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

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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.

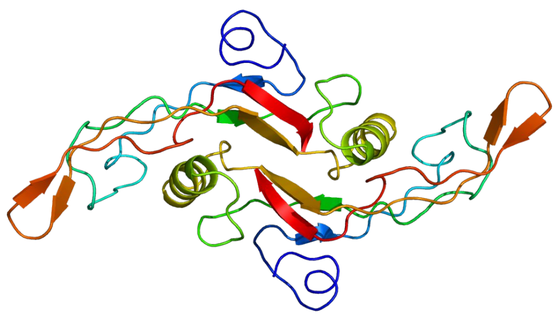

Figure 1. Crystal structure of human TGF-β 1. Source: RCSB Protein Data bank.

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 airway remodelling
subepithelial fibrosis
extracellular matrix deposition
goblet cell proliferation
smooth muscle and mucosal gland hypertrophy
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 MFB-F11 cell line consists of murine fibroblasts engineered to detect and quantify TGF-β in cell cultures. MFB-F11 cells were stably transfected with a reporter plasmid consisting of TGF-β-responsive Smad binding elements linked to a secreted embryonic alkaline phosphatase (SEAP) reporter gene. In the presence of TGF-β, MFB-F11 cells secrete SEAP into the culture medium.

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.

Colorimetric p-nitrophenyl phosphate assay:
MFB-F11 cells were seeded into 96-well plates. 24 hours after cell seeding, the culture medium was removed and replaced with fresh culture medium or a solution of TGF-β inhibitors in the culture medium. After 6 hours of incubation with inhibitors, 20 μM TGF-β was added to the wells. After 24 h of incubation, supernatant from all wells was transferred to a plate containing 2mg/mL p-nitrophenyl phosphate solution.

SEAP catalyses the hydrolysis of p-nitrophenyl phosphate to p-nitrophenol and phosphate. As a result from this hydrolysis, the solution changes colour from colourless to yellow, at a rate proportional to SEAP concentration.
Measuring absorbance values at 405 nm allows for quantitative estimation of SEAP concentration- and thus TGF-β activity, and the effectiveness of the tested peptide inhibitors. Absorbance values were read using a standard plate reader with spectrophotometer functionality.

Results - inhibitor selection

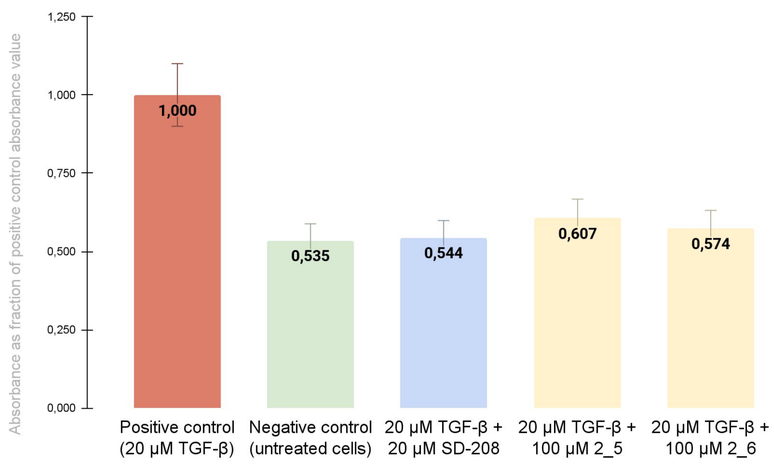

Figure 2. P-nitrophenyl phosphate assay absorbance values of peptide inhibitors 100 μM 2_5 and 100 μM 2_6 and control samples, expressed as fractions of positive control absorbance values.

A number of p-nitrophenyl phosphate assays was carried out using each of the 14 peptide inhibitors at concentrations of 100 μM in order to select the most effective inhibitors to be used in the next stages of research.

Inhibitors were selected based on the comparison of absorbance reduction between samples incubated with the control inhibitors SD-208 or SB-431542 and samples incubated with peptide inhibitors.

Absorbance reduction was determined by comparing the absorbance of the test sample to the absorbance of the positive control sample which was incubated with TGF-β only, without the addition of inhibitors.

Inhibitors which caused the greatest reduction in absorbance were selected for further stages of the research. Two most effective inhibitors named 2_5 and 2_6 were selected.

Results - inhibitor IC50 estimation

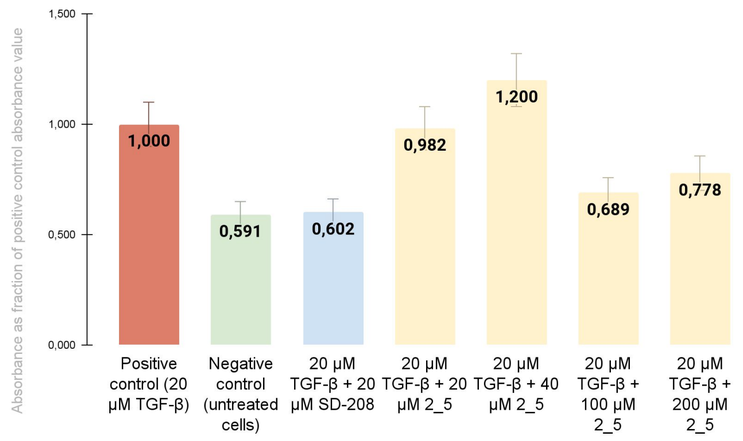

Figure 3. P-nitrophenyl phosphate assay absorbance values of peptide inhibitor 2_5 at concentrations of 20 to 200 μM and control samples, expressed as fractions of positive control absorbance values. Some concentrations omitted for visual clarity.

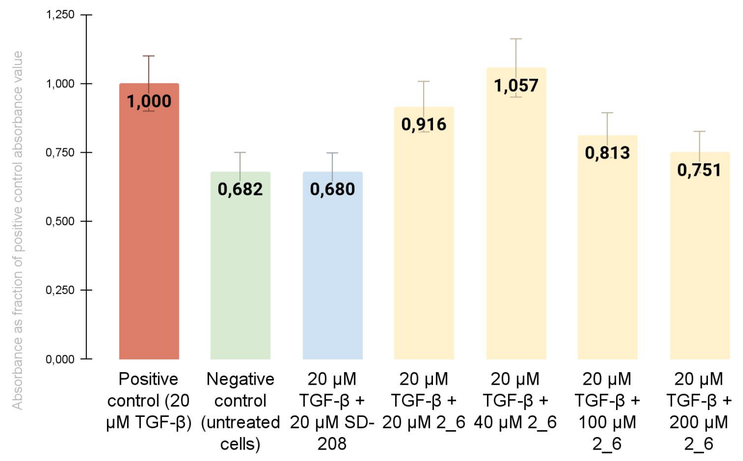

Figure 4. P-nitrophenyl phosphate assay absorbance values of peptide inhibitor 2_6 at concentrations of 20 to 200 μM and control samples, expressed as fractions of positive control absorbance values. Some concentrations omitted for visual clarity.

The next stage of the research entailed determining IC50 values for 2_5 and 2_6. For this purpose, a number of colorimetric tests were carried out using inhibitors in concentration ranges from 10 nM to 500 μM. A concentration range of 20 to 200 μM was specified as the concentration range at which the tested inhibitors are effective.

Within this concentration range, the results for both inhibitors were similar. Concentrations of 20 to 60 μM did not change, or increased absorbance levels by 10 to 20% compared to the positive control. Importantly, an increase in the level of absorbance was present after adding the inhibitors to the cell culture regardless of the presence of TGF-β.

Concentrations of 80 to 200 μM resulted in a 15-30% decrease in absorbance compared to the positive control. In the concentration range from 80 to 200 μM, there were samples in which a significant percentage of cells detached from the substrate, disrupting the results. Because of these unexpected effects, IC50 values for the 2_5 and 2_6 inhibitors have not yet been established.

Conclusions

Peptide inhibitors 2_5 and 2_6 effectively inhibit TGF-β receptors at concentrations of 80 to 200 μM. Unfortunately, inhibitors 2_5 and 2_6 also cause an increase in the level of absorbance at 20 to 60 μM and in some cases loss of adherence by cells at 80 to 200 μM. The causes behind these undesirable effects are yet to be elucidated.

The experimental protocol should be modified to remove the undesirable effects. In the near future, experiments with a modified protocol will be carried out. Cells will be plated in a medium containing 10 μM SB-431542, which will be removed after 24 hours, wells rinsed three times with PBS. A solution of poly-l-lysine will also be used to prevent cells from losing adherence.

Once accurate IC50 values for 2_5 and 2_6 are established, Western blotting and qPCR testing will be carried out to measure MAPK activation and gene expression levels and following TGF-β receptor inhibition.