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MULTITARGET TYROSINE KINASE INHIBITOR TREATMENT IN NOVEL PATIENT-DERIVED MESOTHELIOMA CELL LINES

**(Új mezotelióma sejtvonalak érzékenységeinek
vizsgálata dasatinib és nintedanib kombinált
kezelés hatására)**

OTDK Research Essay

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1 INTRODUCTION

Tumorous malignancy is a complicated state that is influenced by numerous factors. I have a belief that every research related to tumour biology contributes to the understanding of this enigma that demands the life of many people.

Malignant mesothelioma is a rare malignancy that has a particularly poor prognosis. Despite the intensive research of the field, at present, this malignancy cannot be effectively treated. The development of effective therapies is hindered by the rarity of the disease together with its high molecular heterogeneity. Although various strategies are designed to target mesothelioma, based on different molecular mechanisms, just a small percentage of these drugs reach the clinical trial phase, and among them, just a few seems to be effective. Repurposing accepted drugs that proved to be effective in other forms of malignancies is an attractive strategy. As angiogenesis seems to be a critical step in the pathogenesis of mesothelioma, the interest is increased towards anti-angiogenics, including tyrosine kinase inhibitors.

Both the multitarget angiokinase inhibitor nintedanib¹, and Src tyrosine kinase inhibitor dasatinib² seemed to be effective against malignant mesothelioma during the *in vitro* examinations, but they failed during the clinical trials^{3,4}. Despite their moderate efficacy as a single agent, it is hypothesized that their application in combination can have a therapeutic benefit in certain cases.

The main objective of the work was the examination of the efficacy of the nintedanib and dasatinib alone, and in combination, in novel mesothelioma cell lines. To disclose the possibilities in the treatment strategies, the interaction between the studied cell lines and the applied drugs in different concentrations was examined in several aspects, including short-term viability assays, cell cycle analysis, and immunoblot assay.

I have done the experiments in that belief my results – as a small building stone – will have a minor contribution to the innovation of personalized medicine.

The aims of the work were the following:

- Calculation of half-maximal inhibitory concentrations (IC₅₀ values) of nintedanib and dasatinib in 6 different mesothelioma cell lines via **single-drug SRB assay**.
- Calculation of the combination index (CI) in every concentration pair of nintedanib and dasatinib, in the observed cell lines via SRB assay
- Examination of the changes in the distribution of the cell cycle stages responding to the different concentrations of the applied drugs by **cell cycle analysis**.
- Examination of the changes in the expression and phosphorylation of proteins from the Ras/Raf and PI3K/AKT signalling pathways induced by the different concentrations of dasatinib and nintedanib by **western blot immunoblot assay**.

2 SCIENTIFIC BACKGROUND

2.1 Characteristics and epidemiology

The mesothelium is a membrane that forms the lining of body cavities, including the thoracic cavity (pleura), abdominal cavity (peritoneum), and heart sac (pericardium), or it forms the membranous cover for the internal male reproductive organs (tunica vaginalis of testis)⁵. The neoplasm that arises from the surface serosal cells of the mesothelium is referred to as malignant mesothelioma (MM).

Malignant mesothelioma is considered a rare disease that accounts for less than 1% of all cancer deaths in the world, nevertheless it is highly lethal^{5,6}. After prognosis, the reported median survival is between 9-12 months in both untreated and treated patients⁷. On the one hand, the poor outcome can be explained by the slow development of the malignancy; the time between the first exposure and the diagnosis is usually 20 to 50 years. The characteristic symptoms develop in the advanced stage of the disease, which delays the diagnosis^{5,8}. The slow development also contributes to the high molecular heterogeneity of the disease between patients and within individual tumours. The intratumour heterogeneity implies the combination of longitudinal and spatial heterogeneity, which derives from the progression of the tumour over time, and the differences between different parts of the same tumour. This molecular heterogeneity hinders the development of effective therapeutic strategies^{9,10}. On the other hand, the poor prognosis can be explained by the lack of effective treatments. Mesothelioma generally responds insufficiently to conventional therapies. The rarity of the disease and the limited number of patients in individual trials makes it more challenging to identify the novel therapeutic approaches¹¹.

Malignant mesothelioma can be classified into different histological subtypes. Identification of the variants has prognostic significance and contributes to therapy decisions¹². Among the subtypes, epithelioid mesothelioma is the most frequent, giving 50-60% of all cases¹³. Generally, it is associated with a better prognosis – with a median survival rate of 12-24 months after the diagnosis¹² – as the patients may benefit from surgical treatment in a multimodal therapy approach¹⁴. In contrary to the features of the epithelioid subtype, sarcomatoid mesothelioma is known for its aggressive phenotype, and it has a remarkably poor prognosis^{12,14}. This variant is particularly rare, described in 10-20% of all malignant mesothelioma cases¹³. Biphasic mesothelioma has a mixed type that contains at least 10% of each epithelioid and sarcomatoid component, and it occurs in 25-35% of all mesothelioma cases^{13, 14}. Due to the different features of the histological cell types, the individual outcomes highly depend on the ratio of the two subtype¹².

2.2 Mesothelioma inducing effects

2.2.1 Asbestos

The name asbestos is a collective term for six mineral fibres (chrysotile, actinolite, tremolite, anthophyllite, amosite, crocidolite) found in nature^{15,16,17}. The specific thermal and chemical resistance of these materials made their application worldwide popular until the early 1980s in many forms, including construction materials and various products, such as protective clothing and textured paints^{18,19}. Despite the fact that all forms of asbestos are currently banned in 67 countries, the incidence of asbestos-related diseases remains a major problem even in developed countries. This tendency can be explained by the due to the low public awareness and insufficient abatement^{20,21}.

Despite the intensive research in the field, the mechanism of mineral fibres induced carcinogenesis is still elusive. It has been suggested that the fibres can directly interfere with chromosome segregation during mitosis, but more recently, the role of fibres-induced chronic inflammation was found to be more prominent²².

2.2.2 Other factors in tumourigenesis and frequent genetic alterations

Even though mesothelioma has been considered as a ‘sentinel’ or ‘signal’ tumour for asbestos exposure, various other environmental factors are prone to have similar tumour-inducing effects. The malignancy is associated with mineral fibres and fibrous materials with asbestos-like physical properties and low-persistence. Furthermore, certain studies identified radiation and the presence of Polyomavirus simian virus 40 (SV40) as a potential risk factor²³.

Clinical studies predominantly target those pathways that are altered due to activation of oncogenes. In malignant pleural mesothelioma (MPM) so far no driver mutations was identified, only inactivation of tumour suppressor genes. In MPM cells, the most commonly BRCA-associated protein 1 (BAP1), neurofibromatosis type 2 (NF2) protein, and cyclin-dependent kinase inhibitor 2A (CDKN2A) are mutated or lost²⁴.

2.2.3 Kinase targets in the mesothelioma

Kinases are enzymes that transfer a phosphate group from adenosine triphosphate (ATP) to a specific residue. In proteins, the target amino acids can be serine, threonine, or tyrosine residues. In cell signalling pathways, regulation through phosphorylation is one of the main working mechanisms. Accordingly, protein kinases have a crucial role in carcinogenesis and cancer metastasis. The fact that they are the second-most-targeted group among the drug targets after the G-protein-coupled receptors demonstrate their importance in cancer formation²⁵. As in other cancer types, in the treatment of malignant mesothelioma, the interest shifted towards protein kinases.

2.2.3.1 Growth factor receptors

Receptor tyrosine kinases (RTKs) represent an important group among protein kinases. These are transmembrane proteins with intrinsic tyrosine kinase activity that got activated upon cytokine-induced dimerization or oligomerization. As essential compounds of signal transduction pathways, their mutation and aberrant activation is a common event in cancer formation and progression²⁵. Certain receptors – including vascular endothelial growth factor receptor (VEGFR), fibroblast growth factor receptor (FGFR), platelet-derived growth factor receptor (PDGFR), and transforming growth factor- β 1 receptor (TGFB β 1) – became important oncological targets because they convey signals that enhance angiogenesis²⁶. This form of neovascularization is critical for tumour survival, local invasion, and metastasis. After intensive research, the FDA approved several small-molecule inhibitors against RTKs, including imatinib, sorafenib, lapatinib, and nintedanib^{1,25,26}.

2.2.3.2 Src-family kinases

The Src-family kinases (SFKs) are a group of non-receptor tyrosine kinases, including ten members (c-Src, Blk, Fgr, Fyn, Frk, Hck, Lck, Lyn, Yes, and Yrk). SFKs act downstream of receptor tyrosine kinases (RTKs) and integrins in the regulation of tumour cell proliferation and survival, and they were found to be overexpressed or hyperactivated in various cancer types. The elevated level of Src, Lyn, and Yes proteins was also described in malignant mesothelioma^{2,27}.

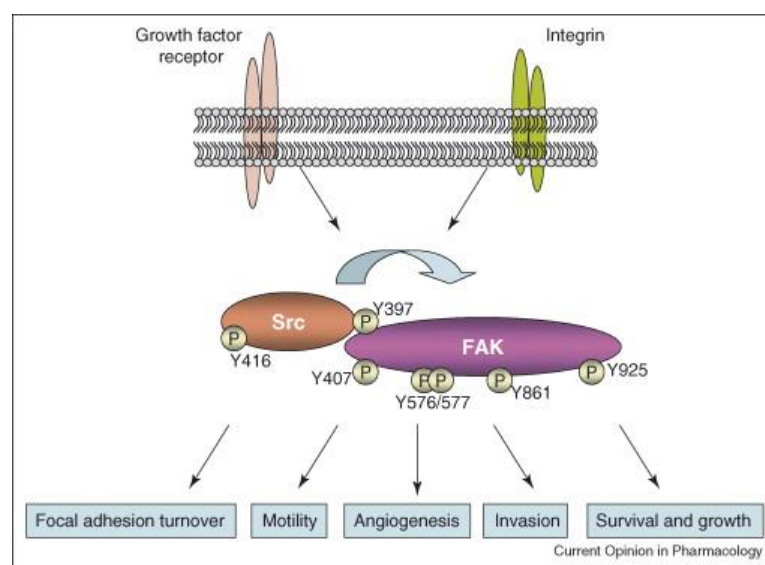


Figure 1 Activation of the Src-FAK multiprotein complex²⁸; Y416 - Auto-phosphorylation site on Src; Y397 - Integrin-induced auto-phosphorylation site on FAK; Y407, Y576/577, Y861, Y925 - Src-specific phosphorylation sites on FAK^{28,29,30}

Src proteins gain their full activity by autophosphorylation at tyrosine 419 (Tyr⁴¹⁹) residue (avian tyrosine 416) on their Src homology 1 (SH1) tyrosine kinase domain, which sequentially leads to the activation of SH2 and SH3 protein-binding domains (Figure 1). Through its SH2

domain, activated Src binds to the focal adhesion kinase (FAK) and forms the Src-FAK multiprotein complex. This complex plays an important role in the regulation of cell motility, migration, invasion, survival, and apoptosis^{2,28,31} (Figure 1).

2.2.3.3 Focal Adhesion Kinase (FAK)

Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that is frequently overexpressed in human cancers, including mesothelioma. FAK is primarily associated with integrin signalling, but also various transmembrane receptors can transmit extracellular signals through it³².

Prior to the formation of the Src-FAK multiprotein complex, FAK should be activated. Upon integrin-engagement, or as a secondary event of the signal transduction of growth factors, chemokines, and G-protein-coupled receptors, FAK is released from autoinhibition, and its tyrosine 397 (Tyr³⁹⁷) residue becomes auto-phosphorylated. The phosphorylated Tyr³⁹⁷ serves as a high-affinity binding site for the SH2 domain of Src, which promotes the association of the Src-FAK multiprotein complex (Figure 2). FAK contains five further tyrosine phospho-acceptor sites (Tyr⁴⁰⁷, Tyr⁵⁷⁶, Tyr⁵⁷⁷, Tyr⁸⁶¹, Tyr⁹²⁵) whose activation is Src-dependent. These special residues serve as a docking site for several signalling intermediates that eventually lead to changes in the cell behaviour^{2,28,29,30}. Consequently, FAK has a critical role in signal transduction and controls fundamental cellular processes, such as cell adhesion, migration, and proliferation. It promotes important malignant features in cancer progression, like epithelial to mesenchymal transition (EMT) and tumour angiogenesis³².

As a member of different signalling cascades of transmembrane receptors, FAK has an important scaffold function. It has gained great importance in the regulation of cell migration through multiple signalling pathways, including the activation of p130^{CAS} and the phosphorylation of Grb7³³.

Src in complex with FAK phosphorylates p130^{CAS} (also known as CAS), which associates with several SH2 domain-containing proteins and subsequently activates Rap1-, Rac- and ERK-signalling. In this way, the process is linked to the regulation of cell motility, growth, and survival^{33,34,35}.

FAK can also form a complex with phosphatidylinositol 3-kinase (PI3K) and stimulate cell migration through Rac protein. The activated PI3K indirectly induces conformational changes in growth factor receptor-bound protein 7 (Grb7). Grb7 is an adaptor protein, which due to its special domain structure, interacts with various proteins and influences mitogenic signalling^{33,36,37}.

2.2.3.4 Raf/MAPK/ERK pathway

The Raf/MAPK/ERK signalling pathway gets activated upon extracellular stimuli, including growth factors and stress. The first element of the kinase cascade is Raf protein (mitogen-activated protein kinase kinase kinase), which generally gets activated by the Ras small GTPase, but it is also under the influence of Src. Raf activates MEK (MAP kinase kinase) by phosphorylation, which conveys the signal through the activation of ERK (MAP kinase). ERK interacts with certain transcription factors that increase the intracellular level of those proteins that influence cell proliferation and cell cycle progression³¹.

2.2.3.5 PI3K/Akt pathway

The active form of PI3K phosphorylates phosphatidylinositol 4,5-biphosphate (PIP₂) to produce phosphatidylinositol (3,4,5)-triphosphate (PIP₃). The conversion of PIP₂ into PIP₃ consequently leads to the activation of protein kinase B (PKB, also known as Akt). Due to the various downstream targets of Akt – such as mTOR, Bcl-2, GSK3 β , FOXO – the signalling pathway has a pivotal role in the regulation of protein synthesis, cell survival, cellular metabolism, and gene transcription^{31,38,39}.

2.3 Modalities for mesothelioma

2.3.1 Current standard of care

The standard therapeutic strategy in the treatment of malignant mesothelioma primarily is chemotherapy. Until recently, the frontline therapy approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) was the combination of cisplatin-pemetrexed⁴⁰. In resectable tumours, additional radiotherapy is applied together with radical surgery to decrease local recurrence rates^{7,40}. Since 2021, also the combination of immune checkpoint inhibitors ipilimumab (CTLA-4 inhibitor) and nivolumab (PD-1 inhibitor) got approval, however a substantial portion of patients show only limited response to both therapies⁴¹. Therefore, there is an extensive effort to find predictive biomarkers to treatment response and to identify potential new therapeutic modalities^{43, 44}.

2.3.2 Novel therapeutical approaches

Based on the tendencies of the recent years, cytotoxic therapies probably reached a plateau in the treatment of malignant mesothelioma, which directed the attention towards new approaches based on deregulated pathways and targeted therapies⁸.

Targeted therapy aims to inhibit those proteins that have a role in tumour formation, growth, and spread⁴⁴. However, the insufficient molecular characterization of MPM hinders the development of these strategies. In this tumour type, the loss of tumour suppressor genes is

dominant, including the mutation of BAP1, NF2, and CDKN2A. Until this time, no oncogene driver mutation was discovered that might have been responsive to targeted therapies^{24,45}.

2.3.2.1 Anti-angiogenic drugs

Compared to normal tissues, the tumour vasculature is characterized by highly abnormal tortuous vessels, which results in heterogeneity in the tumour blood flow. These conditions contribute to hypoxia, a characteristic feature of the malignancies. Hypoxia is a special condition that leads to changes in gene expression with reduced apoptosis, enhanced receptor tyrosine kinase signalling, promoted metastasis, and invasion, together with immunosuppression. It also affects the generation of the tumour vasculature by contributing to the upregulation of VEGF and other pro-angiogenic molecules⁴⁶. Clinical evidence suggests that these processes may be a critical step in the pathogenesis of malignant pleural mesothelioma²⁴. Due to the prominent role of angiogenesis, many studies are focused on anti-angiogenic strategies, which include the development of anti-VEGF antibodies and angiokinase inhibitors⁴⁶.

Anti-VEGF antibodies

To this date, the most effective antiangiogenic agent against mesothelioma is a VEGF-A targeting monoclonal antibody, called bevacizumab. It inhibits cell proliferation by hampering the attachment of VEGF to its receptor. In the Mesothelioma Avastin Cisplatin Pemetrexed Study (MAPS), this agent, in addition to the cytotoxic chemotherapy, significantly prolonged the patient's overall survival (OS)^{1,46}.

Angiokinase inhibitors

The deviations in the signalling pathway are often derived from the abnormal activation of the vascular endothelial growth factor receptor (VEGFR). It can be caused by autocrine activation, chromosomal translocation, overexpression, and gain-of-function mutations. Accordingly, along with the direct targeting of the growth factor, its receptor also can be blocked. VEGFR is a receptor tyrosine kinase (RTK) which group is a popular target in cancer therapy. Different tyrosine kinase inhibitors were designed against VEGFR, like cediranib and nintedanib⁴⁶.

Besides the inhibition of RTKs, the direct targeting of non-receptor kinases is also an intensively researched area. As it was above described, in mesothelioma, Src and FAK emerged as notable targets for cancer therapy. These kinases are involved in angiogenesis and further processes that influence the malignant phenotype together with the prognosis²⁸.

The activity of the receptor and non-receptor tyrosine kinases is most frequently blocked by small-molecule inhibitors (SMIs). A certain class of SMIs inhibits phosphorylation (Figure 2) by blocking the attachment of the main phosphate group donor (ATP). Normally, ATP binds to the kinase to its ATP-binding pocket located at the highly conserved kinase domain. These SMIs act as ATP-competitors. The inhibitors bind to the active conformation of the kinase, resulting in a structural conformation that is unfavourable to phosphor-transfer^{25,26}. Due to the conservation of the kinase domain, small-molecule agents often have a multikinase inhibitor profile, which offers the possibility of disturbing several independent biological pathways that are vital for tumour proliferation and metastasis^{46,47}. Because of the little selectivity, the inhibitors may interfere with the activity of other kinases, which increases the potential for off-target side effects like cardiotoxicity and deterioration in cardiac function. In the novel generations of SMIs, the potency and tumour bioavailability is improved, coupled with reduced adverse events^{25,26}.

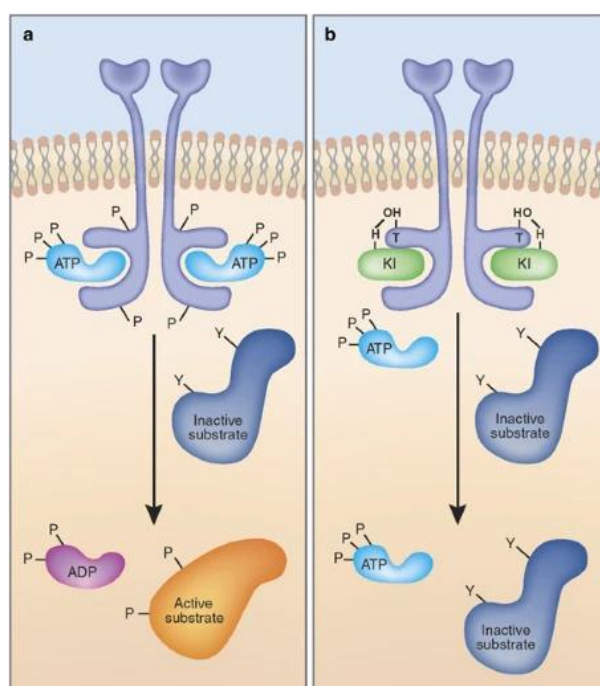


Figure 2 Mechanism of small-molecule inhibitors⁴⁷; (a) Schematic representation of oncogenically activated receptor tyrosine kinase; **(b)** Inhibition of the receptor tyrosine kinase by small-molecule inhibitor (KI)

2.4 Compounds used in the current research

Due to the high molecular heterogeneity among MPM tumours, the treatment options should be identified individually after the molecular characterization of the tumour. Since similar molecular alterations can be identified as in other tumour types, repurposing accepted drugs can be an attractive strategy in treatment development. In the vast majority, those agents are studied that have already proved their efficiency in other types of diseases, predominantly in malignancies. The experiences from the previous approval of the repurposed drug lead to

reduced treatment costs and faster time-to-market for patients because the clinical treatments can start directly with phase II trials. As a further advantage, these drugs have tolerable side effects compared to classical anticancer agents, which limits the need for supportive care⁴⁸.

Due to the established role of angiogenesis in the pathogenesis of malignant mesothelioma, those agents that target the VEGF signalling became prominent candidates in the treatment development. The success of the anti-VEGF bevacizumab affirmed the position of the anti-angiogenics³, and the interest increased towards those agents that target more than one antiangiogenic pathway. One of the promising candidates was nintedanib, a multitarget angiokinase inhibitor³.

2.4.1 Nintedanib

Nintedanib (BIBF 1120) is a triple angiokinase inhibitor, which has an inhibitory effect on VEGFR 1-3, fibroblast growth factor receptor (FGFR) 1-3, and platelet-derived growth factor receptor (PDGFR)- α , and - β ¹. Moreover, it shows activity against the Src and Abl kinase signalling⁴⁹.

Nintedanib received its first global acceptance in 2014, when it earned approval for the treatment of idiopathic pulmonary fibrosis by the Food and Drug Administration (FDA). The European Medicines Agency (EMA) also accepted its application in the treatment of systemic sclerosis-associated interstitial lung disease^{50,51}. In Europe, it is also applicable as a second-line treatment of locally advanced, metastatic, or locally recurrent lung adenocarcinoma in combination with docetaxel¹. These results confirmed the anti-migratory and anti-angiogenic activity of the drug and provoked interest in the treatment of malignant mesothelioma.

After the promising results of the *in vitro* studies, the effectivity of nintedanib in combination with cisplatin plus pemetrexed was studied by the randomized, double-blind, placebo-controlled LUME-Meso clinical trial (ClinicalTrials.gov Identifier: NCT01907100). Based on the progression-free survival (PFS) data, in the second phase of the study, the addition of nintedanib improved the clinical efficacy, especially in patients with epithelioid histology^{3,50}. In light of the phase II results, patients with solely epithelioid characteristics were enrolled in the confirmatory phase III³. Unfortunately, the effectiveness of nintedanib was not confirmed in the final phase of the trial. There was no difference between the progression-free survival of the nintedanib arm (median 6.8 months [95% CI 6.1–7.0]) and the placebo group treated with the cisplatin plus pemetrexed monotherapy (7.0 months [6.7–7.2]; HR 1.01 [95% CI 0.79–1.30], $p=0.91$)^{3,47}.

The reason for the failure of LUME-Meso-III after the promising phase II results is not clearly defined. The individual inhibition of certain tyrosine kinases might be ineffective

because other kinases can replace them by inducing the same cell proliferation and survival pathways. Therefore, the therapeutic efficiency may be enhanced by the combination of different tyrosine kinase inhibitors² (Figure 3).

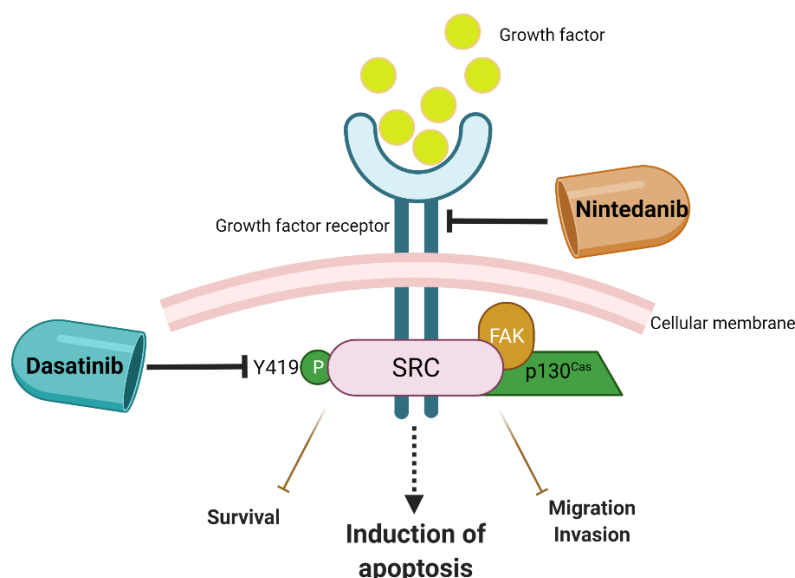


Figure 3 Possible therapeutical targets in the VEGF-signalling pathway (Modified by the Author⁵²)

2.4.2 Dasatinib

Dasatinib (BMS-354825, SPRYCEL) is a second-generation, multitarget small-molecule kinase inhibitor of five oncogenic tyrosine kinase families, including Src-family kinases (SFKs), BCR-Abl fusion protein, stem cell factor receptor (c-Kit), platelet-derived growth factor receptor (PDGFR), and ephrin receptor kinases (Eph A/B). Several studies suggest that it also binds to other tyrosine and serine/threonine kinases, such as the mitogen-activated protein kinases (MAPK), receptor tyrosine kinases (RTKs), and discoidin domain receptors (DDR)^{4,52}. It has earned a Food and Drug Administration (FDA) approval as a second-line drug in the treatment of chronic myeloid leukaemia (CML). The European Commission authorized its application in the treatment of imatinib-resistant or intolerant CML and Philadelphia chromosome-positive acute lymphoblastic leukaemia⁵².

The activity of dasatinib against Src-family kinases (SFKs), vascular endothelial growth factor (VEGF), and platelet-derived growth factor- β (PDGF- β) receptor suggested its potential in the treatment of the malignant mesothelioma⁴³. In preclinical studies, dasatinib derivatives inhibited the activating phosphorylation of SFKs by targeting the phosphorylated Tyr⁴¹⁹ residue of Src (Figure 3). These findings suggested that it also affects the activity of other members downstream in the signalling pathway. In studies, dasatinib has decreased FAK phosphorylation at Src target site Tyr⁸⁶¹, transiently reduced STAT3 activation, and lowered the presented active form of Akt kinase. Through the collective inhibition of Akt and Src, dasatinib contributes to

the cell cycle regulation through the nuclear stabilization of the tumour suppressor p27. It can decrease cell proliferation and angiogenesis by affecting the PDGF/PDGFR pathway^{2,4}.

As a tyrosine kinase inhibitor that targets the Src/FAK signalling pathway, dasatinib seemed to be a good candidate in the treatment of malignant pleural mesothelioma. After the promising *in vitro* results, in the Biomarker-Integrated Neoadjuvant Dasatinib Trial (ClinicalTrials.gov Identifier: NCT00652574), patients with resectable, non-sarcomatoid type malignant mesothelioma were treated with neoadjuvant dasatinib for four weeks before surgical resection. The median progression-free survival of the 24 treated patients was 7.5 months, and the median overall survival was 19.1 months. Regarding these data, dasatinib-monotherapy has failed to show adequate clinical activity^{4,50,53}.

2.4.3 Combination of nintedanib and dasatinib

Notwithstanding the inadequate clinical activity of nintedanib and dasatinib as single agents⁴⁹, they might be effective in combination. The inhibition of a single tyrosine kinase might be ineffective because alternative signal transduction processes can be activated within the cells, and other kinases can induce the same cell proliferation- and survival pathways. The multitarget tyrosine kinases can reduce this factor by affecting multiple pathways. The utilization of combined treatment with different tyrosine kinase inhibitors may enhance the therapeutic efficiency². Nintedanib and dasatinib act at different levels of the same signalling pathway (Figure 3). In non-small cell lung cancer, researchers have found the therapeutic benefit of the vertical inhibition of a Src-related pathway after treating the cells with insulin-like growth factor-1 receptor (IGF-1R) tyrosine kinase inhibitor and dasatinib⁵³. Based on this result, it was hypothesized that the combination of nintedanib and dasatinib might have an increased combined effect in malignant mesothelioma cells.

3 MATERIALS AND METHODS

3.1 Cell lines

The study included six novel malignant pleural mesothelioma cell lines (Table 1), established in the Translational Thoracic Research Laboratory of University Medicine Essen - Ruhrlandklinik, Germany, from the pleural effusion samples of five MPM patients. The PF588 and PF655 cell lines (highlighted in grey in Table 1) originated from the same patient before and after chemotherapy treatment with cisplatin plus pemetrexed. All cell lines were subjected to Multiplex Cell Line Authentication (Multiplexion, Heidelberg, Germany).

Cells were grown in monolayer cultures in DMEM (Catalog. No.: 12-604F; Lonza Walkersville, Inc., Walkersville, MD, USA) – containing 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin-Amphotericin B mixture (Lonza Walkersville, Inc., Walkersville, MD, USA) – at 37°C in a humidified atmosphere with 5% CO₂.

The measurements were done in independent triplicates at different passage numbers.

Table 1 Examined novel malignant pleural mesothelioma cell lines The highlighted PF588 and PF655 cell lines were established from the same patient before and after chemotherapy treatment.

Cell lines	Histotype
PF142	biphasic
PF434	sarcomatoid
PF531	sarcomatoid
PF588	epithelioid
PF626	epithelioid
PF655	epithelioid

3.2 Drugs

Dasatinib was purchased from Selleck Chemicals (Houston, TX, USA). It was dissolved in DMSO in 10 mM concentration and stored at -80°C. Nintedanib (BIBF1122) was provided by Boehringer Ingelheim (Germany). It was dissolved in DMSO in 100 mM concentration and kept at -20°C.

3.3 Chemosensitivity assay

The sensitivity of the cell lines to the examined drugs was measured by the Sulforhodamine B (SRB) assay. The method developed by Skehan and his co-workers served as the base for the procedure⁵⁴.

The assay measures the total cellular protein amount by utilizing the characteristics of the amino-xanthene SRB dye. It binds to basic amino acid residues under mild acidic conditions and dissociates from them under basic conditions. Due to the stoichiometric binding, the amount of dye extracted from the stained cells is directly proportional to the cell mass⁵⁵.

Evaluating the effect of a single drug

For the experiments, 5000 cells/well were plated on the inner wells of 96-well plates and incubated for 24 hours at 37°C, 5% CO₂. The number of the living cells was determined by the NucleoCounter™ NC-3000 system (Chemometec, Allerød, Denmark). For the measurement, 1 µL AO (acridine orange) – DAPI (4',6-diamidino-2-phenylindole) fluorescent dye mix (Solution 13, Chemometec) was added to 19 µL of the cell suspension and loaded into an 8-well NC-slide. Then, the cells were treated with dasatinib (25 nM, 50 nM, 0.1 µM, 0.4 µM, 1 µM), and nintedanib (0.3 µM, 1 µM, 3 µM, 5 µM, 10 µM) in increasing concentrations for 72 hours. After the treatment, the cells were fixed with 6% trichloroacetic acid (TCA) for 1 hour at 4°C, washed with distilled water, and then dried for 1-2 days. Subsequently, the wells were stained with 0.4% SRB dye (Sigma-Aldrich, St. Louis, MO, USA). To remove excess dye, the wells were washed with 1% acetic acid, and then the plate was dried for 1-2 hours. The protein-bound dye was dissolved with 10 mM Tris-puffer, and the optical density was read at 570 nm with a plate reader (EL800, BioTec Instruments, Winooski, VT, USA).

The results were averaged from three independent experiments. The effect of the treatment was expressed as a percentage of the control.

Based on the results, half-maximal inhibitory concentration (IC₅₀) was calculated with CompuSyn software (ComboSyn Inc., Paramus, NJ, USA).

Combined treatment

The combined effect of dasatinib and nintedanib was evaluated by a modified version of SRB assay. For this purpose, cells were treated with the different concentrations of dasatinib (25 nM, 50 nM, 0.1 µM, 0.4 µM) and nintedanib (1 µM, 3 µM, 5 µM) in all combinations. In the 96-well plates, the cells were treated with each concentration pair in duplicates. The SRB assay was performed as described above.

The combination index (CI) was determined in every concentration with CompuSyn software (ComboSyn Inc., Paramus, NJ, USA) based on the Chou-Talalay Method⁵⁶. To the calculation of the CI values, the calculated average of the viabilities from the three independent measurements were loaded into the CompuSyn software.

3.4 Cell cycle measurement

Alterations in the cell cycle caused by the treatments with dasatinib or nintedanib were analysed by the NucleoCounter NC-3000™ system (Chemometec, Allerød, Denmark). The procedure is based on the measurement of the fluorescent nuclear stain (DAPI), which enables the quantification of the nuclear DNA content that changes during cell cycle progression⁵⁷.

Based on the description of Rittler et al. 2019, tumour cells were seeded into 6-well plates ($1.5\text{-}3\cdot 10^5$ cells/well)⁵⁸. After 24 hours of culture, the cells were treated for 72 hours with dasatinib (50 nM, 300 nM), nintedanib (1 μ M), or with a combination of the two drugs in the given concentrations. In the control well, a 3 ml DMEM culturing medium was applied. After the incubation period, both the floating and adherent cells were collected with trypsinisation and centrifugated for 5 minutes with 500G at room temperature. Then the cells were treated for 5 minutes at 37°C with the lysis buffer (Solution 11, Chemometec) that contained DAPI (4',6-diamidino-2-phenylindole) in 20 μ g/mL concentration (Solution 10, Chemometec). The reaction was stopped with the stabilizing buffer (Solution 12, Chemometec), and the samples were analysed by the NucleoCounter NC-3000TM system (Chemometec, Allerød, Denmark).

3.5 Immunoblot

Cell seeding

On the cell lines, western immunoblot assay was performed to study the effect of the applied drugs on the expression and phosphorylation of certain proteins (tSRC, pSRC, tFAK, pFAK(Tyr³⁹⁷), pFAK(Tyr^{576/577}), tERK, pERK, tAKT, pAKT). Based on the description of Laszlo et al., 2018, the cells were seeded into 6-well plates ($1.5\text{-}3\cdot 10^5$ cells/well) and incubated for 24 hours¹. Cells were treated for 24 hours with dasatinib (50 nM, 300 nM), nintedanib (1 μ M), and in a combination of the two drugs with the marked concentrations. In the case of PF588 and PF655 cell lines, dasatinib was applied only in 50 nM concentration during these measurements.

Protein isolation

After the drug treatment, cells were washed twice with 2 mL PBS buffer, and the cellular protein was precipitated with ice-cold 6% TCA solution that was added in 1 mL volume/well, and incubated at least for 1 hour at 4°C. The precipitate was collected in 1.5 mL tubes and centrifugated at 8000 RPM at 4 °C for 10 minutes. The pellet was resuspended in electrophoresis sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5 mM EDTA, 125 mg/mL urea, 100 mM dithiothreitol) by gentle shaking. The pH of the mixture was adjusted by 1.7M TRIS solution (pH 8.8). The concentration of the isolated protein was determined by the Qubit Protein Assay Kit (Qubit 3.0 fluorometer, Thermo Fisher Scientific, WA, USA).

Western blot analysis

20-30 μ g of total suspended protein were loaded on 10% polyacrylamide gel to separate the proteins by size. Vertical SDS-PAGE (sodium dodecyl-sulphate polyacrylamide gel

electrophoresis) was executed on 100V in the stacking gel and on 130V in the resolving gel. The process was stopped when the front left the resolving gel.

After the separation of the proteins, the samples were transferred into Immun-Blot[®] polyvinylidene-difluoride (PVDF) membranes (Bio-Rad Laboratories, Inc., USA) for 1.5-2 hours on 250 mA.

Immunoblot

For increasing the accuracy of protein analysis, the membranes were firstly blocked with a 5% milk solution (5% w/v non-fat dry milk, 1x TBS, 0.1% Tween-20). After the blocking, the membranes were incubated with target-specific primary antibodies (Table 2) for an hour at room temperature or overnight at 4°C. For the detection of the protein-primary antibody binding complex secondary antibodies were applied (Table 2).

For the development, luminography was performed, and ECL Western Blotting Substrate (Thermo Scientific, Waltham, MS, USA) was applied.

Table 2 Primary and secondary antibodies applied in Western blot immunoassay

Applied primary antibodies (produced by Cell Signaling Technology, Inc., Danvers, MA, USA)			
1.	Rabbit monoclonal anti- β -actin (clone 4970T, dilution: 1:1000)	6.	Rabbit anti-tFAK (dilution: 1:1000)
2.	Rabbit anti-tAKT (clone 9272S, dilution: 1:1000)	7.	Rabbit anti-pFAK (Tyr397) (clone D20B1, dilution: 1:1000)
3.	Rabbit anti-pAKT (Ser473) (193H12) (clone 4058S, dilution: 1:1000)	8.	Rabbit anti-pFAK (Tyr576/577) (clone 3281T, dilution: 1:1000)
4.	Mouse anti-tERK (L34F12) (clone 4696S, dilution: 1:2000)	9.	Rabbit anti-tSRC (32G6) (clone 2123T, dilution: 1:1000)
5.	Rabbit anti-pERK (Thr202/Tyr204) (clone 4370S, dilution: 1:2000)	10.	Rabbit anti-pSRC (Tyr416) (clone 2101S, dilution: 1:1000)
Applied secondary antibodies (produced by Jackson ImmunoResearch Laboratories, Inc., Pennsylvania, USA)			
1.	HRP-conjugated AffiniPure Donkey Anti-Rabbit IgG antibody (dilution 1:10'000, LOT number: 128921)	2.	HRP-conjugated AffiniPure Donkey Anti-Mouse IgG antibody (dilution 1:10'000, LOT number: 130080)

3.6 Statistical analysis

The results of the measurements were plotted by GraphPad Prism 5 (GraphPad Software Inc, San Diego, CA, USA). In the case of the combined SRB assay, the statistical differences between the groups were tested by two-way ANOVA with Bonferroni post hoc test. The statistical analysis was performed in GraphPad Prism 5 (GraphPad Software Inc, San Diego, CA, USA).

3.7 Author's contribution to the experiments

As I have executed the measurements as a member of a research group, the results presented below are part of a comprehensive research project. I applied alone all the methods that are described below, and the results presented in this work are solely my work.

4 RESULTS

In this study, the efficacy of nintedanib and dasatinib treatment alone, and in combination was examined on six novel malignant mesothelioma cell lines. Among them, one represented the biphasic histological subtype (PF142), two had sarcomatoid (PF434, PF531), and three had epithelioid (PF588, PF626, PF655) features.

4.1 Effect of nintedanib treatment on Src activation

Previously, it was demonstrated that nintedanib treatment strongly inhibits the migration of MPM cells both *in vitro* and *in vivo*¹. Src plays an important role in the regulation of cell migration, and it is a non-receptor, membrane-associated tyrosine kinase that is a known target of nintedanib⁵⁹. For this reason, first, the effect of nintedanib on Src activation was examined (Figure 4).

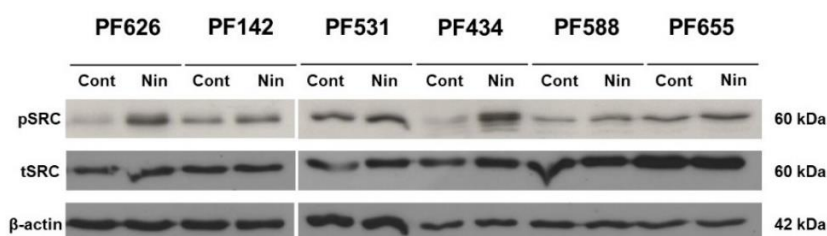


Figure 4 Effect of nintedanib treatment on the Src activation in 6 different MPM cell lines. The cells were treated for 72 hours with 1 μ M nintedanib and, its effect was studied on the proteinlysates by western blot immunoassay. (Cont=control, Nin=1 μ M nintedanib)

Unexpectedly, the phosphorylated Src (pSRC) level was found to be elevated in response to nintedanib treatment in four (PF626, PF142, PF531, PF434) of the six cell lines, and it was unaltered in the other two (PF588, PF655). In order to better understand this paradoxical effect, it was hypothesized that the addition of a Src-inhibitor might reverse Src activation, and the dual inhibition of RTKs and Src might have an increased combined effect in MPM cells. For this purpose, nintedanib was combined with dasatinib in further measurements.

4.2 Evaluating the effect of nintedanib and dasatinib treatment as single agents

The changes in the cell viability in response to nintedanib and dasatinib treatments were investigated with Sulforhodamine B (SRB) assay. Based on the gained data, the half-maximal inhibitory concentrations (IC_{50} values) of both drugs were calculated for each cell line. The IC_{50} value refers to the concentration of the given drug that is required to reduce the cell viability by 50%⁶⁰.

Among the cell lines, PF531 followed by PF142 showed the greatest sensitivity against the nintedanib treatment with IC_{50} 0.9 μ M and IC_{50} 1.9 μ M. In these cells, the viability has been strongly reduced even at lower concentrations. PF434 and PF655 cell lines were also sensitive to nintedanib, with 2.9 μ M and 3.3 μ M IC_{50} values, respectively. Meanwhile, PF588 and PF626

cells showed only moderate sensitivity to the drug (Table 3). The cell viability of the PF588 cells was not decreased by the lowest applied nintedanib concentration (0.3 μ M), but even a slight increase was observable (Figure 5).

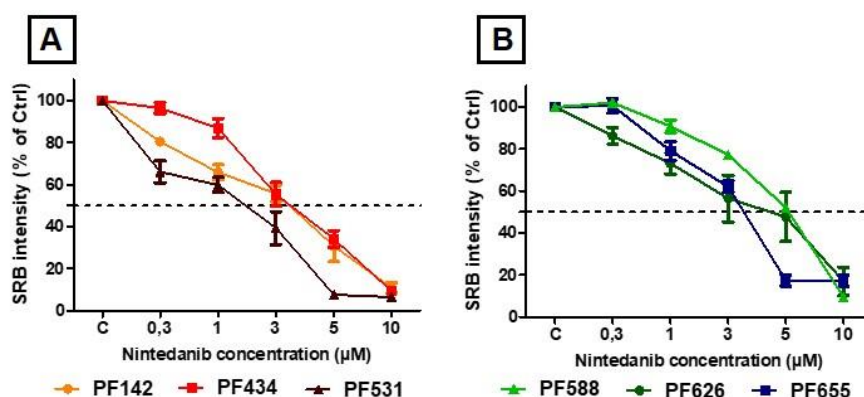


Figure 5 Effect of nintedanib treatments on the viability of malignant mesothelioma cells measured. (A-B) The cells were treated with different concentrations of nintedanib for 72 hours, and their viability was studied by SRB assay. (C=control) (A) Cell lines that show relative sensitivity to nintedanib; (B) Cell lines that show moderate sensitivity against nintedanib. Data are shown as average $\pm$ SEM of the minimum 3 independent measurements.

The PF588 and PF655 cell lines originate from the same patient before and after cisplatin treatment. As an interesting finding, these cells showed different sensitivity to nintedanib, and PF655 was substantially more sensitive to the drug than PF588.

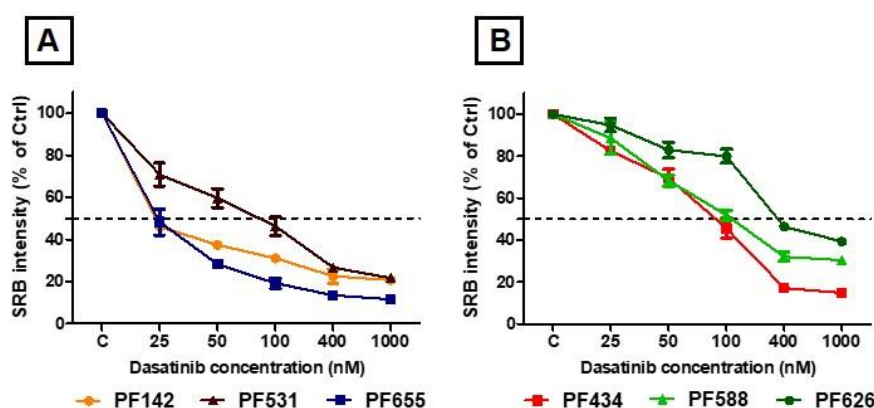


Figure 6 Effect of dasatinib treatments on the viability of malignant mesothelioma cells measured. (A-B) The cells were treated with different concentrations of dasatinib for 72 hours, and their viability was studied by SRB assay. (C=control) (A) Cell lines that show relative sensitivity to dasatinib; (B) Cell lines that show moderate sensitivity against dasatinib. Data are shown as average $\pm$ SEM of the minimum 3 independent measurements.

The sensitivity of the cell lines to dasatinib treatments was also analysed by SRB assay (Figure 6). The PF655 and the PF142 cells were the most sensitive, their viability was strongly reduced even at the lowest applied concentration (Table 3). With the IC_{50} values 94.0 nM and 108.6 nM, the viability of PF531 and PF434 cells was also strongly reduced by dasatinib treatment. Interestingly, PF588 and PF626 cells were the least sensitive, similarly to the nintedanib treatment.

Based on the results of the single-drug SRB assays (Table 3), the PF142 cell line was highly sensitive to both nintedanib and dasatinib treatments. The PF434, PF531, PF655 cell lines also showed substantial sensitivity against both drugs. Meanwhile, in the case of the PF588 and PF626 cells, only a moderate response was induced.

Table 3 Determined half-maximal inhibitory concentration (IC₅₀) based on the results of the SRB assays

IC ₅₀ values		
<i>Cell line\Drug</i>	<i>Nintedanib</i>	<i>Dasatinib</i>
PF142	1.9 μ M	11.1 nM
PF434	2.9 μ M	108.6 nM
PF531	0.9 μ M	94.0 nM
PF588	4.2 μ M	196.8 nM
PF626	4.4 μ M	458.0 nM
PF655	3.3 μ M	10.6 nM

4.3 Effect of nintedanib and dasatinib on the Src/FAK signalling pathway

To investigate the effect of nintedanib and dasatinib treatments on the Src/FAK signalling pathway, the activation of Src and FAK proteins was analysed by western blot immunoassay. In the Src/FAK signalling pathway, the autophosphorylation of the tyrosine 397 (Tyr³⁹⁷) of FAK promotes the formation of the Src-FAK multiprotein complex, and Src mediates further phosphorylation of FAK. In the observed cell lines, the changes in the phosphorylation on the Tyr³⁹⁷ and Tyr^{576/577} residues and the total FAK (tFAK) level were examined (Figure 7).

Dasatinib alone completely inhibited the phosphorylation of Src (pSRC) already at 50 nM concentration in half of the cell lines (Figure 7A-C), while in PF434, PF531, PF626 cells (Figure 7D-F), the same activity was detectable just in 300 nM concentrations. In four cell lines (PF142, PF434, PF531, PF626), as showed previously (Figure 4), nintedanib increased phosphorylated Src level (pSRC). In all four cell lines, the paradox increase in pSRC activation was compensated in those samples where both dasatinib and nintedanib treatment was applied (Figure 7C-F), and the level of pSRC was similar as in the single dasatinib treatment samples. This result supported our hypothesis, and the dasatinib was able to compensate for the pSRC increasing activity of nintedanib. While the applied drugs influenced the phosphorylation of the Src, they did not modify its expression level, as the total Src (tSRC) level remained constant.

In the phosphorylation pattern of FAK, similar tendencies were observable as of Src. After the nintedanib treatment, FAK phosphorylated on Tyr^{576/577} remained unchanged in every cell line. In the PF588, PF655, and PF142 cell lines (Figure 7A-C), dasatinib inhibited the phosphorylation of FAK even at 50nM concentration. In PF434, PF531, and PF626

(Figure 7D-F), the lower dasatinib concentration decreased the intensity of the phosphorylation of Tyr^{576/577}, but just the 300nM concentration was capable of complete inhibition. In every combination treatment, dasatinib counteracted the activity of nintedanib, and it reduced or even inhibited the phosphorylation of FAK on Tyr^{576/577}. While the applied treatments influenced the level of pFAK^{576/577}, phosphorylation of the Tyr³⁹⁷ residue did not change or even slightly increased. This finding is unsurprising and can be explained by the general activation mechanism of FAK. Tyr³⁹⁷ serves as an autophosphorylation site that can be activated independently of Src. In this way, Tyr³⁹⁷ can be phosphorylated even in those cases when Src is inhibited^{28,61}.

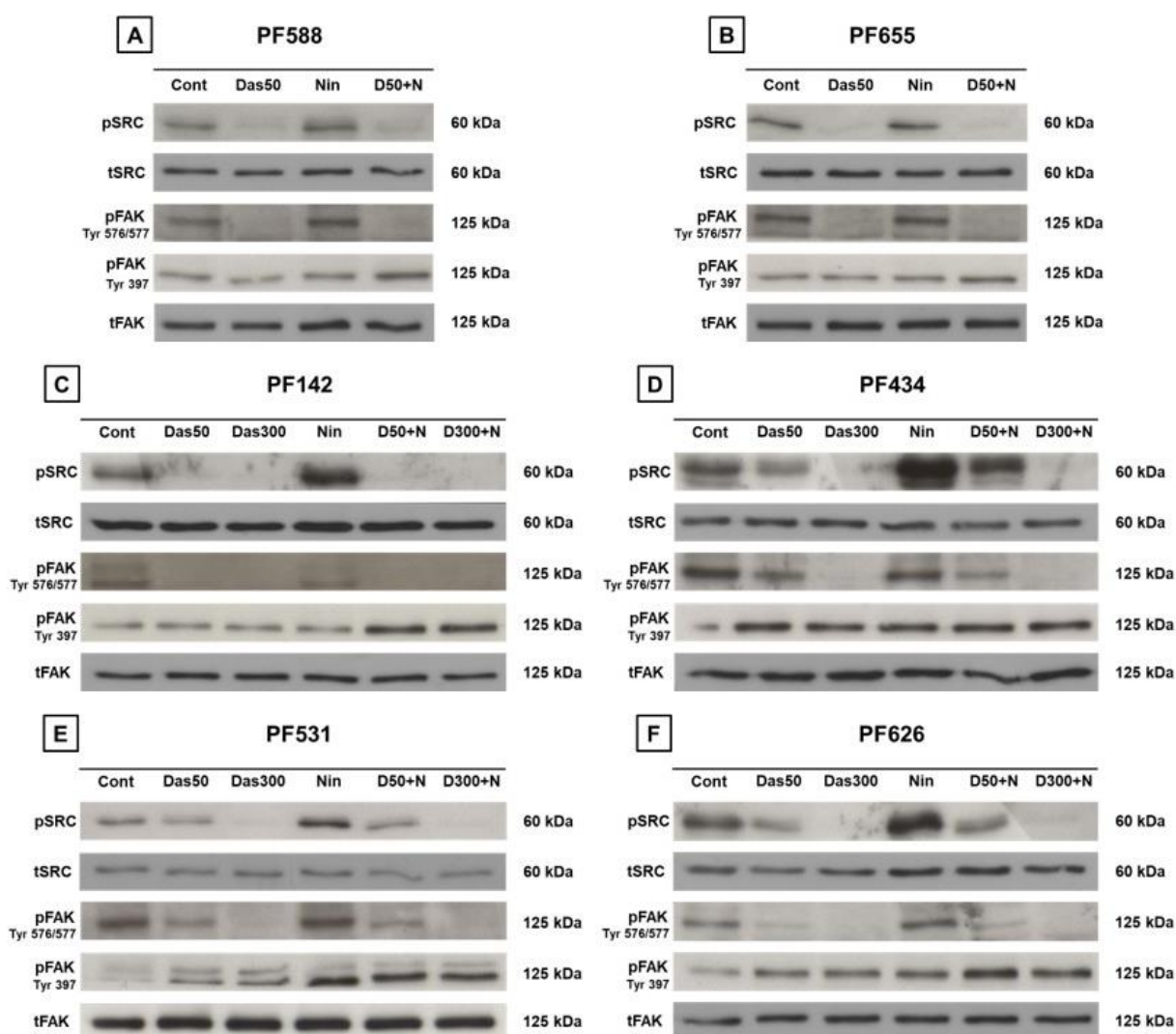


Figure 7 Effect of nintedanib and dasatinib alone, and in combination in the Src/FAK signalling pathway. (A-F) The cells were treated with nintedanib and dasatinib for 24 hours. Since the total Src and FAK expression was not altered by the treatments, these stainings were considered as loading controls. (Cont=control, Das50=50 nM dasatinib, Das300= 300 nM dasatinib, Nin= 1 μ M nintedanib, D50+N=50 nM dasatinib combined with 1 μ M nintedanib, D300+N= 300 nM dasatinib combined with 1 μ M nintedanib)

In the case of the PF588 and PF655 cell lines, the pre-experiments showed the efficacy of 50 nM dasatinib in the inhibition of pSRC and pFAK^{576/577}. As this effect could not have been further increased, these cell lines were not treated with increased dasatinib concentration.

4.4 Evaluating the combined effect of nintedanib and dasatinib on cell viability

After the evaluation of the efficacy of nintedanib and dasatinib alone, their combined effect on cell viability was determined by the SRB chemosensitivity assay. For this purpose, dasatinib, in 25 nM, 50 nM, 0.1 μ M, 0.4 μ M concentrations and 1 μ M, 3 μ M, 5 μ M nintedanib were applied in all combinations. The relation between the two drugs was determined by the calculated CI (combination index) values, which quantitatively depict synergism ($CI < 0.9$), additive effect (CI is between 0.9 and 1.1), and antagonism ($CI > 1.1$)⁵⁷. The interaction between the two drugs is synergetic if they create a stronger effect than the predicted sum of their individual effects⁶². The additive effect refers to a lower level of cooperation, where their effect simply summates⁶³. The antagonism is the opposite to the synergism, where the combined agents have an overall effect that is weaker than the sum of their individual effects⁶⁴.

The PF142 cells showed high sensitivity to both drugs in the single treatments. The viability curves of PF142 (Figure 8A) showed that the combination of the drugs further decreased cell viability upon higher nintedanib and lower dasatinib concentrations. The calculated CI values (Figure 8D) also represented that the two applied drugs enhanced the efficacy of each other in almost every combination when higher nintedanib concentrations were applied. However, the most pronounced synergetic effect between the two drugs was detectable when dasatinib was used in 50 nM concentration. This result can be explained by the high dasatinib sensitivity of PF142. The effect of dasatinib can be just slightly increased because even the lower dasatinib concentrations have a great impact on cell viability.

In the case of the PF626 cells (Figure 8B) strong additive and synergistic effects were found. At 1 μ M nintedanib concentration, the combined effect was stronger, than in dasatinib alone, and in 3 μ M and 5 μ M concentrations, it reduced the cell viability by 50%. The calculated CI values (Figure 8E) represented an additive effect between the compounds in lower dasatinib concentrations and a synergetic effect in the higher concentrations of the drug.

In the case of the PF655 cell line, the combination of the drugs was the most effective among all the cells. The cell viability was systematically decreased in response to the increasing concentrations of nintedanib (Figure 8C). The calculated CI values (Figure 8F) suggest that the two drugs have a greater efficacy together than alone. The results show that in 50 nM and 100 nM dasatinib concentrations, there was a well-defined synergetic effect between the two drugs.

As a summary, in the PF142, PF626, and PF655 cell lines, synergetic interaction was found between the two drugs. In PF142, this effect was visible in every nintedanib concentrations combined with 50 nM dasatinib. In the other two cell lines, the synergism was visible when 1 μ M, 3 μ M, and 5 μ M nintedanib were combined with 50 nM, and 100 nM dasatinib.

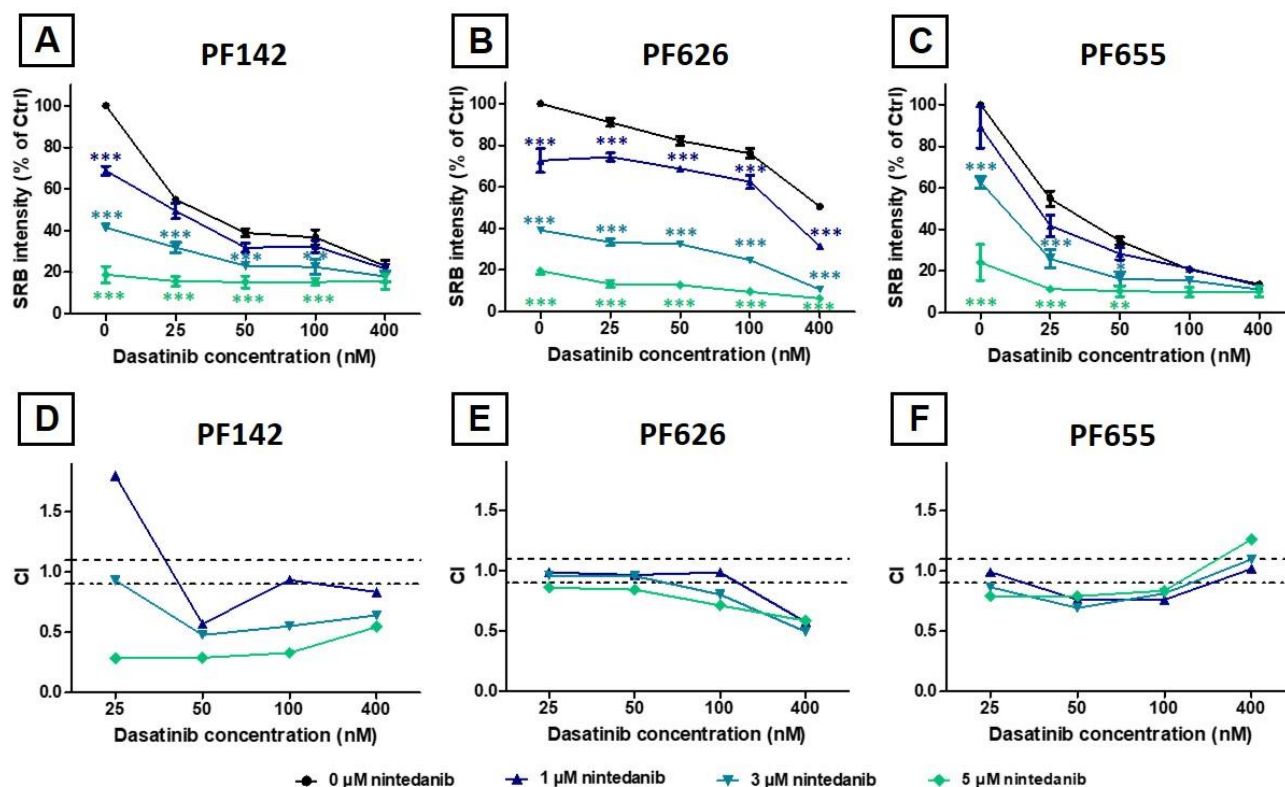


Figure 8 Evaluation of the combined nintedanib and dasatinib treatments in PF142, PF626 and PF655 cell lines. (A-C) Changes in the cell viability in response to different nintedanib and dasatinib concentrations measured by SRB assay. The cells were treated with the drugs for 72 hours. (D-F) Combination index (CI) was calculated for each combination based on the measured cell viability. CI < 0.9: synergism, 0.9 < CI < 1.1: additive effect, CI > 1.1: antagonism. Asterisks designate significant differences from the control calculated by two-way ANOVA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

In the PF434 cells, the drug combination had no synergistic effect. The viability curves (Figure 9A) showed that the lowest nintedanib concentration had no additional effect on the single application of dasatinib, while higher nintedanib concentrations did increase its effect. According to the representation of the calculated CI values (Figure 9C), only in 5 μ M nintedanib concentration was an additive effect observed in every dasatinib concentration. In the case of 50 nM and 100 nM dasatinib concentrations in combination with every nintedanib concentration, the CI value was at the edge of the additive and the antagonistic effect.

The results suggest that the viability of the PF531 cells could be decreased by increasing the concentration of nintedanib (Figure 9B). Different ranges of the CI values (Figure 9D) were found for different nintedanib concentrations. A synergetic effect was detected in every case when 5 μ M nintedanib was applied. The interaction between the drugs was slightly antagonistic

when the nintedanib was presented in 1 μM concentration. The cooperation between the drugs was enhanced, in the case of 3 μM nintedanib, by the application of a higher dasatinib dosage.

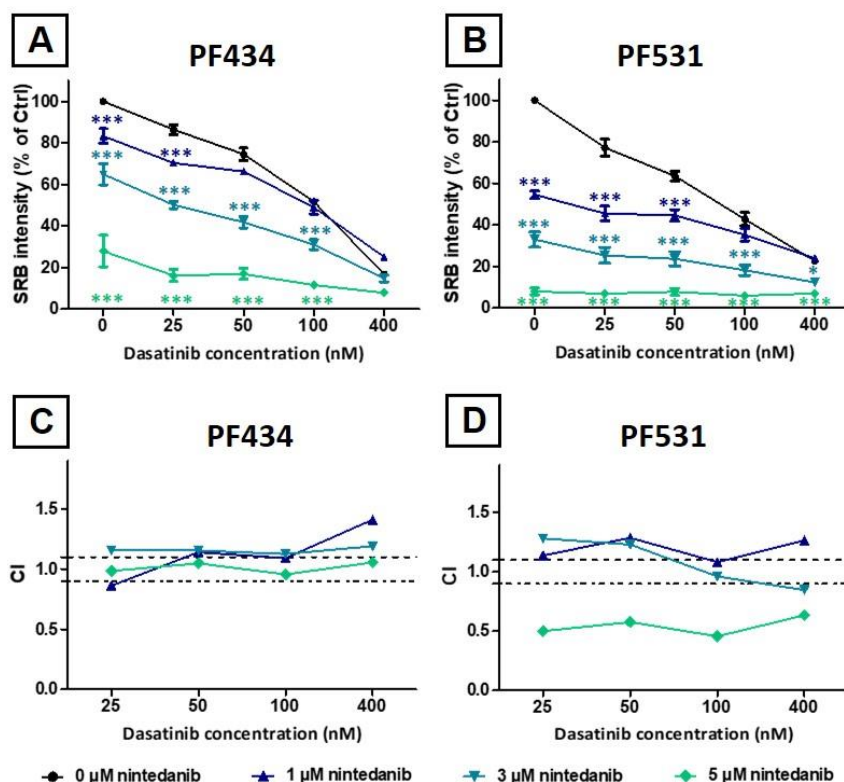


Figure 9 Evaluation of the combined nintedanib and dasatinib treatments in PF434 and PF531 cell lines.

(A-B) Changes in the cell viability in response to different nintedanib and dasatinib concentrations measured by SRB assay. The cells were treated with the drugs for 72 hours. (C-D) Combination index (CI) was calculated for each combination based on the measured cell viability. $\text{CI} < 0.9$: synergism, $0.9 < \text{CI} < 1.1$: additive effect, $\text{CI} > 1.1$: antagonism. Asterisks designate significant differences from the control calculated by two-way ANOVA: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

In brief, even though, the PF434 and PF531 sarcomatoid cell lines were relatively sensitive to both drugs as single agents, there was no synergetic effect between them in the physiologically relevant drug concentrations (nintedanib: $\sim 1 \mu\text{M}$; dasatinib: $\sim 50 \text{ nM}$ - 100 nM).

The measured viability of PF588 cells, treated with $1 \mu\text{M}$ nintedanib, was even higher than in the applied control. For this reason, the combination index could not be calculated, and this cell line was excluded from this analysis.

Based on the results of the short-term cell viability assays could determine those nintedanib and dasatinib concentrations that can be applied in further experiments. In most of the cases, a synergetic or an additive interaction was observable between the two drugs in 50 nM and 100 nM dasatinib, and $1 \mu\text{M}$, $3 \mu\text{M}$, and $5 \mu\text{M}$ nintedanib concentrations. Based on these results, the measurements continued with $1 \mu\text{M}$ nintedanib – as most physiologically relevant – along with dasatinib in 50 nM and 300 nM concentrations.

4.5 Cell cycle analysis

Since both dasatinib and nintedanib treatments strongly influenced the viability of the cells, their effect was further analysed by performing a cell cycle analysis. The sub-G1 phase represents the ratio of the dead cells. The cells in the G0/G1 phase are in the intermediate phase between mitosis and DNA replication. The S phase indicates those cells that undergo DNA replication. The dividing cells are presented in the G2/M phase of the cell cycle.

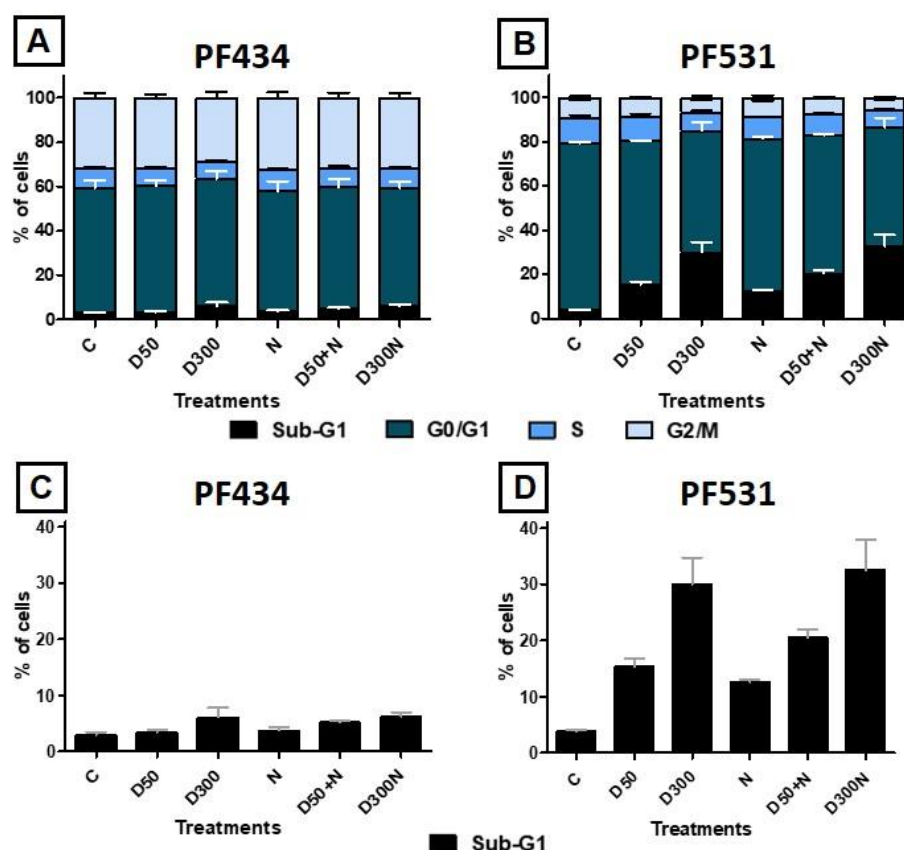


Figure 10 Result of the cell cycle analysis in PF434 and PF531 cell lines. (A-B) Distribution of the cell cycle stages in response to different concentrations of nintedanib and dasatinib. (C-D) Changes in the ratio of the cells in sub-G1 phase in response to the drug treatments. The cells were treated with nintedanib and dasatinib for 72 hours. Data are shown as average $\pm$ SEM of the minimum 3 independent measurements. (C=control, D50= 50 nM dasatinib, D300= 300 nM dasatinib, N= 1 μ M nintedanib, D50+N= 50 nM dasatinib combined with 1 μ M nintedanib, D300+N= 300 nM dasatinib combined with 1 μ M nintedanib).

In the PF434 and PF531 cell lines, increased cell death was observable in response to the drug treatments (Figure 10).

In PF434 cells, dasatinib increased the proportion of dead cells pronouncedly in 300 nM concentration alone and the combination (Figure 10C). PF434 cells can be characterized by a generally higher proportion of dividing cells compared to other cell lines. However, no change was found in the amounts of cells in the S- and G2/M phases after either treatment (Figure 10A).

The most significant effect in the cell cycle distribution was detected in the PF531 cell line (Figure 10B). Compared to the control, the ratio of the cells in the sub-G1 phase was strongly increased in the treated cells (Figure 10D), which suggests the cell death-inducing effect of the applied drugs. This phenomenon was more pronounced in the case of the dasatinib treatment. Furthermore, the proliferation of the cells was also decreased by both treatments (Figure 10B).

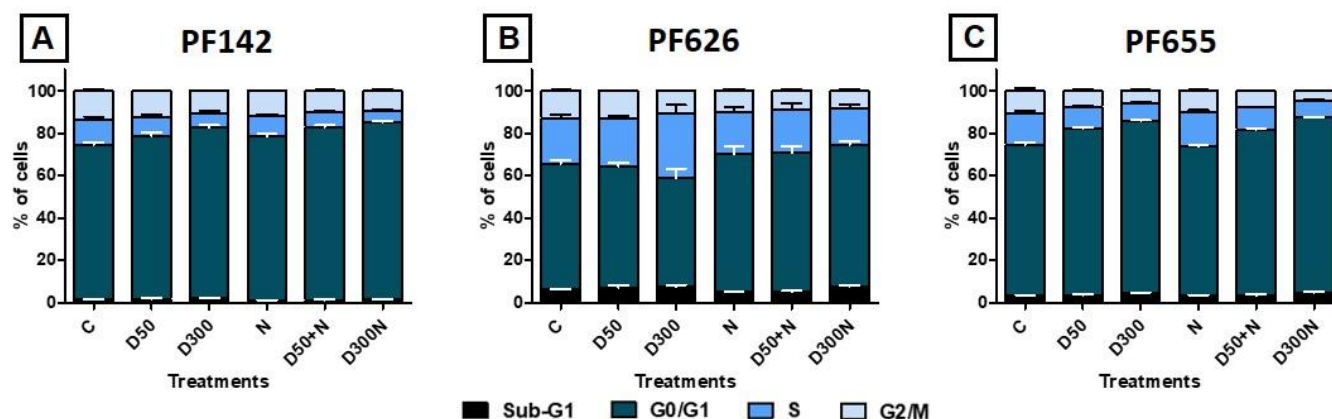


Figure 11 Result of the cell cycle analysis in PF142, PF626 and 655 cell lines. (A-C) Distribution of the cell cycle stages in response to different concentrations of nintedanib and dasatinib. Cells were treated with nintedanib and dasatinib for 72 hours. Data are shown as average $\pm$ SEM of the minimum 3 independent measurements. (C=control, D50= 50 nM dasatinib, D300= 300 nM dasatinib, N= 1 μ M nintedanib, D50+N= 50 nM dasatinib combined with 1 μ M nintedanib, D300+N= 300 nM dasatinib combined with 1 μ M nintedanib).

In the case of PF142 and PF655 cell lines, the ratio of the cells in the S-phase was reduced by dasatinib treatments, and this effect was increased at the higher drug concentration (Figure 11). Cell death was only slightly induced in the PF655 cells (Figure 11C). Meanwhile, in the PF142, the ratio of the cells in the sub-G1 phase was relatively constant during the different treatments (Figure 11A).

In the case of the PF626, just a moderate effect was observable (Figure 11B). In those cases where nintedanib was applied, cell proliferation decreased, as the ratio of the cells in the S-phase and the G2/M phase was reduced compared to the control. The treatment with 50 nM dasatinib did not notably modify the cell cycle profile. Meanwhile, in higher concentrations, it initiated cell cycle arrest in the S-phase.

According to the results of the chemosensitivity assays, the PF588 cell line was found to be insensitive to the different concentrations of the applied drugs. Based on these results, it was hypothesized there will be no remarkable changes in the distribution of the cell cycle stages. The pre-experiments supported this assumption, and this cell line was excluded from the measurement.

In summary, in the PF434 and PF531 cell lines, an elevated ratio of dead cells was observable in response to the drug treatments. In PF142 and PF655, dasatinib reduced the ratio

of the cell in the S-phase alone and by the combined treatments. In the PF626, the ratio of the cells in the S-, and G2/M phases were reduced by the application of nintedanib alone, and in combination.

4.6 Effect of nintedanib and dasatinib treatments on ERK and Akt signalling

In order to examine the effect of nintedanib and dasatinib treatments on signal transduction processes, other than the Src/FAK pathway, the expression, and phosphorylation of Akt and ERK proteins were analysed in all cell lines (Figure 12).

In the case of the PF588 and PF655 cell lines, 50 nM dasatinib has inhibited the phosphorylation of Src. Based on these results, these cell lines have not been treated with 300 nM dasatinib during the examination of the expression of Akt and ERK proteins.

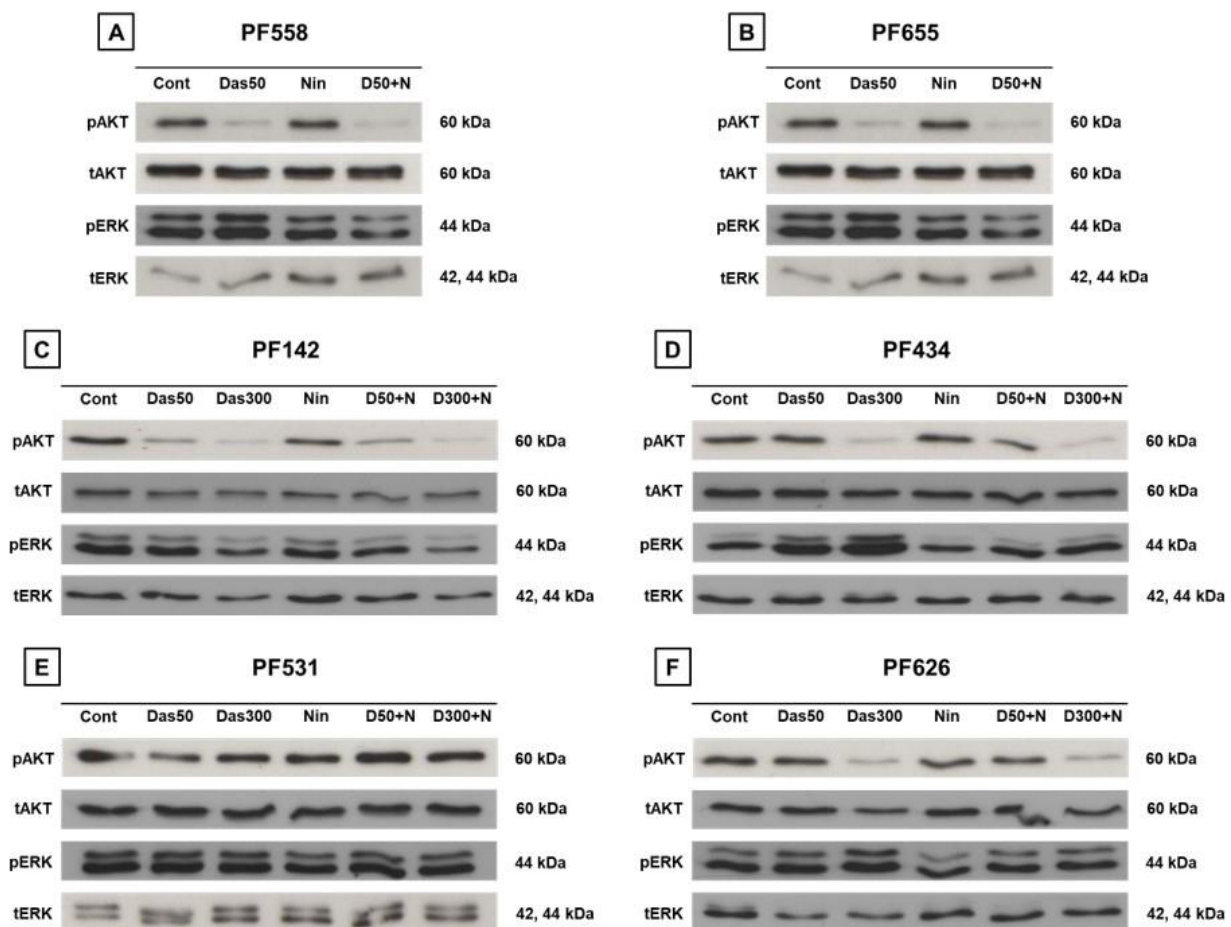


Figure 12 Effect of nintedanib and dasatinib treatments alone, and in combination on Akt and ERK activation. (A-F) The cells were treated with nintedanib and dasatinib for 24 hours. Since the total Akt and ERK expression was not altered by the treatments, these stainings were considered as loading controls. (Cont=control, Das50= 50 nM dasatinib, Das300= 300 nM dasatinib, Nin=1 μM nintedanib, D50+N= 50 nM dasatinib combined with 1 μM nintedanib, D300+N= 300 nM dasatinib combined with 1 μM nintedanib)

In the phosphorylation pattern of Akt protein (pAKT), similar tendencies were observable as by the pSRC and pFAK^{576/577}. In PF588, PF655, and PF142 cell lines (Figure 12A-C), its phosphorylation was already reduced, or even inhibited, by the 50nM concentration of dasatinib applied alone or in combination with nintedanib. In PF434 and PF626 cells

(Figure 12D, F), the dasatinib treatment was effective just in 300nM concentration. Meanwhile, nintedanib alone was unable to change the intensity of its activation. As an exception to the previous observations, in PF531 (Figure 12E), the pAKT level remained constant despite the treatments. The applied drugs did not modify the total Akt level (tAKT) in any of the cell lines.

ERK (also known as MAP-kinase) is a downstream member of the Mitogen-Activated Protein Kinase Cascade, and it has a crucial role in cell proliferation and cell cycle progression³¹. Based on the results of all the observed cell lines, dasatinib and nintedanib affected the phosphorylation of ERK neither in a single application nor in combination, as both the phosphorylated ERK (pERK) and its total (tERK) level remained unchanged after the treatments (Figure 12).

5 DISCUSSION

Malignant mesothelioma is a highly aggressive malignancy. The disease is associated with poor prognosis and general insufficient response to conventional therapies^{3,10}. The lack of effective treatment approaches emphasizes the need for the generation of novel preclinical models. The comprehensive *in vitro* studies contribute to the investigation of possible therapeutical targets and novel biomarkers that may be implemented later into patient care⁶⁵.

The aim of this work was the examination of the therapeutic sensitivity of the combined nintedanib and dasatinib treatment. For this purpose, six novel patient-derived cell lines were examined.

In the applied cell lines, the efficacy of nintedanib and dasatinib treatment was studied by short-term viability assays. Against nintedanib, the sarcomatoid PF531 (IC₅₀ 0.9 µM) showed the greatest sensitivity. Nintedanib had just a moderate effect on the epithelioid PF588 (IC₅₀ 4.2 µM) and PF626 (IC₅₀ 4.4 µM) cell lines. Contrary to the expectations, the sarcomatoid and biphasic cell lines – which are generally considered less treatable – were found to be more sensitive to nintedanib than the ones with epithelioid features. This observation is in contrast with the findings of the second phase of the LUME-Meso trial, where the clinical benefit in patients with epithelioid mesothelioma was found to be evident⁴⁹. The patients with non-epithelioid features were excluded from the third phase of the trial. According to some investigators, this decision may have contributed to the final negative outcome⁴⁶. Accordingly, my results emphasize the effectiveness of nintedanib in the sarcomatoid histotype.

The cells generally showed higher sensitivity to the dasatinib than to nintedanib, however, the cell response was more variable among the cell lines. Dasatinib decreased the most remarkably the viability of the biphasic PF142 (IC₅₀ 11.1 nM) and the epithelioid PF655 (IC₅₀ 10.6 nM). Similar to the nintedanib treatment, the epithelioid PF588 (IC₅₀ 196.8 nM) and PF626 (IC₅₀ 458.0 nM) showed the lowest sensitivity. The relation between the sensitivity and histological subtype was similar as for the nintedanib treatments. The epithelioid PF655 serves as an exception from this observation, as it was highly responsive to the drug, with the lowest IC₅₀ value.

Interestingly, the PF588 and PF655 cell lines responded differently to dasatinib treatments, despite their common origin. While PF655 showed an increased sensitivity to the drug, the PF588 presented as a relatively resistant cell line. In contrary to this finding, the difference in the response of the two cell lines was less remarkable during the nintedanib treatment. The distinct response to dasatinib suggests a change in the molecular mechanism inside

the tumour cells triggered by the first-line therapy. This observation cannot be generalized, and further cell line pairs should be examined to draw conclusions.

As a summary of the findings of the short-term viability assays, the biphasic PF142 cell line was found to be highly sensitive to both drugs, whereas the epithelioid PF588 and PF626 represented the other end of the spectrum, with a moderate response to the treatments. The insufficient responsiveness of the epithelioid cell lines was opposed to the previous expectations, as they are generally associated with a better prognosis.

In 2007, Tsao and her co-workers comprehensively studied the effect of dasatinib on four human MPM cell lines obtained from the American Type Culture Collection⁶⁶. In their work, the IC₅₀ values were determined by two different short-term viability assays, which hinders the direct comparison. Although, their result had a similar distribution in terms of histotypes. In their case, also a biphasic cell line showed the highest sensitivity (MSTO-211H, IC₅₀ 25 nM) to the dasatinib treatment. A cell with epithelioid characteristics was insensitive to the drug (NCI-H2452, IC₅₀ 980 nM). The researchers interpreted their results as promising because three (MSTO-211H, IC₅₀ 25 nM; NCI-H28, IC₅₀ 60 nM; NCI-H2052, IC₅₀ 80 nM) of the four studied cell lines had IC₅₀ levels below 100 nM, which is physiologically relevant and achievable in serum levels. In this current work, three of the six observed cells had this characteristic (PF142, IC₅₀ 11,1 nM; PF655, IC₅₀ 10,6 nM; PF531, IC₅₀ 94,0 nM), and PF434 was just slightly above 100 nM, with IC₅₀ 108,6 nM.

In a similarly extensive work, Laszlo and her co-workers (2018), studied the effect of nintedanib on 20 malignant mesothelioma cell lines¹. During their experiments, the cell viability was also determined with SRB assay after 72 hours of nintedanib treatment. The studied cell lines showed a wide range of sensitivity to nintedanib, with IC₅₀ varying from 1.6 µM to 5.9 µM. Compared to the result of Laszlo et al.¹, the IC₅₀ values determined in this current work lay within the range of 0.9 µM to 4.4 µM. In this way, the comparison can confirm the effectiveness of nintedanib. Similar to my work, their research team also did not find a significant association between the efficacy of nintedanib and the histological subtype.

The efficacy of the combined agents was tested by short-term viability assays. During these measurements, the same methodology was followed as in the single-drug SRB assay. The interaction of the two agents was determined by the calculated combination index (CI) values. Based on the calculations, in the PF142, PF626, PF655 cell lines, a synergetic effect was found between the physiologically relevant concentrations of the two drugs (nintedanib: ~ 1 µM; dasatinib: ~50 nM-100 nM). It means that the combined effect exceeded their individual impact⁶². In the PF434 and PF531 cell lines, the drug interaction was additive

– or even antagonistic – in the relevant drug concentrations. The study of PF588 did not continue because it responded insufficiently to the combined treatments.

As an unexpected outcome, the combined treatment seems to be less relevant in the studied sarcomatoid cell lines (PF434 and PF531) although they have previously shown sensitivity against both drugs. As a possible explanation, in these cases dasatinib and nintedanib alone had such a strong effect, that could not be further increased by combination. This argument is not supported by the fact that in the biphasic PF142, the interaction was found to be synergetic, despite its high sensitivity to both drugs.

During the experiments, that nintedanib and dasatinib concentration pair, which can sufficiently reduce the viability of the cells, greatly varied. These results suggest that the cell response to the nintedanib and dasatinib treatment is highly affected by molecular mechanisms and characteristics that are currently unexplored.

The differences in the results between the repeated SRB measurements were acceptable. Increased deviations were observable mainly in low drug concentrations, where the cell response is generally variable due to the modest effect of the drugs. As more factors have influenced the results, compared to the single application of nintedanib and dasatinib, the deviations in the combined drug treatments were higher. The results – along with the conclusions – are influenced by the general characteristics of the SRB staining. Namely, it is independent of the mitochondrial activity of the cell, however, it cannot distinguish between viable and dead, but still adherent cells⁵⁶. This factor has a general influence, especially in greater drug concentrations. Due to these arguments, the results should be further evaluated by cell cycle analysis and long-term viability assays.

After studying the effect of nintedanib and dasatinib on cell viability, my work has focused on understanding the underlying mechanisms. First, the changes in the cell cycle upon the drug treatment were analysed. The results showed that nintedanib and dasatinib are able to influence the cell-division cycle in different forms and intensities.

In the sarcomatoid cell lines, a cell death-inducing effect of the treatments is suggested by the elevated ratio of the cells presented in the sub-G1 phase. In PF434, this phenomenon was observable in the higher dasatinib concentration and the combined treatments. Meanwhile, in PF531, it was markedly visible in every experimental setting. This result provides important insights into the molecular mechanisms within the PF531 cells. As the cell death induction was more remarkable when dasatinib was applied, its contribution to the decreased cell viability can be concluded. On the other hand, nintedanib may achieve its effect through other mechanisms.

These cell lines were responsive to both nintedanib and dasatinib, but according to the SRB assays, their combination did not provide further advantages. However, the ratio of dead cells during the cell cycle analysis was elevated in response to the combined therapy. This result underlines the relevance of the combined nintedanib and dasatinib treatment in cells with similar molecular composition as PF531.

In the biphasic PF142 and epithelioid PF655 cell lines, the decreased ratio of the cells in the synthesis phase indicates reduced proliferation by dasatinib- and the combined treatments. The relatively small change in the cell cycle profile of the PF142 is somewhat surprising, concerning its high sensitivity.

In the epithelioid PF626 cell line, there were moderate changes in the cell cycle distribution in response to the drug treatments. This finding corresponds to its modest sensitivity to nintedanib and dasatinib, evaluated by the cell viability assays. PF588 was excluded from the measurement due to its insufficient response to the treatments.

In summary, these results indicate a role of the cell cycle alteration in the mode of action of nintedanib and dasatinib. Even though, it is concludable that additional molecular processes also contribute to their effect.

The role of dasatinib in the cell cycle arrest was also confirmed by Tsao and her co-workers (2007)⁶⁶. In their study, four cell lines were stained with a prerequisite for DAPI, called propidium iodide (PI). The DNA content was analysed on a cytofluorimeter by fluorescence-activated cell sorting (FACS) analysis⁶⁶. In their work, they found changes in the cell cycle distribution, as the ratio of the cells in the S- and G2/M phases were more increased. In their work, apoptosis-induction and cell cycle arrest were detected in the sensitive cell lines. In half of the studied cell lines (MSTO-211H, NCI-H28), an elevated level of dead cells was found. These cells have previously shown sensitivity to dasatinib in the proliferative assays. This is in accordance with my results; however, it did not confirm the relevance of the histotype in the cell response. Their research group also studied the ratio of the apoptotic and necrotic cells. According to these results, dasatinib increased both the ratio of apoptotic and necrotic cells in the sensitive cell lines. These results are noteworthy, as the cell death-inducing effect was less perceptible in the cell cycle analysis. This finding shows the need for other apoptosis assays, for instance the TUNEL assay.

Immunoblot assay was performed to reveal the differences in the cellular signalling to the drug treatments. The measurements revealed a paradoxical pSRC-inducing effect of nintedanib. Dasatinib, as shown before, inhibited Src phosphorylation effectively by itself, and it also counteracted the activity of nintedanib in the combined treatments. In the PF142, PF588, and

PF655 cell, dasatinib was effective even at 50 nM concentration, while, in the PF434, PF531, PF626 cell lines, a higher concentration was required for the full inhibition. The concentration dependency of the inhibition of Src-family kinases (SFK) can give a possible explanation for the differences in the cell response. The different SFKs have different IC₅₀ values, so their activity is being blocked by different concentrations of dasatinib. Moreover, in higher concentrations, dasatinib is capable to inhibit various other kinases⁶⁶. It is possible that in the different cell lines, various members of the SFKs play a dominant role, and in this way, different dasatinib concentrations are required to alter their activity.

In the phosphorylation of the FAK protein on the Tyr^{576/577} residues, similar changes were observed as by pSRC. This similarity was expected because of their strong interaction. The effect of Src on these residues has importance in the signalling cascade. The phosphorylation on Tyr^{576/577} induces further stimulations, which leads to full activation of FAK, and triggers a positive feedback loop for the signal transduction^{29,61}. In this way, Src-inhibition can moderate the signal intensity. In contrary to Tyr^{576/577}, the drug treatments did not affect the phosphorylation on the Tyr³⁹⁷ residue. As a site for autophosphorylation, Tyr³⁹⁷ is a key residue on FAK because it is necessary for its scaffolding functions. It was previously found that the phosphorylation of this site induced by other interactions independently of Src^{28,61}. This observation can explain the absence of the changes in this residue in response to the drug treatments. The total amount of FAK and Src was not affected by the drug.

Similar to the previous findings, the nintedanib treatment did not influence the phosphorylation of Akt protein. The pAKT level was decreased or inhibited by 50 nM dasatinib alone, and in combination in the PF142, PF588, and PF655 cell lines. In the PF434 and PF626 cell lines, this effect was observed just in a higher dasatinib concentration. In the PF531 cell line, the drug treatment did not affect the phosphorylation of Akt. The applied drugs did not modify the expression of the total Akt and ERK proteins and did not influence the phosphorylation of ERK in any of the cell lines.

These results show that further studies are required to explore those molecular characteristics that make the cells responsive to the nintedanib and dasatinib treatment. It is visible that the changes in the phosphorylation of the studied proteins are not directly correlate to the drug sensitivity of the cell lines. Nintedanib was able to potently decrease the cell proliferation in certain cell lines despite its pSRC-inducing effect. On the other hand, dasatinib strongly influenced the protein phosphorylation in the epithelioid PF588 and PF655 and the biphasic 142 cell lines. This activity correlated with the enhanced dasatinib sensitivity of the PF142 and PF655; but not with the moderate response of the PF588. However, there can be a

relation between the decreased activity of these proteins and the effectiveness of the combined treatments. A strong synergism was found between the nintedanib and dasatinib in those two cell lines, where the dasatinib notably affected the phosphorylation.

Nevertheless, there was no direct conclusion found between the changes in the phosphorylation of Src, FAK, and Akt and the viability of the cell lines, these molecular mechanisms still can play important role in other attributes, such as their mobility and capacity to metastasis. The similarities in the changes of the protein phosphorylation patterns confirm the connection between the distinct signal transduction pathways.

In their work, Tsao and her co-workers treated four human MPM cells with 100 nM dasatinib for 24 hours, and examined the phosphorylation of the Src, FAK, Akt, and ERK proteins⁶⁶. Dasatinib inhibited the phosphorylation of Src in every cell line. As the differences in the cell response were observed just in lower dasatinib concentrations, the comparison would not give further information. In their work, the Tyr⁸⁶¹ phosphorylation site of FAK was studied, which has a role in the regulation of invasion²⁸. Compared to my observations, the changes in this phosphorylation site corresponded better to the sensitivity to dasatinib, as it was completely blocked in the sensitive cells and only reduced in the irresponsive NCI-H2452. Similar to my results, there was no direct relationship between the inhibition of pAKT and the dasatinib sensitivity. In these measurements, the total amount of Src, FAK, Akt, and ERK, along with the phosphorylated ERK level remained constant during the drug treatment.

In 2018, Laszlo and her co-workers performed a western blot immunoassay on five different malignant mesothelioma cell lines that were treated with 0.5 μ M nintedanib for 24 hours¹. During these measurements, the phosphorylation of Akt and ERK proteins was studied. In these measurements, the level of pAKT and ERK changed more notably compared to the observations in this current work. The changes in the total cellular amount of the proteins were similar. The level of the tAKT remained constant in the cell lines of both studies.

6 CONCLUSIONS AND FUTURE PERSPECTIVES

The main motive of this current work was the better understanding of malignant mesothelioma, a unique asbestos-related malignancy. Currently, there are no curative therapeutic options for this disease. Despite the various experimental approaches, just a low percentage of the studied drugs reach the clinical trial phase, and among them, just a few of them show real effectiveness. By considering the role of angiogenesis in the pathogenesis of mesothelioma, our research group has focused on the application of anti-angiogenic tyrosine kinase inhibitors. In the early studies, the application of the triple kinase inhibitor nintedanib seemed to be promising. Unfortunately, this agent failed to show effectiveness in the confirmatory phase of the clinical trial. Several factors can contribute to the unpleasant result, including a paradoxical pSRC-inducing. It was hypothesized that the efficiency of nintedanib might be increased with the additional application of a Src-inhibitor. The small-molecule tyrosine kinase inhibitor dasatinib was selected for this purpose. Dasatinib also had a remarkable effect against the mesothelioma cells in the studies *in vitro*, but it has failed to show adequate clinical activity during the clinical trials. According to this information, the main hypothesis of the work was that additional therapeutic benefit can be gained by the combination of nintedanib and dasatinib. For this purpose, six novel cell lines (PF142, PF434, PF531, PF588, PF626, PF655) were applied as a preclinical model that was originated from pleural effusion samples of 5 MPM patients.

We calculated the half-maximal inhibitory concentrations (IC₅₀ values) of nintedanib and dasatinib in 6 different mesothelioma cell lines and found that the biphasic PF142 showed great sensitivity to both drugs, whereas the epithelioid PF588 and PF626 cell lines were the least responsive to the treatments. Our findings are in line with earlier results found in the literature^{1,66}. Then we analysed the effect of the combination treatment of dasatinib and nintedanib.

In the PF142, PF626, and PF655 cell lines, there was a synergic interaction of the two drugs in the physiologically relevant concentrations. In the PF434 and PF531 cell lines, the interaction between the drugs in the relevant concentrations was mainly additive. During the experiments, that nintedanib and dasatinib concentration pair, which can sufficiently reduce the viability of the cells, greatly varied. This finding highlights the molecular heterogeneity of mesothelioma cells. According to the cell cycle analysis, the combined treatment induced apoptosis or arrested the cell cycle in the sensitive cell lines. In the sarcomatoid PF434 and PF531 cell lines, the cell death-inducing effect was detectable. Meanwhile, the cell cycle was arrested in the biphasic PF142 and the epithelioid PF655 cells. The cell distribution of the PF626 cells did not change remarkably in response to the drug treatments. Then we examined the changes in the expression

and phosphorylation of key signalling molecules from the Ras/Raf and PI3K/Akt signalling pathways, induced by the different concentrations of dasatinib and nintedanib

These measurements revealed a paradoxical pSRC-inducing effect of nintedanib. Dasatinib was able to counteract nintedanib and decreased, or completely inhibited the pSRC alone, and in combination, either in 50 nM or 300 nM concentrations. The changes in the phosphorylation of pFAK(Tyr^{576/577}) and pAKT were similar as by the pSRC. The PF531 cell line served as an exception, where the drug treatments did not modify the pAKT level. The total cellular amount of tSRC, tFAK, tAKT, tERK proteins, along with the level of pFAK(Tyr³⁹⁷), and pERK remained constant in any of the cell lines.

There was no significant association found between the changes in the protein phosphorylation and the sensitivity of the cells to nintedanib and dasatinib. With this outcome, the study was unable to identify those molecular characteristics that can help to predict the relevance of the combined treatment. The achievement of this goal requires further work, with the involvement of new mesothelioma cell lines and candidate proteins for molecular markers.

Among the observed 6 cell lines, there were great differences within the response to the drug treatments. This finding confirms the previous assumptions about the molecular heterogeneity of the mesothelioma cell lines. Further MPM cell lines should be involved in the study to the extensive exploration of correspondence between the cell sensitivity and the molecular characteristics.

PF588 and PF655 cell lines have been originated from the same patient before and after chemotherapy. The most intriguing result of the work was the difference between the response of these cells to the dasatinib and combined drug treatments. While the PF588 did not respond well to the treatments, PF655 was one of the most sensitive cell lines to dasatinib, and a synergetic effect was found between the two drugs in the combined SRB assays. This finding implies that the chemotherapy can sensitize cells to dasatinib alone, and in combination with nintedanib. It also shows the potential of these drugs as a possible second-line treatment of malignant mesothelioma. Although, the low case number (n=1) inhibits drawing a scientifically relevant conclusion. This hypothesis should be tested on cell lines that are established from patients that underwent chemotherapy.

The unfortunate outcome of the clinical evaluation of nintedanib and dasatinib highlights one of the greatest challenges in oncology: the differences between the *in vitro* and *in vivo* cell response. For a better understanding of the tumorigenesis and therapeutic response, appropriate pre-clinical models should be applied. Cell cultures cannot mimic the tumour microenvironment and the interaction of tumour with other cells, although these factors can

drastically modify the response to treatment. Taking that into account, in the next phase of the experiments, the effect of the combined nintedanib and dasatinib treatment should be studied on xenografts.

The above-presented work is just a portion of the extensive research towards MPM. The results of my assay, along with the most recent findings of the research team of the University Hospital Essen were published this year in *Frontiers in Cell and Developmental Biology*⁶⁷.

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