival of HEK293T line cells were performed, via flow cytometry. Compared to untreated cells, 20 µM SD-208 did not reduce cell viability, while the tested peptide inhibitors at a concentration of 50 µM reduced cell viability by 5-10 percentage points. In the example given above (Figure 2 and Table 2), cells incubated either in culture medium alone, or with 20 µM SD-208 exhibited a viability rate of 82%, while cells incubated with 50 µM 2_6 peptide inhibitor - a viability rate of 73%.

## Results - Luminescence assays

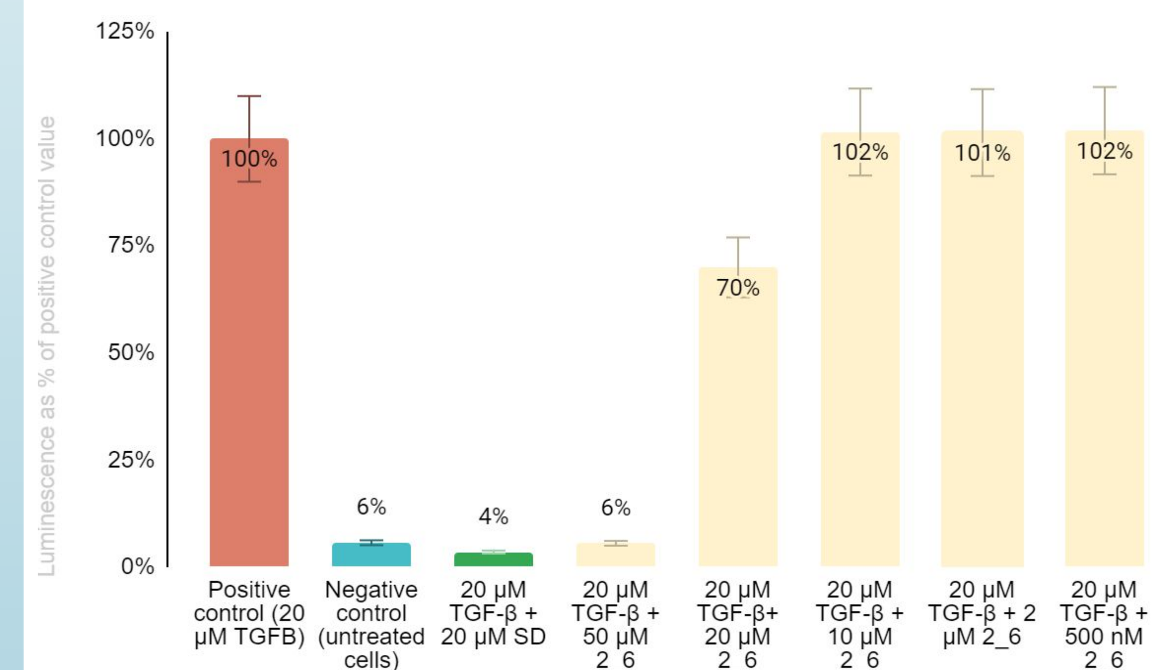


*Figure 3. Luciferase assay luminescence values for peptide inhibitor 2_6 at concentrations of 50 µM to 500 nM and control samples, expressed as percentages of positive control absorbance values. Some concentrations omitted for visual clarity.*

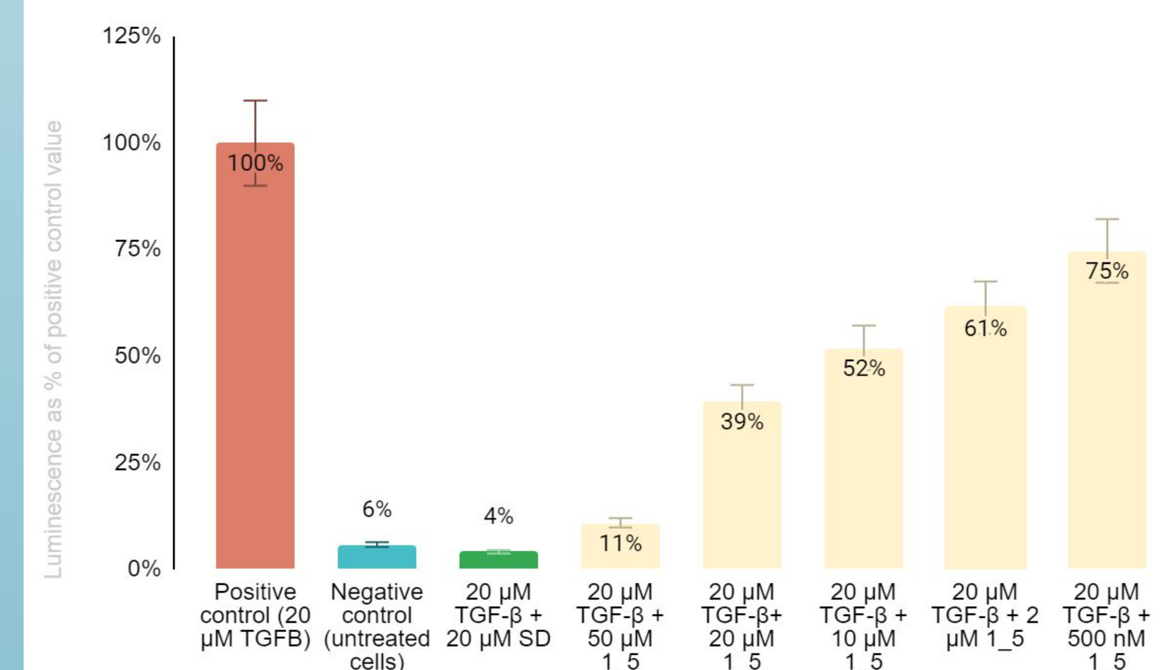


*Figure 4. Luciferase assay luminescence values for peptide inhibitor 1_5 at concentrations of 50 µM to 500 nM and control samples, expressed as percentages of positive control absorbance values. Some concentrations omitted for visual clarity.*

A series of Promega Dual-Luciferase® Reporter Assay System tests were performed using each of the peptide inhibitors (1_1-1_5, 2_1-2_9), to measure inhibition efficacy and determine the IC50 for each peptide.

IC50 values were determined based on reduction in luminescence values for samples incubated with the inhibitor compared to the positive control sample.

Reduction of luminescence to a degree similar to that caused by commercially available inhibitors (SD-208 or SB-431542) indicated effective inhibition of signaling through TGF-β receptors.

Concentrations of peptide inhibitors ranging from 50 µM to 250 nm were used in the experiments. At a concentration of 50 µM, all tested inhibitors reduced luminescence values to an extent equal to or slightly lower than commercial inhibitors. The IC50 values for the tested inhibitors ranged between 10 µM and 35 µM. For the majority of tested peptide inhibitors, concentrations at or below 2 µM had no statistically significant impact on luminescence values.

## Conclusions

The results of luciferase assays indicate that the novel peptide inhibitors we tested inhibitors (1_1-1_5, 2_1-2_9) are effective. IC50 values were also determined for each of the inhibitors, allowing for further research on the biological activity of the tested peptides. Flow cytometry studies have shown that peptide inhibitors at the maximum concentration used in luciferase assays slightly reduce the viability of HEK293T cells (5-10 pp, depending on tested peptide). Analogous measurements will be performed at IC50 concentrations appropriate for each of the tested inhibitors.

The results we obtained are sufficient for the preparation of an original scientific publication and constitute the basis for carrying out the second, and final phase of this research project - assessment of the impact of novel peptide inhibitors on the phosphorylation of proteins of the TGF-β signaling pathways with use of Western Blotting.