Pirfenidone regulates pericellular proteolysis in cancer

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1. Introduction

1.1. Lung cancer

1.1.1. Epidemics

Lung cancer is one of the most common cancer types globally with the highest incidence of all cancers in men and the fourth highest in women in 2008. It is associated with bad outcome and high lethality, which results in 1.4 million (18%) lung cancer related deaths worldwide annually [1]. A vast majority (approximately 80-90%) of the lung cancer cases are related to cigarette smoking [2], but also other risk factors like arsenic [3] or radon exposure [4] as well as air pollution [5] are well known.

1.1.2. Cancer development

Today's explanation of the development of cancer is based on the multiple-hit theory, which describes the change of a "healthy" into a cancer cell as a long-lasting process with an accumulation of multiple DNA mutations and subsequent changes in the behaviour of the cell [6]. DNA mutations can be caused by various environmental or endogenous factors. Examples for environmental carcinogens are radiation [7], especially the UV-radiation from the sun [8] or chemical substances like aromatic amines or polycyclic aromatic hydrocarbons [list with carcinogenic substances at [9]. Also viral infections [10] e.g. with the hepatitis B or C virus [11, 12], Eppstein-Barr virus [13] or the high-risk subtypes of the human papillomavirus [14] can induce mutations. Endogenous mutations, which may trigger spontaneous carcinogenesis, include among others depurination caused by the chemical instability of the DNA, mutagenesis by free radicals of oxygen and errors in the DNA duplication [review [15]]. In a normal cell, mutations are repaired by multiple mechanisms like the direct repair, the base excision repair [16] or the nucleotide excision repair [17]. If the mutations cannot be repaired, the cell undergoes apoptosis [18]. In cancer cells, however, not all mutations in the DNA are repaired and the mutations accumulate.

In the development of cancer, these mutations affect critical cellular functions, which are described as the "hallmarks of cancer" [19]. The hallmarks of cancer include increased pro-proliferative signalling, evasion of growth suppression and apoptosis [20], angiogenesis induction [21] and activated tissue invasion and migration leading to metastasis formation [22, 23]. Although there is no specific blueprint for the order of the

cancer hallmark acquisition, there are some general steps in the cancer development, which can be observed in the most types of cancer.

Firstly, an increase in cell proliferation, which leads to a higher amount of cancer cells and elevated probability of additional mutations, must be observed [23]. Secondly, cancer cells gain the ability to migrate and invade. Thereby the cells must produce or activate proteases, which degrade the surrounding extracellular matrix to create migration tracks in which the cells can move [24, 25]. And finally, the cancer cells themselves must increase their mobility to invade nearby blood or lymphatic vessels. This can lead to detaching from the primary tumor, resulting in growth of lymph node or distant metastases.

To enable a fast and aggressive growth of the tumor, the interactions between cancer cells and surrounding tissue as well as the host immune system must be modified. One of the main interactions with the peritumorous tissue is the induction of angiogenesis to supply the cancer cells with oxygen and nutrients for further growth [21, 26]. The complex interactions between the cancer cells and the host immune system are not fully understood. But it is known that cancers develop evading strategies to escape the host immune system. These evading strategies include immune suppression by regulatory cells of the immune system like regulatory T cells as well as modified antigen presentation on the tumor cell surface [27, 28].

1.1.2.1. Proliferation in cancer

The first step in the development of nearly all types of cancer is an increased proliferation. The most common mutations, which lead to increased proliferation, effect the cell cycle or the regulation of apoptosis. Cell cycle describes the process of the duplication of the cell DNA and the following division into two cells. Therefore, the cell cycle can be divided into the separation phase, also called mitosis, and so-called interphase between two mitoses. The interphase can be further divided into the gap 1 (G1), synthesis (S) and gap 2 (G2) phase. In the G1 phase the cell grows and enhances metabolic processes in preparation for the S phase. In the S-phase the DNA replication takes place. After the DNA duplication, rapid cell growth, as preparation for the mitosis, occurs in the G2-phase [29-31].

To prevent an excessive, uncontrolled proliferation, the cell cycle is strictly controlled. Main regulation points are the transitions between different phases of the cell cycle, which are called checkpoints. To pass such a checkpoint, specific proteins named cyclins must activate cyclin-dependent-kinases (CDKs). First discovered in 1983, there are more than 30 cyclins discovered until today [32, 33]. All cyclins have a common

structural element called cyclin-box, which is responsible for the binding with CDKs [34]. The cyclins, which are regulating the cell cycle, are expressed in varying amounts based on the cell cycle phase. In healthy cells specific cyclins are upregulated at the end of a cell cycle phase and indicate a healthy status of the cell and finished preparations for the next phase of the cell cycle [35]. Then a cyclin can form a complex with its matching CDK, leading to the activation of the CDK. Activated CDK can phosphorylate multiple substrates [36, 37], which in turn are necessary for the transition of the cell into the next phase of the cell cycle. CDKs are generally present in the cell the whole cell cycle, but their kinase activity without the binding of cyclins, is exceedingly small.

The molecular regulation of cyclins and CDKs is incredibly complex and involves numerous proteins and pathways. Cyclin expression is regulated by the many signalling pathways, including the extracellular-signal regulated kinases (ERK)-pathway and the protein kinase B (PKB/AKT) pathway [38, 39]. These pathways are the downstream mediators of extracellular growth factors, like the epidermal growth factor (EGF) [40] and insulin or platelet derived growth factor (PDGF) [41]. Prominent inhibitors of cyclin expression are the so-called tumor suppressor proteins including p53 and the retinoblastoma (RB) protein [42]. Both can inhibit the cell cycle or even induce apoptosis when they are upregulated [43]. The activity of CDKs is mainly regulated by the expression of the cyclins but there are two families of direct CDK inhibitors: the inhibitor of CDK 4 (INK4) family and the CDK interacting protein (Cip/Kip) family [44, 45], which can induce cell cycle arrest in response to certain growth factors [46]. Due to their critical role in the regulation of cell proliferation, cyclins and their regulators are often affected by mutations in tumor cells. The most common mutations are described in cyclin D, which is altered in about 50% of breast cancer cases [47], p53 [48, 49], RB protein [50, 51] and the rat sarcoma (ras)-oncogene, which is a central component of the ERK pathway [52, 53].

The primary effect of the elevated proliferation rate is an increased number of tumor cells, resulting in a growing tumor mass. Such a growing tumor mass cannot be supplied with nutrients and oxygen by diffusion. Therefore, the growth of new blood vessels, a process called angiogenesis, must be induced. The most prominent inducers of angiogenesis are members of the vascular endothelial growth factor (VEGF) and hypoxia induced factor (HIF) families [54, 55]. Partially, these factors are expressed in response to the hypoxic conditions in a central tumor mass. However, mutations in the members of both families have been described in cancer cells [56]. Additionally, activators of angiogenesis, like matrix metalloproteinases (MMPs), plasmin (PLA), kallikreins and heparinases, are often upregulated in cancer [26, 57].

Beside this, an increased proliferation rate along with the suppression of the proteins regulating the cell cycle markedly elevates the risk of mutations in the cancer cells. Natural selection of the most aggressive cells is leading to the next step in cancer development, which is the acquisition of migratory and invasive abilities [58].

1.1.2.2. Migration and Invasion in cancer

The biggest threat of cancer is not represented by the primary tumor, but by the growing of secondary tumor masses, called metastases. To metastasise, cancer cells must migrate and invade into nearby tissue in order to get connection to the blood- or lymphatic vessel system.

The complex process of migration requires many changes in cell behaviour. Thereby tumor cells use mechanisms, which are known from physiological processes like embryogenesis, wound healing or immune cell movement [59]. But these physiological processes are strictly regulated by internal or external stimuli. In cancer, however, the pro-migratory effects are permanently activated and adequate inhibitory mechanisms are inactivated or ineffective due to mutations [review [24]].

The process of tumor cell migration can be divided into several steps [60]: Firstly, the tumor cells must stretch out their leading part, adhere and interact with the extracellular matrix (ECM) components or surrounding cells. These interactions are mostly initiated by transmembrane receptors called integrins. Integrins connect ECM components with intracellular adaptors and signalling proteins [61]. Secondly, pericellular proteases become activated, leading to proteolytic degradation of ECM components. This creates intercellular gaps, in which the cancer cells can move forward. Additionally, cell-cell contacts as well as connections between cells and ECM components can be degraded by proteases. As a result, cancer cells are relieved from their former position and start migration. A prominent role in the pericellular proteolysis is held by MMPs, cathepsin B and serine proteases like urokinase-type plasminogen activator (uPA), PLA or kallikreins [62]. All these proteases are secreted as zymogens and become activated on the cell surface [63-65]. Thereby active proteases are the main activators of zymogens, leading to a positive feedback loop and uncontrolled, overshooting proteolysis around tumor cells [66]. The primary target of these proteases is collagen, the main component of the ECM and the basal membrane, but ECM proteins like fibronectin or laminin are degraded as well [67]. Especially the role of MMP-2 and MMP-9, also known as gelatinases, in cancer progression is established [68]. Finally, the cell must move, which happens mostly by actomyosin contractions in the cell [69]. The regulation of these contractions is similar to the actomyosin contractions in smooth

muscle cells and involves the myosin light chain kinase (MLCK), the intracellular Ca^{2+} concentration [70] and the small G-protein ras-homologue (RHO).

Due to the complexity of ECM-tumor communication, multiple patterns of tumor cell migration and invasion have been described. The pattern is determined by the origin of the cancer cell, the strength of the intercellular binding between tumor cells and the activity of extracellular proteases. Generally, it can be distinguished between single-cell, chain-string and collective migration [60]. Thereby single cell migration is the most aggressive form, which allows a directed migration of a single cell. This migration type is characterized by a polarization of the cancer cell with an actin-mediated adhesion and protrusion at the cell front and a myosin-mediated retraction at the cell end [71, 72]. In chain-string migration, small strands of cancer cells are migrating into the same direction, mostly guided by a chemokine gradient or extracellular tissue structures. Thereby the cell-cell contacts remain intact, but no supracellular coordination and contraction can be observed [73-75]. In collective migration cell-cell junctions are preserved and the tumor mass remains the ability of supracellular coordinated actomyosin contractions with a collective movement of the tumor mass [76]. Most carcinomas prefer this collective migration pattern, especially in early stages of tumor development [77]. However, with an increasing number of mutations upon tumor cell development, the migration and invasion pattern can change, often leading to a more aggressive pattern [78].

All patterns of invasion can lead to metastasis formation, which is defined as the growth of tumor cells in other organs. Development of metastases is the last step in tumor development and responsible for 90 % of cancer related deaths [79]. Therefore, an increased motility and invasion leads to a bad prognosis of cancer patients [80]. Consequently, also high concentrations and increased activity of proteases like MMPs, cathepsin B or serine proteases are associated with a worse clinical prognosis of breast, colon, lung and gastric cancer patients [81-83].

1.2. Plasminogen/plasmin system

1.2.1. Components of the plasminogen/plasmin system

The plasminogen/plasmin (PLG/PLA) system plays a central role in many physiological and pathological processes. First and best described is its role in the intravascular fibrinolysis, where PLA can effectively degrade fibrin clots [84]. But also in embryogenesis [85], ovulation [86], wound healing [87], angiogenesis [88, 89] and tumor invasion [90], the role of PLA is well described.

The central step in the activation of the PLG/PLA system is the generation of PLA by the cleavage of its precursor PLG. Plasminogen is a 92 kDA large protein, which gets synthesized in the liver and circulates in human plasma with an average concentration of 2 µM [91]. It can be converted into plasmin by an enzymatic cleavage at the peptide bond between Arg^{561}-Val^{562} [92]. This activation process can be mediated by two serine proteases tissue-type plasminogen activator (tPA) or uPA and can be accelerated by altering the conformation of PLG through binding to surface-associated PLG receptors. There are various PLG receptors described, e.g. annexin A2 [93, 94], enolase-1 [95], actin, gangliosides [96] or histone 2B [overview [97]]. Although, both tPA and uPA cleave PLG in the same proteolytic reaction, their physiological functions are different. Tissue-type plasminogen activator is a 70 kDa large serine protease, which is produced by endothelial cells as a reaction to local fibrin clot formation and secreted intravascularly. Therefore, tPA-mediated PLA generation is mainly associated with local, intravascular fibrinolysis [98]. Urokinase-type plasminogen activator, on the other hand, is produced from multiple cell types including epithelial cells in the kidney [99], monocytes/macrophages [100, 101], trophoblasts [85, 102] and multiple types of cancer cells [103]. It is secreted as a single chain uPA (scuPA), which has little to no intrinsic activity [104]. To become active, scuPA must be converted into a two-chain uPA (tcuPA) by a proteolytic cleavage at the position Lys^{158}-Ile^{159} [105]. The main activator of scuPA is PLA [90], but also kallikrein [105], cathepsines [106, 107] and other proteases [listed in [63]] have been described as activators of scuPA. For both reactions, the activation of PLG by uPA and the conversion of scuPA to tcuPA, the binding of scuPA/uPA to the uPA receptor (uPAR) seems to be crucial [108]. Urokinase-type plasminogen activator receptor is a specific receptor for uPA and vitronectin [109], which is mostly anchored in the cell membrane with glycosylphosphatidylinositol (GPI) [110], but also exists in soluble form [111]. As a result, the uPA mediated PLG activation is located near to the cell surface and is associated with pericellular effects of PLA like pericellular proteolysis and activation of growth factors. There are physiological inhibitors of the PLG/PLA system. Firstly, there are direct PLA inhibitors, most importantly α_2-antiplasmin, which can bind and inactivate free PLA [112]. Also α_2-macroglobuline can act as a PLA-inhibitor, but it is less specific than α_2-antiplasmin [113]. Secondly, the activity of PLG activators can be inhibited by plasminogen activator inhibitor (PAI)-1 and -2. Plasminogen activator inhibitor-1 is a 52 kDa serine protease inhibitor (SERPIN), which under physiological conditions is mostly secreted by endothelial cells and circulates in blood at the concentration of 400 pM [114]. Plasminogen activator inhibitor-2 is predominantly

produced by the placenta [115] and monocytes [116]. Therefore it is detectable in the blood only under specific conditions, like pregnancy or immune responses [117, 118].

1.2.2. Regulation of urokinase-type plasminogen activator and plasminogen activator inhibtor-1

Urokinase-type plasminogen activator was first discovered in the human urine, which can be seen in its name until today [119]. Because of the very low activity of uPA in plasma as compared to the activity of tPA, its functions remained unclear for a long time. In the 1980s it became more and more clear, that the main function of uPA is the activation of pericellular proteolysis leading to increased cell motility. Consequently the expression of uPA has been shown to be a crucial factor in cancer invasion and metastasis [review [63]]. The expression of the uPA-gene, which is located on the long arm of chromosome 10 [120], is regulated by a plethora of growth factors including hepatocyte growth factor (HGF) [121], VEGF, basic fibroblast growth factor (bFGF) [122], colony stimulating factor 1 (CSF-1) [123] or insulin-like growth factors 1 and 2 (IGF1/2) [124]. But also pro-inflammatory agents like lipopolysaccharides [125] or intracellular events like microtubuli disassembly can lead to an increased expression of the uPA gene [126]. Most of these stimulators activate ERK1 and ERK2 in the cell [review [127]]. This event triggers binding of the activator protein 1 (AP-1) to an enhancer region located 2 kb upstream of the transcription initiation site of the uPA gene, and increased expression of uPA mRNA [128-130]. Another mechanism to induce uPA expression is mediated by the nuclear factor κ-B (NF-κB), which has two binding elements in the promoter of the uPA gene [131]. Nuclear factor κ-B belongs to the family of transcription factors, which is known for its central role in the regulation of inflammation, immune responses and oncogenesis [132, 133]. Suppression of the uPA mRNA expression has been described for glucocorticoids, which inhibit AP-1 and NF-κB [134, 135].

Under physiological conditions, Plasminogen activator inhibitor-1 (PAI-1) is the main inhibitor of uPA and the pericellular PLA activity [136]. Plasminogen activator inhibitor-1 is mostly synthesized and secreted by vascular endothelial cells, but also by hepatocytes [137], adipocytes [138] and fibroblasts [139]. The pericellular activity of PAI-1 is regulated by the conversion from active PAI-1 into its latent form and by the regulation of the PAI-1 synthesis. The native form of PAI-1 is also the active form, in which the reactive centre loop (RCL) is accessible for uPA. The RCL contains a specific peptide bond (P1P1´), which mimics the natural target of uPA and acts as a “bait” for the serine protease [140]. After the proteolytic cleavage of the P1P1´ bond,

the RCL gets inserted into the protein and the conformation of PAI-1 changes [141]. As a result, a stable complex between PAI-1 and uPA is formed and both proteins are inactivated [140]. However, the active form of PAI-1 is not very stable and quickly converts under physiological conditions into its latent form leading to a short half-time of active PAI-1 in blood (~ 10 min) [142-144]. The active conformation of PAI-1 can be stabilized *in vitro* by low pH [145] or high chloride concentration [146]. Under physiological conditions, vitronectin (VN) seems to be the most important stabilizer of active PAI-1 [147, 148].

The expression and synthesis of PAI-1 mRNA and protein is regulated by multiple factors. In healthy tissue only minimal levels of PAI-1 mRNA and protein are displayed, but following exposure to inflammatory stimuli, like tumor necrosis factor (TNF)-α, interleukin 1, or bacteria, strong induction of PAI-1 expression can be observed [149]. Therefore PAI-1 is considered as an acute phase protein. However, the strongest stimulator of PAI-1 mRNA and protein expression is transforming growth factor (TGF) β1 [150]. Members of the TGFβ1 signalling pathway, like Smad 2 or 3 with their co-factor Smad 4 have direct binding sites in the PAI-1 promoter and can lead to 50-fold increase in the expression of PAI-1 mRNA and protein [151]. Other inducers of PAI-1 expression are hormones like glucocorticoids [152] or angiotensin [153].

1.2.3. The plasminogen/plasmin system in cancer

Effects of the PLG/PLA-system on the cancer development have been described since the 1970s [154] and an unknown plasminogen activator, which was later named uPA, was associated with this new function of the PLG/PLA-system [155]. There are multiple ways how the members of the PLG/PLA-system interact with the cancer cells, mostly leading to cancer progression. Not all these ways are properly understood.

The most prominent and best described mechanism is associated with cell migration and invasion. Here uPA/uPAR leads to uncontrolled activation of pericellular PLG into PLA [review [156]]. Plasmin itself, activates multiple other proteases including the gelatinases MMP-9 [157] and MMP-2 [158], the collagenases MMP-3 and MMP-13 [159] and cathepsin B [160]. Considering that cathepsin B can activate scuPA into tcuPA [107] a positive feedback loop with uncontrolled activation of the pericellular proteolytic activity is created. The activation of this “proteolytic network” [66] leads to degradation of the ECM and basal membrane components [161]. This increases the motility of the cancer cells and enables the migration into nearby tissue [162]. Furthermore, the proteases can activate various growth factors, such as PDGF C/D [163], TGFβ [164], VEGF C/D [165] or EGF [review [166]]. As a result these growth

factors can: 1) activate pro-proliferative signals in the surrounding cancer cells; 2) increase the production and secretion of proteases [167-169]; 3) induce angiogenesis to support the growing tumor with nutrients and oxygen [55, 167].

In addition, PLA can bind to the cell-surface receptors like the plasmin receptor, PLG-R_{KT}, or to the protease activated receptors (PAR) to activate intracellular signalling in the surrounding cancer cells. Different functions have been described for PLG-R_{KT}. On the one hand PLG-R_{KT} seems to regulate the cell surface PLG activation [170]. On the other hand, PLG-R_{KT} is involved in the recruitment of macrophages to the inflamed tissue [171]. Protease activated receptors are a group of four G-protein-coupled receptors (PAR1-4), which can be activated be serine proteases, and play a crucial role in haemostasis and thrombosis, but also promote tumor proliferation and migration [reviews [172, 173]]. Protease activated receptor 1 activation is associated with enhanced cancer cell proliferation, migration and metastasis formation in various cancer types [listed in [174]]. Furthermore, it has been shown, that PAR1 is required for the PLA induced migration in melanoma cells [175, 176]. Protease activated receptor 4 is also associated with increased proliferation in hepatocellular carcinoma, but these effects are thrombin-dependent [177]. Interestingly, all types of PLA activated receptors trigger similar intracellular pathways, which include the ERK 1/2, the RHO-kinase pathway and the NF-κB pathways [178, 179].

Based on all these effects of PLG and PLA, it is not surprising, that upregulated activity of the system has been observed in various types of cancer and is associated with a bad prognostic outcome in most of the cases [review [81]]. Most prominent is the role of uPA in breast cancer, where an increased concentration of uPA in the tumor tissue is an independent prognostic marker for a poor prognosis [180]. But also in colorectal [181, 182], prostate [183] or gastric cancer [184], increased concentrations of uPA and uPAR are associated with poor prognosis.

1.2.4. Plasminogen activator inhibitor-1 in cancer

In comparison to the unambiguous pro-tumorigenic effects of PLA, uPA and uPAR, the function of PAI-1 in cancer development is contradictory [185, 186]. One the one side, PAI-1 has been shown to inhibit tumor growth and tumor associated angiogenesis of prostate cancer cells [187] and to reduce the invasion of bladder cancer cells [188] as well as the metastasis formation of fibrosarcoma cells [189]. The anti-cancer properties of PAI-1 have been mainly attributed to its ability to inhibit the pericellular activity of uPA and its binding to VN [190, 191]. As a result the PLG activation at the cell surface is inhibited and the pericellular proteolysis is reduced. Following this idea, it has been

also described, that PAI-1 can inhibit angiogenesis in *in vivo* tumor models with mouse keratinocytes [192].

On the other side, it has been observed, that higher levels of PAI-1 are associated with a poor prognosis in multiple types of cancer [overview [193]], including breast [194, 195], gastric [196], lung [197, 198] and colon cancer [199]. There are multiple theories about the molecular mechanism, by which PAI-1 exerts its pro-tumorigenic properties. One theory is, that the elevated PAI-1 production is a physiological reaction on the increased activity of proteases and inflammatory markers in the cancer tissue. This idea is supported by the fact that PAI-1 is either produced in tumor stroma or by cancer cells [200, 201].

Another theory proposes, that PAI-1 plays an important role in detachment of cancer cells from the ECM [62]. Thereby, PAI-1 disrupts the binding of VN to a uPAR/integrin-complex on the cell surface [202] resulting in the detachment of the cell from the ECM components like fibronectin or collagen [203]. Additionally, PAI-1 can regulate the level of integrins on the cell surface by triggering a low density lipoprotein receptor-related protein-1 (LRP1)-dependent internalization of the molecules [204].

The last theory about the pro-tumorigenic properties of PAI-1 focuses on the intracellular signalling triggered by PAI-1. Through the binding to LRP-1 on the cell surface, PAI-1 can stimulate the Janus kinase (JAK)/ signal transducer and activator of transcription (STAT) pathway in the cancer cell [205]. Interestingly, not only the active, but also the latent and cleaved PAI-1 are able to trigger the signalling [206]. The JAK/STAT pathway regulates the transcription of various genes, which are involved in cell proliferation (e.g. cyclin D1/D2 [207, 208] or myc [209]), angiogenesis (e.g. VEGF [210]) and cell survival (e.g. p53 [211] or b-cell lymphoma 2 (BCL2) [212]) [review [213]]. For example it has been shown, that the LRP1-dependent stimulation of the JAK/STAT pathway by PAI-1 can increase cell migration of cancer cells [205, 213]. Another signalling pathway, which can be activated by PAI-1, is the ERK pathway, which can be activated by PAI-1 *via* LRP-1 and β-catenin [214] or uPAR [190]. Both ways lead to phosphorylation of ERK, activation of the pathway [215] and finally to the induction of proliferation, survival and differentiation of cancer cells [216, 217].

Despite all described molecular and cellular effects of PAI-1 on cancer cells, it still remains unexplored, which factors are responsible for the anti- or pro-tumorigenic properties of PAI-1.

1.3. Pirfenidone

1.3.1. Pirfenidone in lung fibrosis

Pirfenidone (5-methyl-1-phenyl-2-(1H)-pyridone; PFD) is an orally available drug, which is used in the treatment of idiopathic pulmonary fibrosis (IPF). Orally applied PFD is absorbed rapidly and the peak blood concentration of PFD is reached within one hour in fastened patients [218]. A simultaneous food ingestion can delay the absorption of PFD significantly [219]. Pirfenidone gets mainly metabolized by the cytochrome P450 (CYP) 1A2, but also by CYP 2C9, 2C19, 2D6 and 2E1 [220]. The main product of this metabolization is the inactive 5-carboxy-PFD, which gets excreted into the urine [219]. Due to fast metabolization in the liver and a significant first pass effect after oral administration, the half-time of active PFD in the blood is approximately 2-2.5 h [218]. Therefore, PFD must be administered three times per day with a total dose of 2403 mg/d.

Pirfenidone was approved for the treatment of mild to moderate IPF in Europe in 2010 and in the US in 2014. This approval is based on four randomised placebo-controlled phase III studies [221-224]: The CAPACITY 1 and 2 as well as the ASCEND trials showed reduced disease progression under PFD treatment, which was reflected by reduced decline of the forced vital capacity (FVC), less dyspnoea and better results in the six-minute-walk test [223, 224]. Additionally, a pooled analysis of all four trials demonstrated a reduced all-cause mortality under PFD treatment [225].

The molecular effects of PFD were mainly investigated in animal models with bleomycin-induced pulmonary fibrosis. It has been demonstrated that PFD reduces the accumulation of collagens [226] and inflammatory markers, including IL-1β, IL-6 and monocyte chemoattractant protein (MCP)-1 [227], in the lungs of bleomycin-treated animals. Additionally, multiple interactions between PFD and members of the signalling pathways, like PDGF [228], Hedgehog [229] and TGFβ [230, 231], have been described. Although these pleiotropic effects of PFD are known on a cellular level, it still remains elusive, how PFD inhibits the progression of IPF.

1.3.2. Pirfenidone in cancer

Alongside its effects in fibrosis, PFD has also a significant impact on cancer cells. It has been observed that the incidence of lung cancer is reduced in PFD-treated IPF patients in comparison to untreated IPF patients [232]. Additionally, multiple *in vitro* studies demonstrated reduced proliferation, differentiation and migration of non-small

cell lung cancer (NSCLC) [233], mesothelioma [234] and pancreatic [235] cancer cells after the exposure to PFD. These results were further confirmed by *in vivo* studies, in which PFD alone or in combination with classical chemotherapeutic drugs like cis-platin or gemcitabine significantly decreased lung mesothelioma and pancreatic cancer cell growth by inducing changes in the tumor microenvironment [235, 236]. The underlying molecular mechanisms of the PFD mediated effects in cancer cells is just partially understood. Studies with multiple lung adenocarcinoma cell lines showed, that PFD can interact with TGFβ- and fibroblast growth factor-2 related pathways. As a result, PFD can reverse the epithelial-to-mesenchymal transition (EMT) in these cancer cells [237, 238]. Since the EMT is a crucial process in the development of tumor invasion and closely linked to changes in the tumor microenvironment, PFD could exert its effects partially through this process [239, 240]. Additionally, it has been shown that PFD reduces the expression of collagen and the heat-shock protein 47 in the peri tumorous tissue of mammary tumor models [241], as well as mesothelioma [234] and lung adenocarcinoma cell lines [242].

Altogether, it has been shown, that PFD can directly influence behaviour of different types of tumor cells and, in addition, influence the matrix architecture of the tumor stroma. However, it remains unclear, how PFD exerts its effects on tumor cells and the tumor microenvironment.

2. Objective

Since previous studies showed anti-tumorigenic effects of PFD in *in vivo* and *in vitro* cancer models, the objective of the present study was to evaluate the molecular mode of action of PFD in cancer.

In detail, the following objectives were addressed:

- Does PFD regulate proliferation, 2D-migration, 3D-migration or colony formation of cancer cell lines?

- Which molecular pathways, known to regulate cancer cell motility, are affected by the PFD treatment?

- What are the potential molecular targets of PFD in cancer?

3. Materials and methods

3.1. Materials

3.1.1. Apparatuses and Equipment

Axiovert 200 M light microscope	Carl Zeiss Microscopy GmbH, Jena, Germany
Cell culture inserts	Ibidi, Martinsried, Germany
Cell culture plates	Greiner Bio-One, Frickenhausen, Germany
Cell culture insert companion plates	Falcon, Corning, NY
Centrifuges	Mikro20, Hettich, Tuttlingen, Germany
	Heraeus Labofuge 400R, Functional line, ThermoFisher Scientific, Waltham, MA
ChemiDoc Imaging system	Bio-Rad, Munich, Germany
Electrophoresis power supply	Power Pac 1000, Bio-Rad, Munich, Germany
Electrophoresis chamber	Biometra, Göttingen, Germany
Falcon tubes	Greiner Bio-One, Frickenhausen, Germany
Film cassette	Kodak, Rochester, NY
Filters (22µm pore size)	Roth, Karlsruhe, Germany
Pipets	Eppendorf, Hamburg, Germany
Pipet tips	Eppendorf, Hamburg, Germany
Polyvinylidenfluorid (PVDF)-membrane	Roth, Karlsruhe, Germany
Radiographic film	Amersham Bioscience, Little Chalfront, UK
Shakers	VWR, Darmstadt, Germany
Thriller Thermoshaker-incubator	PeQlab, Erlangen, Germany

Spectral Cell Analyzer SP6800	Sony, Tokyo, Japan
SpectraMax plate reader	Molecular Devices, San Jose, CA
StepOne Real time PCR-System	Life Technologies, Carlsbad, CA
Transwell filter inserts	Falcon, Corning, NY
Vortex machine	VWR, Darmstadt, Germany

3.1.2. Reagents

[^{3}H]thymidine	PerkinElmer Life Sciences, Waltham, MA
2-mercaptoethanol	Sigma-Aldrich, St. Louis, MO
2-Propranol	Roth, Karlsruhe, Germany
Acetic acid	Roth, Karlsruhe, Germany
Acetone	Roth, Karlsruhe, Germany
Acrylamide solution	Roth, Karlsruhe, Germany
Agarose (low-melting)	PeQlab, Erlangen, Germany
Agarose	Roth, Karlsruhe, Germany
Ammonium persulfate	Sigma-Aldrich, St. Louis, MO
BC 11 hydrobromide	R&D Systems, Minneapolis, MN
BCA protein assay kit	ThermoFisher Scientific, Waltham, MA
Bovine serum albumin	Sigma-Aldrich, St. Louis, MO
Calcium chloride	Sigma-Aldrich, St. Louis, MO
Complete protease inhibitor cocktail	Roche Applied Science, Indianapolis, IN
Coomassie Brilliant Blue	Serva, Heidelberg, Germany
Crystal violet	Sigma-Aldrich, St. Louis, MO
Dulbecco's modified Eagle's medium (DMEM)	Invitrogen Life Technologies, Carlsbad, CA
ECL Prime WB Detection Reagent	Amersham Bioscience, Little Chalfront, UK
Ethanol	Roth, Karlsruhe, Germany
Ethiduim bromide	Sigma-Aldrich, St. Louis, MO
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, St. Louis, MO

Fetal calf serum (FCS)	Hyclone, Cramlington, UK
Fluorescein isothiocyanate (FITC)	Sigma-Aldrich, St. Louis, MO
Gelatine	Sigma-Aldrich, St. Louis, MO
Glutamax	Invitrogen Life Technologies, Carlsbad, CA
Glycerol	Roth, Karlsruhe, Germany
Glycine	Roth, Karlsruhe, Germany
Lys-plasminogen (PLG)	ThermoFisher Scientific, Waltham, MA
Methanol	Roth, Karlsruhe, Germany
Milk powder	Roth, Karlsruhe, Germany
Pefachrome®uPA 8294	Pentapharm, Basel, Switerland
Penicillin/Streptomycin	Invitrogen Life Technologies, Carlsbad, CA
Phenylmethylsulfonylfluoride	Sigma-Aldrich, St. Louis, MO
Pirfenidone (PFD)	InterMune, Brisbane, CA
Potassium chloride	Roth, Karlsruhe, Germany
Potassium dihydrogenphosphate	Sigma-Aldrich, St. Louis, MO
Protein marker (PageRuler, Prestained Protein Ladder)	ThermoFisher Scientific, Waltham, MA
Roti®-Block	Roth, Karlsruhe, Germany
RPMI 1640 Medium powder	Gibco Life Technologies, Carlsbad, CA
RPMI 1640 Medium	Invitrogen Life Technologies, Carlsbad, CA
	Sigma-Aldrich, St. Louis, MO
Sodium azide	Sigma-Aldrich, St. Louis, MO
Sodium bicarbonate	Sigma-Aldrich, St. Louis, MO
Sodium carbonate	Sigma-Aldrich, St. Louis, MO

Sodium chloride	Sigma-Aldrich, St. Louis, MO
Sodium deoxycholate	Sigma-Aldrich, St. Louis, MO
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich, St. Louis, MO
Sodium hydrogen phosphate	Sigma-Aldrich, St. Louis, MO
Sodium hydroxide	Sigma-Aldrich, St. Louis, MO
Sodium orthovanadate	Sigma-Aldrich, St. Louis, MO
Tetramethylethylenediamine (TEMED)	Sigma-Aldrich, St. Louis, MO
Tiplaxtinin (TPX)	Tocris, Bristol, UK
Transforming growth factor beta 1 (TGFβ1)	R&D Systems, Minneapolis, MN
Tris	Roth, Karlsruhe, Germany
Triton X-100	Sigma-Aldrich, St. Louis, MO
Trypsine	Sigma-Aldrich, St. Louis, MO
Tween 20	Sigma-Aldrich, St. Louis, MO
Ultra-pure water	B. Braun, Melsungen, Germany
Urokinase-type plasminogen activator (u-PA)	MyBioSource, San Diego, CA
Zinc chloride	Sigma-Aldrich, St. Louis, MO

3.2. Methods

3.2.1. Cell culture

Human non-small cell lung cancer cell line A549 was purchased from American Type Culture Collection (Manassas, VA) and cultured in DMEM containing 10% heat-inactivated FCS, 1% Penicillin/Streptomycin (Invitrogen Life Technologies, Carlsbad, CA). Human MDA-MB-435 breast carcinoma, human MCF-7 breast adenocarcinoma, human SK-BR3 breast adenocarcinoma and human MDA-MB-231 metastatic breast carcinoma (all kindly provided by Dr. Magdolen, Clinical Research Unit, Department of Obstetrics and Gynecology, Technical University of Munich, Munich, Germany) cell lines were maintained in Roswell Park Memorial Institute (RPMI)1640 Medium supplemented with 10% FCS, 2mM Glutamax and 1% Penicilin/ Streptomycin. All cell cultures were maintained in humidified atmosphere of 5% CO_2 at 37°C.

3.2.2. Pirfenidone preparation

A stock solution of the PFD, which was bought from InterMune, was prepared by dissolving 3 mg/ml powder in serum-free DMEM and heating for 30 min at 60°C under continuous shaking. The solution was then filtered under sterile conditions using a filter with a pore size of 22 µm. The PFD solution was used directly or stored at 4°C for maximum 3 days. The key findings of the cell culture, binding assays and kinetic experiments were also performed with PFD bought from Sigma-Aldrich.

3.2.3. Cell stimulation

Prior stimulations, the cells were growth-arrested in serum-free DMEM for 12-16 h. Afterwards, the medium was exchanged for a serum-free medium containing 0.8 mg/ml PFD, 10 ng/ml TGFβ1, 10 µM TPX and/or 20 µM GANT61. After indicated time points the cells or the cell culture supernatants were collected. The cell culture supernatants were centrifuged for 10 min at 170 g at 4°C and carefully pipetted to a new vessel to remove cell debris.

3.2.4. Proliferation Assay

Proliferation of the cancer cells was determined by a DNA synthesis assay based on the uptake of [^{3}H]thymidine. Briefly, the cells were cultured in a 48-well plate, growth-arrested for 8-12 h in serum-free medium and subsequently stimulated with different concentrations of PFD in the presence or absence of 10 µM TPX. Simultaneously with PFD, the cells were pulsed with 0.2 µCi/ml [^{3}H]thymidine for 16 h. Afterwards the cells

were washed extensively with phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) and then solubilized in 0.5 M NaOH. The [^{3}H]thymidine incorporation was measured by a liquid scintillation spectrometry.

3.2.5. 2D-migration

2D-migration was measured by a wound healing assay with cell culture inserts. Briefly, equal amounts of cells (between 15,000 and 25,000) were added into both chambers of the insert and left until cells reached ~90% confluency. Afterwards the medium and the inserts were removed, the cells were washed with PBS and stimulated with either increasing concentrations of PFD or with 0.8 mg/ml PFD in the absence or presence of 10 µM TPX or 10 µM of the uPA inhibitor BC11. Lipopolysaccharide (LPS) in a concentration of 1 µg/ml was used a stimulator for migration for SK-BR-3 cells. Pictures at time points 0 and 16 h after stimulation were taken and cells that migrated into the gap were counted using the LabImage 1D software (Kapelan Bio-Imaging, Leipzig, Germany).

3.2.6. 3D-migration

3D-migration was performed using transwell inserts containing a 8 µm pore size polycarbonate membrane (Falcon, Corning, NY) in a specific 12-well plate (Corning, Kennebunk, ME). Serum starved cells (5×10^4) were added into the upper chamber of the insert with 200 µl DMEM containing either 0.8 mg/ml PFD alone or in combination with 10 µM TPX. Five hundred µl of DMEM containing 2% FCS was added into the lower chamber of the transwell. Cells were then cultured for 16 h at 37°C. Afterwards, cells on the upper surface of the polycarbonate membrane of the transwell were removed with a cotton swab and the cells that migrated onto the underside of the membrane were fixed for 10 min with an ice-cold aceton/methanol (1:1) solution at 4°C, washed with PBS and stained with 0.5% crystal violet for 30 min at room temperature. Cells that migrated to the lower surface of the filter were photographed under the light microscope and counted using the LabImage 1D software (Kapelan Bio-Imaging).

3.2.7. Soft-agar Assay

Untreated cells and cells (2.5×10^3 each) treated with 0.8 mg/ml PFD alone or in combination with 10 µM TPX were mixed at 40°C with 0.4% agar in RPMI medium containing 10% FCS and gelled at room temperature for 20 min over a previously gelled layer of 0.7% agar in RPMI medium in 6-well plates. The two layers of agar were

covered with medium containing 0.8 mg/ml PFD and/or 10 µM TPX, which was exchanged every day. Every second day 10% FCS was added to the medium. After 21 days, the medium was removed and the colonies were stained with a crystal violet dye (0.04% crystal violet in 2% ethanol). Colonies were counted using an Axiovert 200 M light microscope and sorted into small (2-5 cells) and large colonies (more than 5 cells). Images of representative colonies were taken.

3.2.8. Molecular Analysis

3.2.8.1. RNA isolation

Isolation of RNA from cultured cells was performed using a peqGOLD Total RNA kit (Peqlab, Erlangen, Germany). Firstly, 200 µl lysis buffer were put onto the cells. Then the cells were scratched off and run through a DNA column at 14,000 g for 1 min at room temperature. Two hundred µl of ethanol were added to the flow through and vortexed. The mixture was placed on an RNA column and centrifuged at 9,700 g for 1 min at room temperature, followed by two washing steps with solutions, provided by the kit. The columns were dried by spinning at 9,700 g for 2 min at room temperature and then transferred on a sterile collection tube. To release the RNA from the filter, 10-20 µl RNA free-water was applied to the filter in the column and incubated for 3 min. Through a centrifugation at 8,000 g for 1 min at room temperature, the RNA was transferred into a collecting tube. The RNA concentration was determined with a spectrophotometer (NanoDrop 2000, ThermoFisher Scientific, Waltham, MA).

3.2.8.2. Reverse transcriptase reaction

The reverse transcriptase (RT) reaction was performed to convert the RNA into cDNA. The master mix for the reaction was prepared like described in table 3.1 (all ingredients from Applied Biosystems, Life Technologies, Carlsbad, CA). For each sample, 10 µg RNA were diluted in 10 µl water and added to 10 µl of the Master Mix. Afterwards the reaction was performed in a thermocycler (TGradient Thermocycler, Biometra, Göttingen, Germany) at 25°C for 10 min, 37°C for 2 h and 85°C for 5 min.

Table 3.1 Ingredients of the reverse transcriptase reaction

Master Mix Ingredients	Volume (µl)
MultiScribe™ RT[a)] (50 U/µl)	1.0
10x RT Buffer	2.0
10x RT Random Primers (25 µM)	2.0
dNTP[b)] (100 mM)	0.8
RNase[c)] Inhibitor (20 U/µl)	0.5
RNase free water	3.7
Total	**10.0**

[a)] RT, reverse transcriptase; [b)] dNTP, desoxy nucleoside triphosphate;
[c)] RNase, ribonuclease

3.2.8.3. Real-time PCR

The real time polymerase chain reaction (qPCR) was performed using the Platinum® SYBR Green qPCR Super Mix (Invitrogen, Karlsruhe, Germany). All components of the reaction are represented in table 3.2.

Table 3.2 Ingredients of the qPCR reaction

qPCR Ingredients	Volume (µl)
Reverse Primer (10 µM)	0.5
Forward Primer (10 µM)	0.5
Sybr Mix	12.5
RNase[a)] free water	10.5
cDNA[b)]	1.0
Total	**25.0**

[a)] RNase, ribonuclease; [b)] cDNA, complementary DNA.

Real-time PCR was used to quantify transcripts of the human glioma-associated oncogene homolog (*GLI*) 1, human *GLI2*, human α-smooth muscle actin (*ACTA2*), human vimentin (*VIM*), human E-cadherin (*CDH1*), human zonula occludens-1 (*TJP1*), human *MMP-2*, human *MMP-9*, human uPA (*PLAU*), human uPAR (*PLAUR*), and human PAI-1 (*SERPINE1*) genes (primer sequences are listed in table 3.3). Porphobilinogen deaminase (*PBGD*) was used as a reference gene. qPCR conditions were as followed: 95°C for 10 min, followed by 40 cycles with 95°C for 15 s and 60°C for 60 s. Melting curve analysis and gel electrophoresis of the qPCR products were performed to confirm the specificity of the primers. The qPCR data were evaluated with StepOneTM Software (Life Technologies) and are presented as ΔC_T value, defined by subtracting the C_T value of the gene of interest from the C_T value of the reference gene. Alternatively fold change in the target mRNA expression was determined. Therefore, $\Delta\Delta C_T$, which is the difference between the ΔC_T values of two samples, was calculated. The fold change in expression of the gene of interest between the two samples is then equal to $2^{(-\Delta\Delta CT)}$.

Table 3.3 Primer sequences

Gene	Accession number	Nucleotide sequence (5´-3´)
GLI1	NM_005269.3	F[a)]: TCTGGACATACCCCACCTCCCTCTG R[b)]: ACTGCAGCTCCCCCAATTTTTCTGG
GLI2	NM_005270.4	F: TGGCCGCTTCAGATGACAGATGTTG R: CGTTAGCCGAATGTCAGCCGTGAAG
ACTA2	NM_001141945.2	F: GGGACTAAGACGGGAATCCT R: CAAAGCCGGCCTTACAGAG
VIM	NM_003380.5	F: TGCAGGAGGAGATGCTTCAG R: ATTCCACTTTGCGTTCAAGG
CDH1	NM_004360.5	F: GCCGAGAGCTACACGTTCAC R: ACTTTGAATCGGGTGTCGAG
TJP1	NM_003257.4	F: AGACAAGATGTCCGCCAG R: TCCAAATCCAAATCCAGGAG
MMP-2	NM_004530.6	F: CTTCCAAGTCTGGAGCGATGT R: TACCGTCAAAGGGGTATCCAT
MMP-9	NM_004994.3	F: TGGGCAGATTCCAAACCTT R: CAAAGGCGTCGTCAATCAC
PLAU	NM_002658.5	F: ATTCCTGCCAGGGAGACT R: GACTCTCGTGTAGACGCC
PLAUR	NM_002659.4	F: CGCTTGTGGGAAGAAGGA R: ACACAACCTCGCTAAGGC
SERPINE1	NM_000602.4	F: CAAGCAGCTATGGGATTCAA R: TGGTGCTGATCTCATCCTTG
PBGD	NM_000190.4	F: ACCCTAGAAACCCTGCCAGAGAA R: GCCGGGTGTTGAGGTTTCCCC

[a)] F, forward; [b)] R, reverse.

3.2.9. Western Blot

For quantification of the proteins in cell lysate and cell supernatant, Western blotting was performed.

3.2.9.1. Protein isolation

Cells were washed once with PBS and then lysed in ice-cold lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton-X- 100, 1% Sodium Deoxycholate and 0.1% SDS supplemented with 1 mM Na_3VO_4 , 1 mM and 1 µg/ml Complete Protease Inhibitor Cocktail). Cells lysates were incubated on ice for 30 min and afterwards centrifuged at 12000 g for 10 min at 4°C. Supernatant was collected and the protein concentration was determined using a Pierce BCA Protein Assay Kit (Thermo FisherScientific, Waltham, MA) according to the manufacturer´s instruction.

3.2.9.2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis

To separate the protein based on their molecular weight, a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) was performed. For sample preparation, 5x SDS-loading buffer (0.25 M Tris-HCl,, pH 6.8, 10% (w/v) SDS, 50% (v/v) glycerol, 10% (v/v) β-mercaptoethanol) was added to 40 µl cell supernatant or 100 µg protein from cell lysate. The mixture was then heated at 98°C for 10 min and afterwards centrifuged. Then, a two-layered SDS polyacrylamide gel was prepared, composed of a lower separating gel [10% acrylamide: bisacrylamide, 375 mM Tris-HCl, pH 6.8, 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulfate (APS), 0.1% (v/v) tetramethylethylenediamine (TEMED)] and an overlaying stacking gel (4% acrylamide: bisacrylamide, 375 mM Tris-HCl, pH 6.8, 0.1% (w/v) SDS, 0.1% (w/v) APS, 0.1% (v/v) TEMED). After polymerization, the prepared samples and 5 µl of protein marker (Page ruler, Prestained Protein ladder, ThermoFisher Scientific, Waltham, MA) were loaded on the gel. The proteins were separated on the gel. The gel was run in SDS-running buffer [25 mM Tris, 250 mM glycine, 0.1% (w/v) SDS] via electrophoresis at a voltage of 100 V (Power Pac 1000, BioRad, Hercules, CA).

3.2.9.3. Immunoblotting

After separation, the proteins were electrotransferred from the gel to a PVDF membrane (Roth, Karlsruhe, Germany). The transfer was performed in ice-cool blotting buffer [25 mM Tris, pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween 20] at a voltage of 100 V for 1 h. The membrane was blocked with Roti®-Block (Roth) for at least 90 min. After

washing with TBS-T (5 mM TRIS-Cl, 150 mM NaCl, 0.1% Tween 20, pH 7.5), the membrane was incubated with one of the antibodies listed in table 3.4. Afterwards the membrane was washed and then incubated with a peroxidase-labelled secondary antibody (dilution 1:5000; all from Dako, Gostrup, Denmark). Finally, the proteins were detected with an ECL Plus Kit (Amersham Biosciences, Freiburg, Germany) or a Pierce® ECL Western Blotting Substrate (Thermo Fisher Scientific). β-actin, detected with a mouse anti-β-actin antibody (see table 3.4), was used as a loading control for cell lysate samples. For the loading control of cell supernatants, the SDS-PAGE gels were stained with silver (Bio-Rad Silver Staining Kit, Bio-Rad, Hercules, CA).

Table 3.4 Primary antibodies used for western blotting in this study

Protein	Abb.[a)]	Company	Cat. No.[b)]	Dilution
Zonula occludens 1	ZO-1	Invitrogen Life Technologies, Carlsbad, CA	40-2200	1:500
E-cadherin	E-cdh	Epitomics, Burlingame, CA	1702-1	1:1000
Vimentin	VIM	Santa Cruz Technology, Santa Cruz, CA	sc-58901	1:1000
Matrix metalloproteinase-2	MMP-2	Cell Signaling Technology, Danvars, MA	4022	1:1000
Glioma-associated oncogene 1	GLI-1	Cell Signaling Technology, Danvars, MA	2643	1:1000
Glioma-associated oncogene 2	GLI-2	Santa Cruz Biotechnology, Santa Cruz, CA	sc-271786	1:1000
Urokinase-type plasminogen activator	uPA	R&D Systems, Minneapolis, MN	MAB9185	1:1000
uPA receptor	uPAR	kindly provided by Dr. Magdolen, Clinical Research Unit, Department of Obstetrics and Gynaecology, Technical University of Munich, Munich, Germany		1:1000
Plasminogen activator inhibitor 1	PAI-1	kindly provided by Dr. Preissner, Department of Biochemistry, Faculty of Medicine, Justus-Liebig-University, Giessen, Germany		1:1000
β-actin	β-actin	Sigma-Aldrich, St. Louis, MO	A1978	1:10000

[a)] Abb, abbreviation; [b)] Cat. No., catalogue number.

3.2.10. Gelatinase zymography

Forty-eight µl cell supernatants were separated on a SDS-PAGE gel under non-reducing conditions with a gel containing 8% poly-acrylamide and 10% gelatine (Sigma-Aldrich). The gel was washed 3x for 15 min with washing buffer (2.5% Triton X-100, 50 mM TRIS pH 7.6, 10 mM $CaCl_2$, 1 µM $ZnCl_2$) to remove SDS and subsequently incubated in incubation buffer (15 mM NaN_3, 1% Triton X-100, 50 mM TRIS, pH 7.6, 10 mM $CaCl_2$, 1 µM $ZnCl_2$) at 37 °C for 72 h. Finally, the gel was stained with Coomassie Brilliant Blue (Serva, Heidelberg, Germany) for 1 h and afterwards destained in 30% 2-Propranol and 5% acetic acid for 1 h. The uncolorized areas show the activity of MMP-2 and MMP-9. The pictures of the lysis zones were taken and the size of the lysis zones was determined using the LabImage 1D software (Kapelan Bio-Imaging).

3.2.11. Urokinase-type plasminogen activator/ plasminogen activator inhibitor-1 zymography

Thirty-six µl cell supernatants were subjected to SDS-PAGE with a 10% polyacrylamide gel under non-reducing conditions. Afterwards, the gel was washed twice with 2.5% Triton X-100 in water for 10 min and two more times with PBS for 10 min. Meanwhile, a second gel containing 1.5% non-fat dry milk (Roth), 0.01% NaN_3, 40 µg/ml Lys-PLG (Thermo Fisher Scientific) and 8.3 mg/ml low-melting agarose (PeqGOLD Low Melt Agarose, Peqlab) was prepared. The first gel was placed on the second gel, both were wrapped in wet paper towels and stored at 4°C overnight. On the next day, the gels were incubated at 37°C until zones of lysis in the underlying gel, which represent the activity of uPA, were visible. The pictures of the lysis zones were taken and the size of the lysis zones was determined using the LabImage 1D software (Kapelan Bio-Imaging). For PAI-1 reverse zymography, the samples (36 µl) were mixed with 4 µl of 5% SDS and 4 µl of 5% β-mercaptoethanol and incubated for 1 h at 37°C prior to the SDS-PAGE. The second gel was supplemented with recombinant uPA in a final concentration of 0.05 U/ml (MyBioSource, San Diego, CA).

3.2.12. Tiplaxtinin activity

The activity of Tiplaxtinin (TPX) was determined by its ability to inhibit the complex formation between PAI-1 and uPA. Recombinant PAI-1 (kindly provided by Dr. Andreasen, Department of Molecular Biology, Danish-Chinese Centre for Proteases and Cancer, University of Aarhus, Aarhus, Denmark) in a final concentration of 8.5 µg/ml was added to 0–10 µM TPX and incubated for 15 min at room temperature. Afterwards recombinant uPA (MyBioSource) in a final activity of 1250 U/ml was added

and the samples were incubated at 37 °C for 30 min. The mixture was then separated on a SDS-PAGE gel under reducing conditions and the proteins were visualized by the silver staining. Recombinant PAI-1 and uPA were used as positive controls.

3.2.13. Microscale thermophoresis (MST)

The binding of PAI-1 wild type (WT) and PAI-1 R346A (both kindly provided by Dr. Andreasen) to PFD and the binding of PAI-1 WT and PAI-1 R346 preincubated with PFD to uPA were performed using a Nano Temper (NanoTemper Technologies, Munich, Germany) as previously described [243]. Briefly, PAI-1 was labeled with the red fluorescent dye NT-647 using a Monolith Protein Labelling Kit NHS-Red 2nd Generation (NanoTemper Technologies). A 14-fold titration series of PFD (500 nM to 0.061 nM) diluted 1:1 in PAI-1 stabilizing buffer (1 M NaCl, 20 mM sodium acetate, 0.01% Tween 20, pH 5.6) were performed. The concentration of NT-647–labeled PAI-1 was kept constant (5 nM). The binding of cooked PAI-1 to PFD as well as binding of albumin to PFD (Thermo Fisher Scientific) served as controls. Alternatively, 25 nM PAI-1 WT or PAI-1 R346A was first preincubated with 50 nM PFD and then mixed with serially diluted uPA (1000 nM to 0.061 nM). The thermophoretic movement of labeled proteins was monitored with a laser On for 30 s and Off for 5 s at a laser power of 80% with the Monolith NT.115 device. Fluorescence was measured before laser heating ($F_{Initial}$) and after 30 s of laser-on time (F_{Hot}). For both measurements the normalized fluorescence $F_{Norm}=F_{Hot}/F_{Initial}$ was plotted directly and multiplied by a factor of 10, yielding a relative change in fluorescence per mill (parts per thousand, ‰) indicated as F_{Norm} [‰]. F_{Norm} reflects the concentration ratio of labeled molecules. Error bars reflect standard deviation from three measurements. K_d values were determined by using the NanoTemper analysis software (NanoTemper Technologies).

3.2.14. Urokinase-type plasminogen activator activity assay

PAI-1 WT (125 nM) was mixed with PFD and incubated for 15 min at room temperature. Afterwards recombinant uPA (125 nM) and the chromogenic substrate, Pefachrome®uPA 8294 (400 µM, Pentapharm, Basel, Switzerland) were added. The hydrolysis of the chromogenic substrate was measured spectrophotometrically at 405 nm every 30 s at 37 °C for 30 min in a microtiter plate reader (SpectraMax 190; Molecular Devices, San Jose, CA).

3.2.15. Lactate dehydrogenase release assay

To determine the cytotoxicity of the used substances, lactate dehydrogenase (LDH) release was measured. The cells were treated with 0.2, 0.4 or 0.8 mg/ml PFD or with 5, 10 or 20 µM TPX for 24 h, the supernatants were collected and then centrifuged for 10 min at 380 g. The release of LDH was quantified with a Cytotoxicity Detection Kit (Roche Applied Science) according to the manufacturer´s instruction. For a positive control the cells were treated with 1% Triton X-100 for 5 min.

3.2.16. Detection of apoptosis

Cell death was controlled by staining of phosphatidylserine with FITC-Annexin V in combination with Sytox Blue (BD Biosciences, Franklin Lakes, NJ; cat. no.: 556547) according to the manufacturer's instructions. Briefly, after 24 h stimulation with 0.8 mg/ml PFD, the cells were harvested through trypsinization and washed once with PBS. The cells were centrifuged at 170 g for 10 min, then the pellet was resuspended in 1 ml binding buffer (BioLegend, San Diego, CA) with a maximal density of 1×10^7 cells per ml. One hundred µl of the sample solution was transferred to a 5 ml culture tube and incubated with 2.5 µl FITC-Annexin V for 15 min in dark. Afterwards 1 µl Sytox Blue was added and the samples were analysed using the Sony Spectral Cell Analyzer SP6800 (Sony, Tokyo, Japan) and the FlowJo 10.0 (FlowJo LLC, Ashlang, OR).

3.2.17. Statistics

Statistical analysis was performed with the GraphPad 5 for Windows (GraphPad software, La Jolla, CA). All results are shown as mean value ± SD, if not otherwise indicated. To compare two groups a Student´s t-test was used. For the comparison of more than two groups, an analysis of variance (ANOVA) followed by Tuckey´s post hoc test was performed. In all cases p values lower than 0.05 were considered as statistically significant.

4. Results

4.1. Pirfenidone reduces cancer cells proliferation, migration and colony formation

In order to evaluate anti-cancer properties of PFD, we tested the effect of PFD on proliferation and 2D-migration of cancer cells. Human non-small cell lung cancer (A549), human highly metastatic breast carcinoma (MDA-MB-435), human metastatic breast carcinoma (MDA-MB-231) and human breast adenocarcinoma (SK-BR-3) cell lines displayed significantly reduced proliferation when treated with different concentrations of PFD (Fig. 4.1 A-D). Interestingly, this reduction was not seen when a non-invasive breast cancer cell line MCF-7 was exposed to PFD (Fig. 4.1 E).

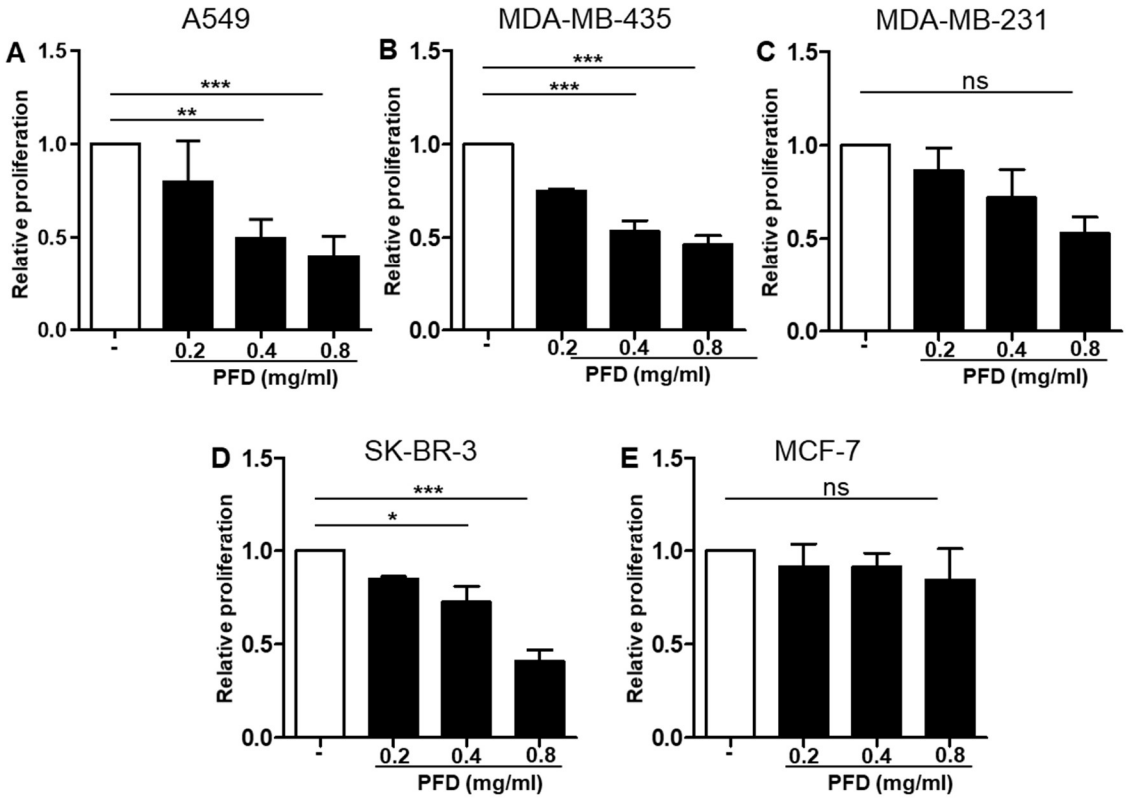

Figure 4.1 Pirfenidone reduces proliferation of lung and breast cancer cells.

Relative proliferation of A549 (A), MDA-MB-435 (B), MDA-MB-231 (C), SK-BR-3 (D), and MCF-7 (E) cells stimulated for 16 h with 0.2, 0.4 or 0.8 mg/ml pirfenidone (PFD). *p≤0.05; **p≤0.01; ***p≤0.001, n=3. ns, not significant

To study the impact of PFD on 2D-cell migration, a wound healing assay was performed. Treatment of A549 cells with PFD reduced migration of the cells in a dose-dependent manner (Fig. 4.2 A). Similar results were observed when MDA-MB-435, MDA-MB-231, and MCF-7 were treated with the drug (Fig. 4.2 B,C,E). SK-BR-3 cells had a very low absolute migration and were not affected by PFD treatment (Fig. 4.2 D). Neither did lipopolysaccharide (LPS) influence the motility of these cells (Fig. 4.2).

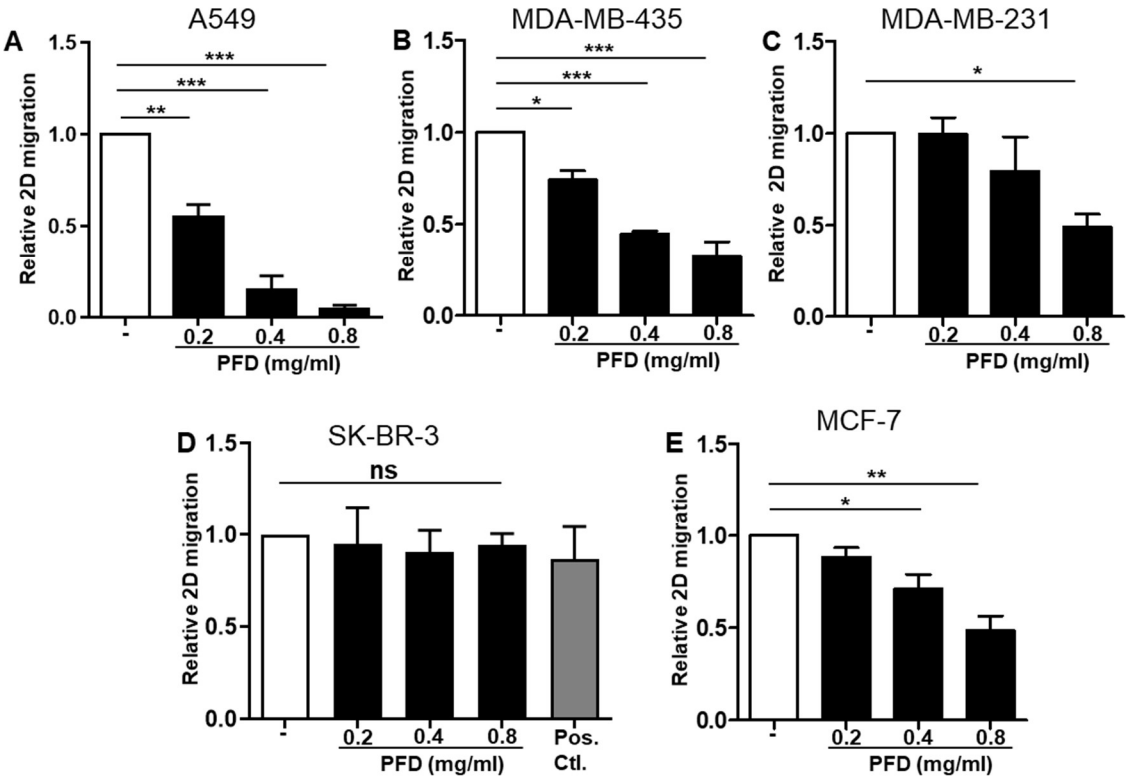

Figure 4.2 Pirfenidone decreases 2D-migration in lung and breast cancer cells.

Relative 2D-migration of A549 (A), MDA-MB-435 (B), MDA-MB-231 (C), SK-BR-3 (D), and MCF-7 (E) cells stimulated for 16 h with 0.2, 0.4 or 0.8 mg/ml pirfenidone (PFD) or 1µg/ml lipopolysaccharide (LPS) as positive control (Pos. Ctl.). *p≤0.05; **p≤0.01; ***p≤0.001, n=3. ns, not significant

To ensure, that the observed effects of PFD were not due to an increased cell death, an Annexin V staining (Fig. 4.3 A) was performed and the LDH release (Fig. 4.3 B) was determined. In these experiments no differences between control and PFD-treated A549 cells were seen (Fig. 4.3 A,B). As the most prominent effects of PFD were visible at the concentration of 0.8 mg/ml on A549 cells, this concentration of the drug and the cell line were used in the further experiments.

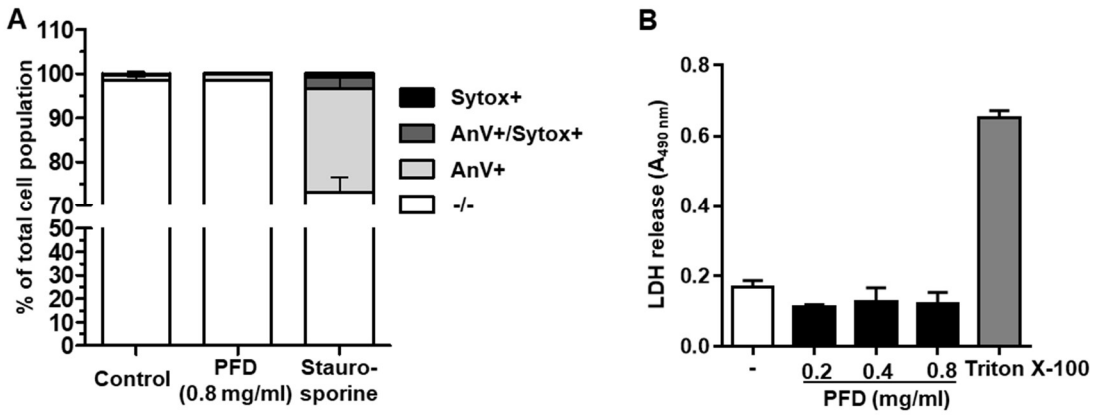

Figure 4.3 Pirfenidone has no cytotoxic effects on A549 cells when used in the concentration of 0.8 mg/ml.

A) Apoptosis of A549 cells treated for 24 h with 0.8 mg/ml pirfenidone (PFD) as measured by Annexin V and Sytox staining. Staurosporine was used as a positive control. The percentage of healthy (−/−), early apoptotic (AnV+), late apoptotic (AnV+/Sytox+) and necrotic (Sytox+) cells is shown. n = 3. B) Lactate dehydrogenase (LDH) release following the stimulation of A549 cells with 0.2, 0.4 or 0.8 mg/ml PFD for 24 h. 1% Triton X-100 was used as a positive control. n=3.

A transwell migration assay and a soft-agar colony formation assay revealed that PFD significantly reduces 3D-migration (Fig. 4.4 A) and growth of small and large colonies (Fig. 4.4 B-D) of A549 cells, respectively.

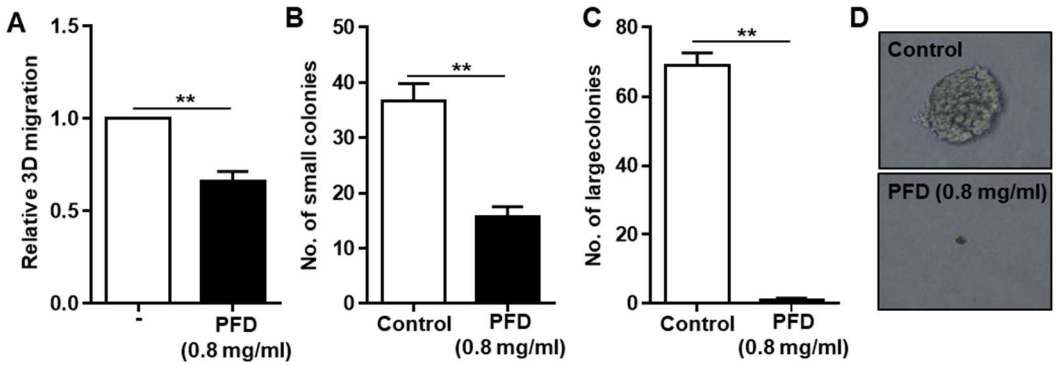

Figure 4.4 Pirfenidone decreases 3D migration and colony formation of A549 cells.

A) Relative 3D-migration of A549 cells 16 h after the application of 0.8 mg/ml pirfenidone (PFD). **p ≤ 0.01. n = 3. B, C) Numbers of small (B) and large (C) colonies 21 days after stimulation of A549 cells with 0.8 mg/ml PFD. **p ≤ 0.01. n=3. D) Representative pictures of a single colony taken at day 21 after exposure to PFD.

4.2. Pirfenidone does not affect expression of proteins involved in cancer cell trans-differentiation and proteins belonging to the pericellular protease system

Highly invasive cancer cells are characterized by the changes in the expression of proteins involved in cancer cell trans-differentiation and proteins belonging to the pericellular protease system such as MMPs, uPA, and uPAR [244]. Thus, we next evaluated the impact of PFD on the expression of these proteins in A549 cells. As depicted in Fig. 4.5 A-C, PFD did not affect mRNA and protein expression of vimentin (VIM), E- cadherin (CDH1) and zonula occludens-1 (TJP1/ZO-1).

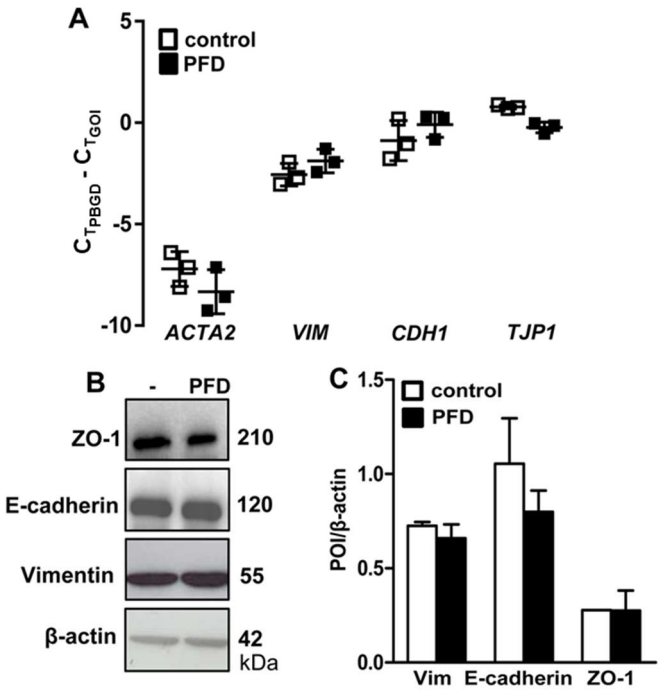

Figure 4.5 Pirfenidone does not affect expression of proteins involved in A549 cell transdifferentiation.

A, B) mRNA (A) and protein (B) expression of alpha smooth muscle actin (ACTA2), vimentin (VIM), E-cadherin (CDH1), and zonula occludens-1 (TJP1/ZO-1) in A549 cells treated for 8 h (for mRNA) or 24 h (for proteins) with 0.8 mg/ml pirfenidone (PFD). The qPCR data are presented as a ΔC_T using PBGD as a reference gene. n=3. For western blotting, β-actin was used as a loading control. C) Densitometry analysis of (B), n=5. GOI, gene of interest; POI, protein of interest.

Neither, the mRNA and protein levels of MMP-2, uPA (PLAU), and uPAR (PLAUR) were changed following the PFD treatment (Fig. 4.6 A-C). α-SMA (ACTA2) and MMP-9, although measureable on the mRNA level, were not detected on the protein level by means of western blotting (Fig. 4.5 A; Fig. 4.6 A).

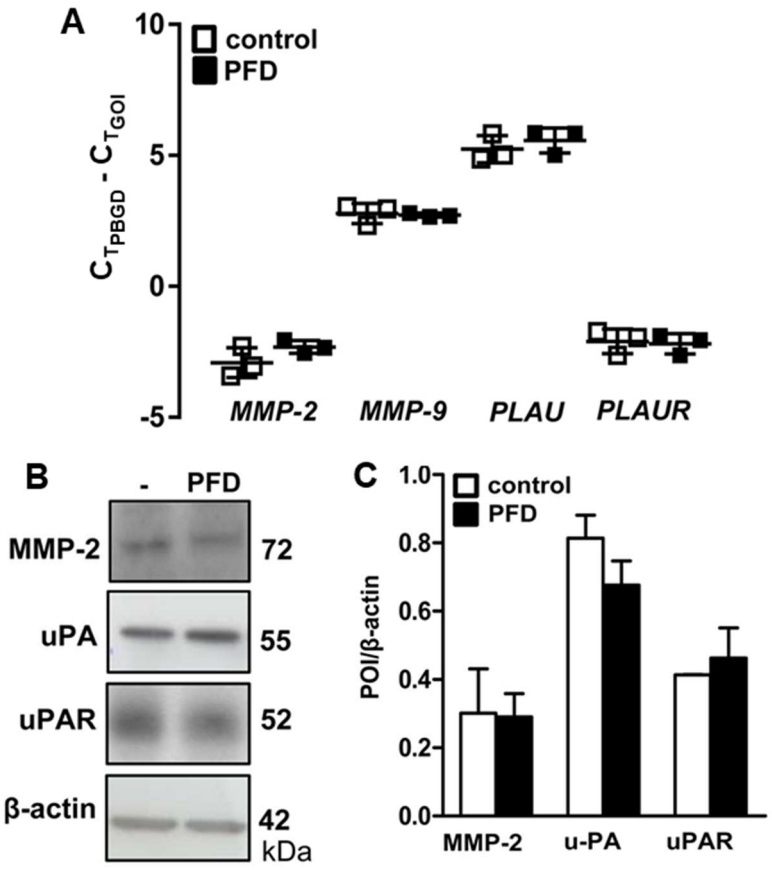

Figure 4.6 Pirfenidone does not alter the expression of proteins regulating pericellular proteolysis in A549 cells.

A, B) mRNA (A) and protein (B) expression of matrix metalloprotease (MMP)-2, MMP-9, urokinase-type plasminogen activator (PLAU/uPA) and uPA receptor (PLAUR/uPAR) in A549 cells exposed for 8 h (for mRNA) or 24 h (for proteins) to 0.8 mg/ml pirfenidone (PFD). The qPCR data are presented as a ΔC_T using PBGD as a reference gene. n = 3. For western blotting, β-actin was used as a loading control. C) Densitometry analysis of (B). n=5. GOI, gene of interest; POI, protein of interest.

Since, the activity of the pericellular protease system is regulated on the multiple levels [244], we next measured the enzymatic activity of MMPs and plasminogen activators (uPA and tPA) in the conditioned media of A549 cells either untreated or treated with PFD. As depicted in Fig. 4.7 A and B, PFD decreased the activity of MMP-2 as visualized by a smaller lysis zone at ~70 kDa. In line with the western blotting results, MMP-9 activity was not detectable in A549 cells. Furthermore, PFD treatment reduced the activity of uPA as revealed by a smaller transparent lysis area at ~50 kDa (Fig. 4.7 C and D, both left panel). The activity of tPA was much lower than the activity of uPA and only visible after longer incubation time (regions of lysis at ~70 kDa, Fig. 4.7 C, right panel). Still, the activity of tPA was diminished following the exposure of A549 cells to PFD (Fig. 4.7 D, right panel).

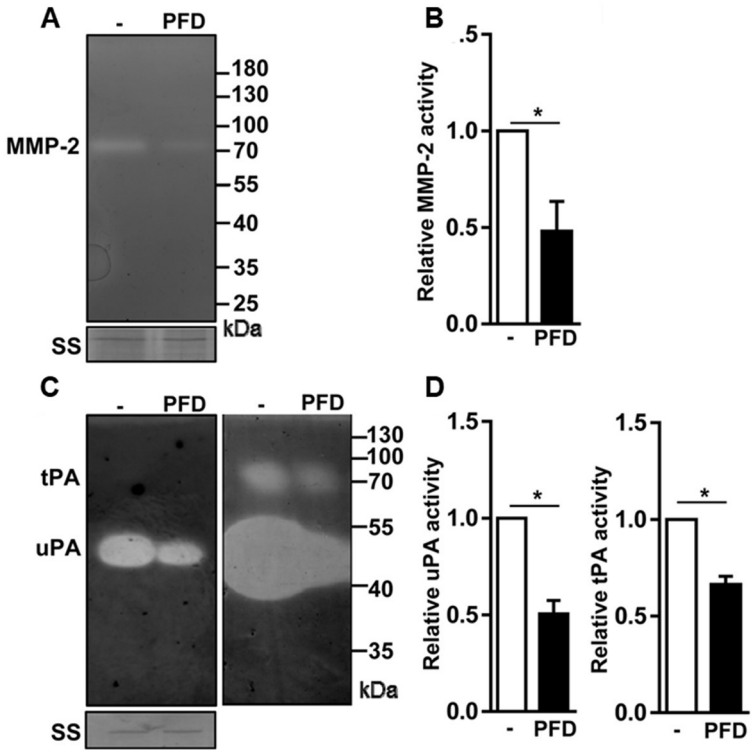

Figure 4.7 Pirfenidone inhibits extracellular proteolytic activity in A549 cells.

A) Matrix metalloprotease (MMP)-2 activity in cell supernatant after the treatment of A549 cells for 24 h with 0.8 mg/ml pirfenidone (PFD) as assessed by a gelatinase zymography. Silver staining (SS) of a SDS-PAGE was used as a loading control. B) The size of the lysis zones (shown in A) was determined. The control was set up as 1. *p ≤ 0.05. n=5. C) Activity of urokinase-type plasminogen activator (uPA; left panel) and tissue-type plasminogen activator (tPA; right panel) in cell supernatant following the exposure of A549 cells for 24 h to 0.8 mg/ml PFD as determined by a casein zymography. SS of a SDS-PAGE was used as a loading control. D) The size of the lysis zones (shown in C) was determined. The control was set up as 1. *p ≤ 0.05. n=5.

4.3. Pirfenidone increases the expression of plasminogen activator inhibitor-1 in A549 cells

Since PAI-1 is one of the main inhibitors of the pericellular protease system, which may directly interfere with the activity of uPA and tPA and indirectly, *via* reduced PLA formation, with the activity of MMPs, we next evaluated whether PFD may affect the expression and the activity of this serpin. Treatment of A549 cells with PFD elevated PAI-1 mRNA expression (Fig. 4.8 A). Concomitantly, the levels of PAI-1 protein in cell culture supernatants were increased after PFD treatment (Fig. 4.8 B and C). Recombinant PAI-1 (rPAI-1), produced in E.coli, was used as a positive control in western blotting (Fig. 4.8 B). The kinetic experiments revealed accumulation of PAI-1 protein in conditioned media of A549 cells exposed to PFD during a 24 h incubation period (Fig. 4.8 D and E). Most importantly, PAI-1 produced in A549 cells in response to PFD displayed inhibitory activity as indicated by the appearance of transparent lysis

zones in a reverse zymography. No higher molecular weight complexes containing active PAI-1 were detected by means of this method (Fig. 4.8 F).

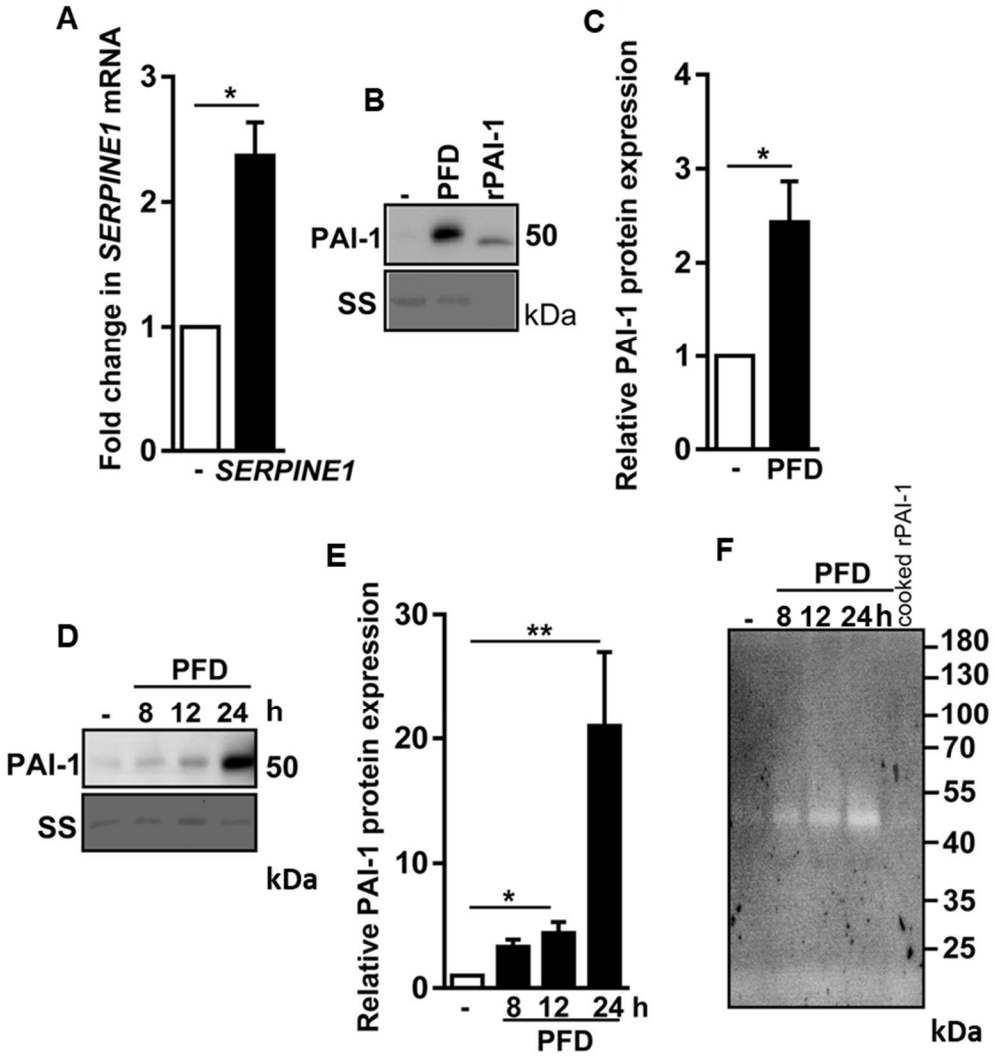

Figure 4.8 Pirfenidone increases PAI-1 mRNA and protein expression in A549 cells.

A, B) mRNA (A) and protein (B) expression of plasminogen activator inhibitor-1 (SERPINE1/ PAI-1) in A549 cells treated for 8 h (for mRNA) or 24 h (for proteins) with 0.8 mg/ml pirfenidone (PFD). The qPCR data are presented as a relative fold change in SERPINE1 expression normalized to the reference gene (PBGD) levels. *p ≤ 0.05. n=5. Silver staining (SS) of SDS-PAGE was used as a loading control for western blotting of cell culture supernatant. Recombinant PAI-1 (rPAI-1) was used as a positive control. C) Densitometry analysis of (B). The control was set up as 1. n=5. D) Time course of PAI-1 expression in A549 cells exposed to 0.8 mg/ml PFD. SS of SDS-PAGE was used as a loading control for western blotting of cell culture supernatant. E) Densitometry analysis of (D). The control was set up as 1. *p ≤ 0.05, **p ≤ 0.01. n=3. F) The activity of PAI-1 in the cell culture supernatant at indicated time points after stimulation of A549 cells with 0.8 mg/ml PFD as assessed by a reverse zymography. Cooked PAI-1 was used as a control. n=3.

Our previous results demonstrated that PFD destabilizes GLI transcription factors [229], thus we next evaluated whether the PFD-triggered induction of PAI-1 expression is a result of the Hedgehog signalling inhibition. As depicted in Figs. 4.9 A-C, PFD did

not change GLI1 and GLI2 mRNA expression, but it did decrease protein levels of these proteins. PFD-induced increase in the PAI-1 protein expression was mimicked, only to a certain extent, by a GLI inhibitor, GANT61 (Fig. 4.9 D and E), thus indicating that PFD targets, in addition to GLIs, other molecules to elevate PAI-1 levels.

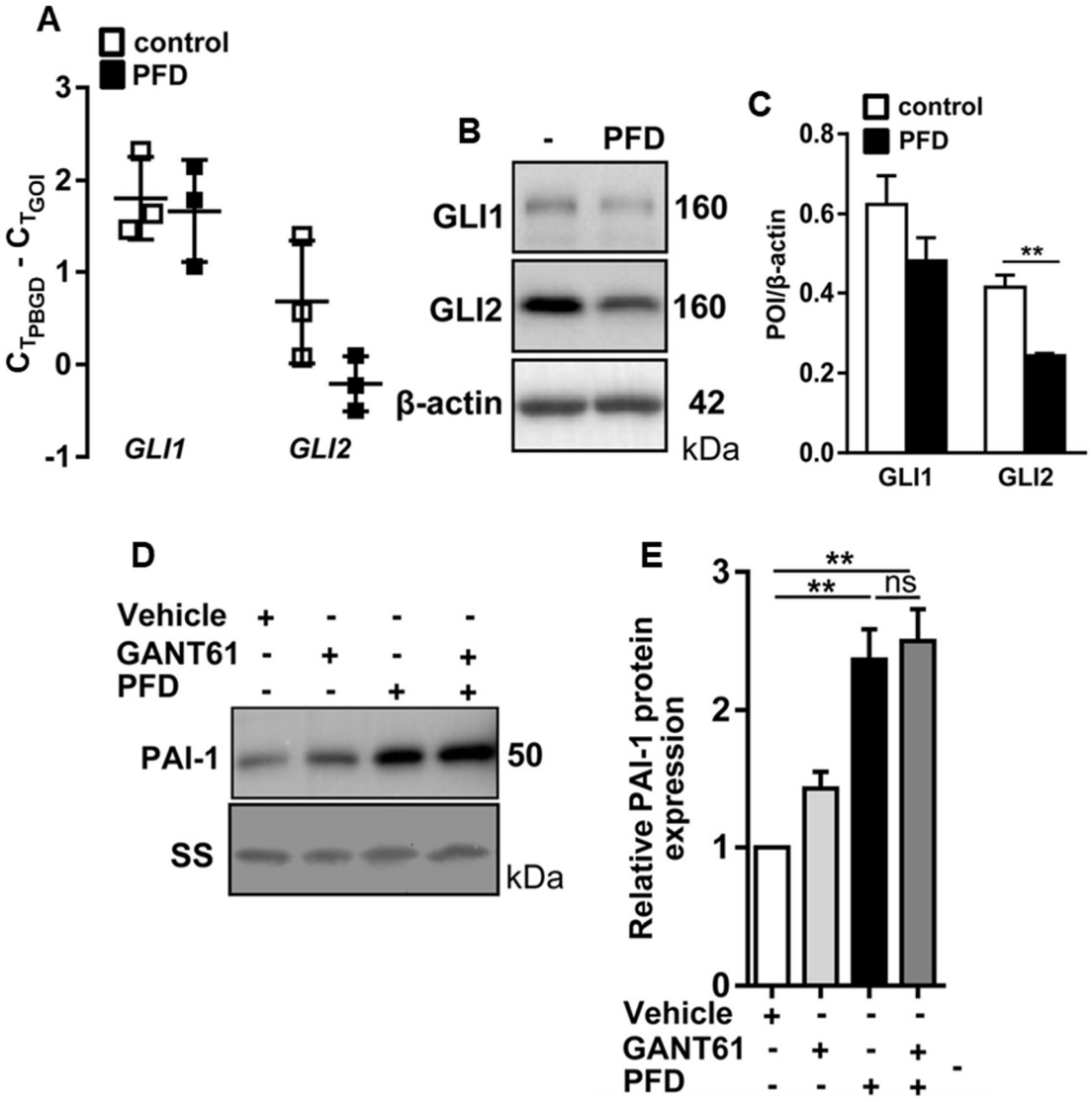

Figure 4.9 Pirfenidone-triggered PAI-1 expression only partially depends on GLI transcription factors.

A, B) mRNA (A) and protein (B) expression of glioma-associated oncogene homolog (GLI) 1 and GLI2 in A549 cells treated for 8 h (for mRNA) or 24 h (for proteins) with 0.8 mg/ml pirfenidone (PFD). The qPCR data are presented as a ΔC_T using PBGD as a reference gene. n=3. For western blotting, β-actin was used as a loading control. C) Densitometry analysis of (B). **p ≤ 0.01. n=5. D) Plasminogen activator inhibtor-1 (PAI-1) expression in A549 cells treated for 24 h with PFD in the absence or presence of a GLI inhibitor, GANT61. Silver staining (SS) of SDS-PAGE was used as a loading control for western blotting of cell culture supernatant. E) Densitometry analysis of (D). The control was set up as 1. **p ≤ 0.01, ns, not significant. n=3. GOI, gene of interest; POI, protein of interest.

4.4. Pirfenidone directly interacts with plasminogen activator inhibitor-1

Previous studies demonstrated that PFD and its derivatives directly interact with proteins, including p38γ [245], thus we next evaluated whether PFD may bind to PAI-1 and change its availability to uPA. The binding interactions between PAI-1 and PFD were analysed by the microscale thermophoresis (MST). As depicted in Fig. 4.10 A PAI-1 wild type (WT) bound to PFD with a K_d of 46.2 ± 11.3 nM. Interestingly, no binding was seen when PAI-1 was cooked or PAI-1 was mutated at the residue 346 (Arg (R)→ Ala (A)) thus suggesting that the conformation of the molecule and Arg-346 are critical for the interaction with PFD (Fig. 4.10 B,C). Albumin was used as a control (Fig. 4.10 D). The Arg residue at position 346 is located in the reactive center loop of PAI-1 and its mutation to Ala leads to a PAI-1 variant that interacts with the active site of a target protease but does not inhibit its activity [246].

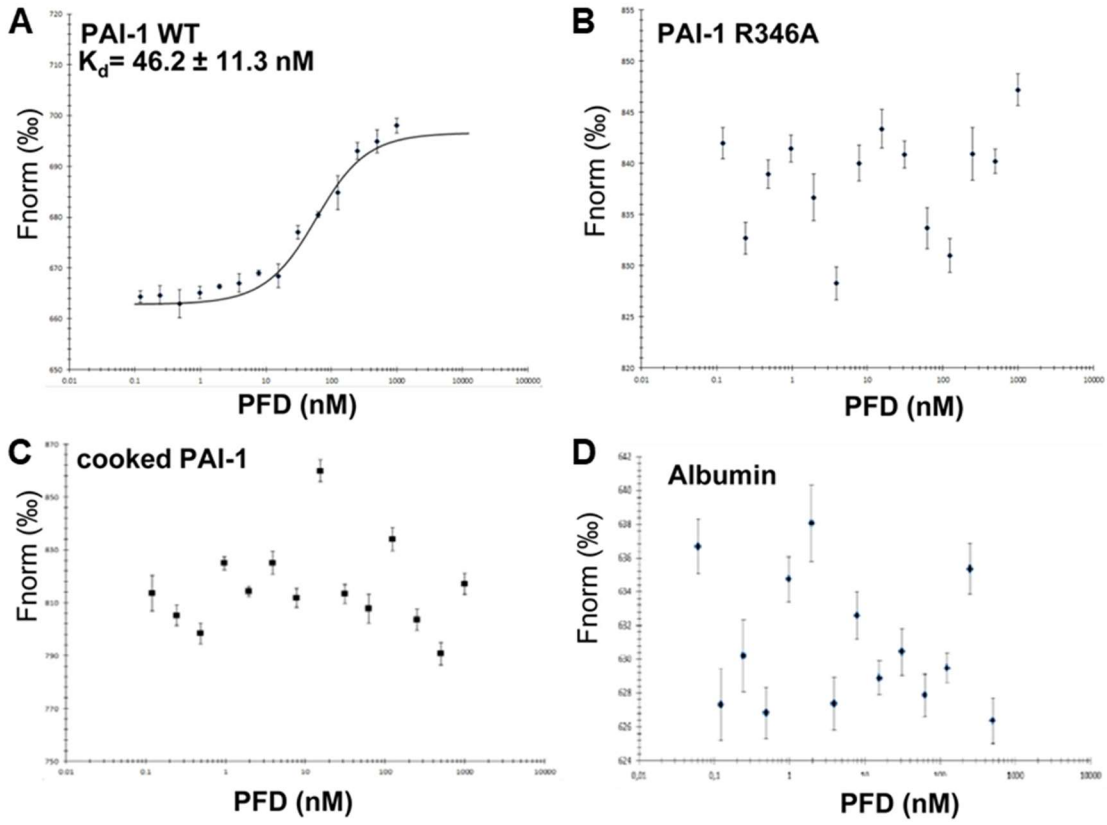

Figure 4.10 Pirfenidone interacts with PAI-1.

Binding of pirfenidone (PFD) to plasminogen activator inhibitor-1 (PAI-1) wild type (WT; A), PAI-1 R346A (B), cooked PAI-1(C) and albumin (D) as assessed by microscale thermophoresis (MST). K_d values were calculated from three independent MST measurements.

Next, we measured whether the association of PFD with PAI-1 affects its binding to uPA. As shown in Fig. 4.11 A preincubation of PAI-1 WT with PFD increased the affinity of PAI-1 WT for uPA by more than 3-fold (K_d of 46.2 ± 11.3 nM *vs* K_d of 14.7 ± 2.28 nM). As expected, the presence of PFD did not influence the affinity of PAI-1

R346A for uPA (K_d of 35.1 ± 3.82 nM *vs* K_d of 33.2 ± 5.26 nM). To examine the capacity of PFD to enhance/block PAI-1 inhibitory activity, a single step chromogenic assay was performed. For this analysis, PAI-1 WT was preincubated with increasing concentrations of PFD followed by the addition of uPA, and the remaining activity of the protease was determined. No effect of PFD, in the concentration used in the functional studies (4.3 mM), on PAI-1 activity was observed, however, at low concentration PFD increased the inhibitory potency of PAI-1 (Fig. 4.11 B). Altogether, our results suggest that PFD may interfere with PAI-1 on the multiple levels and that its impact on PAI-1 activity is concentration dependent.

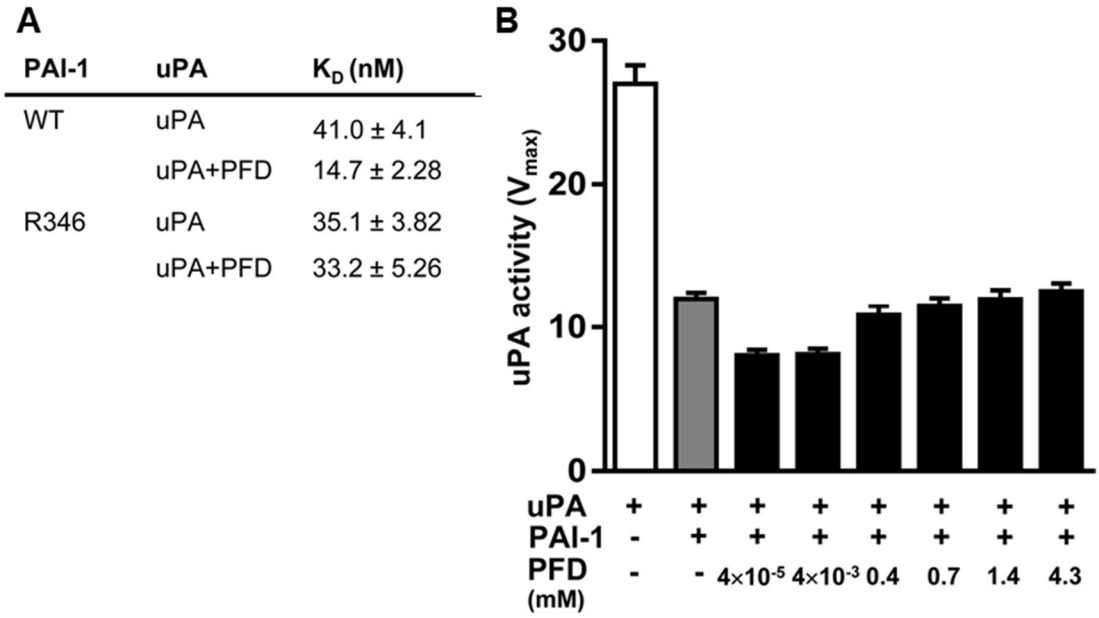

Figure 4.11 Pirfenidone changes the inhibitory potency of PAI-1.

A) K_d for the binding of plasminogen activator inhibitor-1 (PAI-1) wild type (WT) or PAI-1 R346A to urokinase-type plasminogen activator (uPA) in the absence or presence of pirfenidone (PFD). K_d values were calculated from three independent microscale thermophoresis (MST) measurements. B) The effect of PFD on the inhibition of uPA by PAI-1 as assessed by the single step chromogenic assay. A single representative experiment of eight is illustrated.

4.5. Tiplaxtinin reverses the effect of pirfenidone on the plasminogen/plasmin system activity

To evaluate, whether the inhibitory effect of PFD on migration and invasion of A549 cells is PAI-1 dependent, we applied the PAI-1 inhibitor, tiplaxtinin (TPX; PAI-039) [247]. TPX prevents PAI-1-uPA complex formation due to PAI-1 inactivation [248]. Indeed, after pretreatment with TPX at the concentration of 10 μM, the intensity of the higher molecular mass band decreased, indicating a reduction in PAI-1-uPA complex formation caused by TPX-triggered PAI-1 inactivation (Fig. 4.12 A). Associated with the loss of a high molecular weight complex was the concomitant increase in the intensity

of the band representing uPA and cleaved PAI-1 (Fig. 4.12 A). No cytotoxic effect of TPX at the concentration of 10 µM on A549 cells was observed (Fig. 4.12 B).

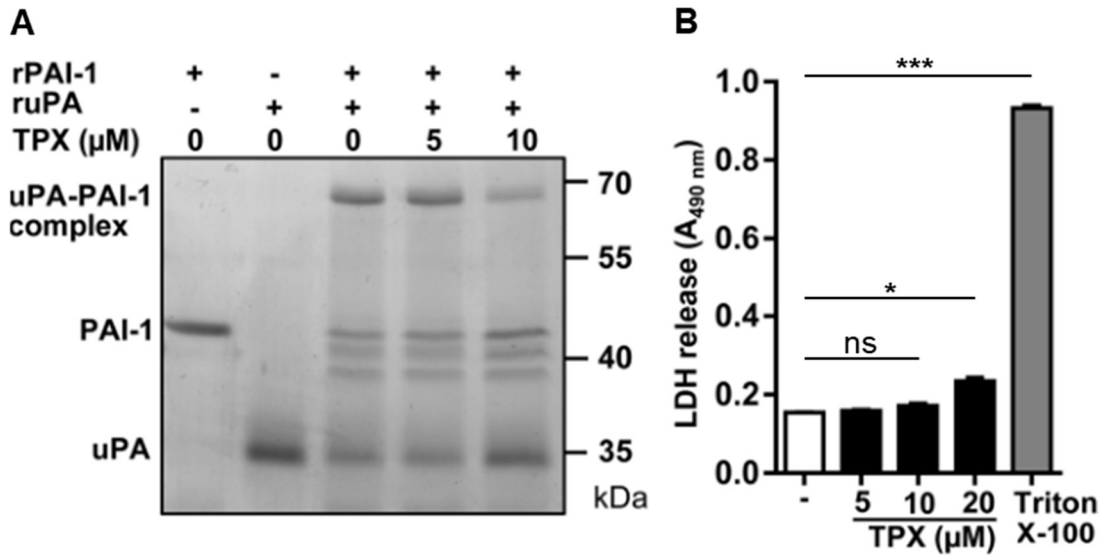

Figure 4.12 Tiplaxtinin inhibits uPA/PAI-1 complex formation.

A) Formation of urokinase-type plasminogen activator (uPA)/ plasminogen activator inhibitor-1 (PAI-1) complexes in the absence or presence of tiplaxtinin (TPX) as assessed by SDS-PAGE and silver staining (SS). B) Lactate dehydrogenase (LDH) release following the exposure of A549 cells for 24 h to TPX. 1% triton X-100 was used as a positive control. *p≤0.05; ***p≤0.001, n=3. ns, not significant

To test whether PFD-induced reduction of MMP-2 and uPA activity depends on the changes in the expression of PAI-1, we incubated A549 cells with PFD alone or in combination with TPX. As depicted in Fig. 4.13 (A,B), PFD reduced MMP-2 activity, however, this effect was not reversed by the addition of TPX.

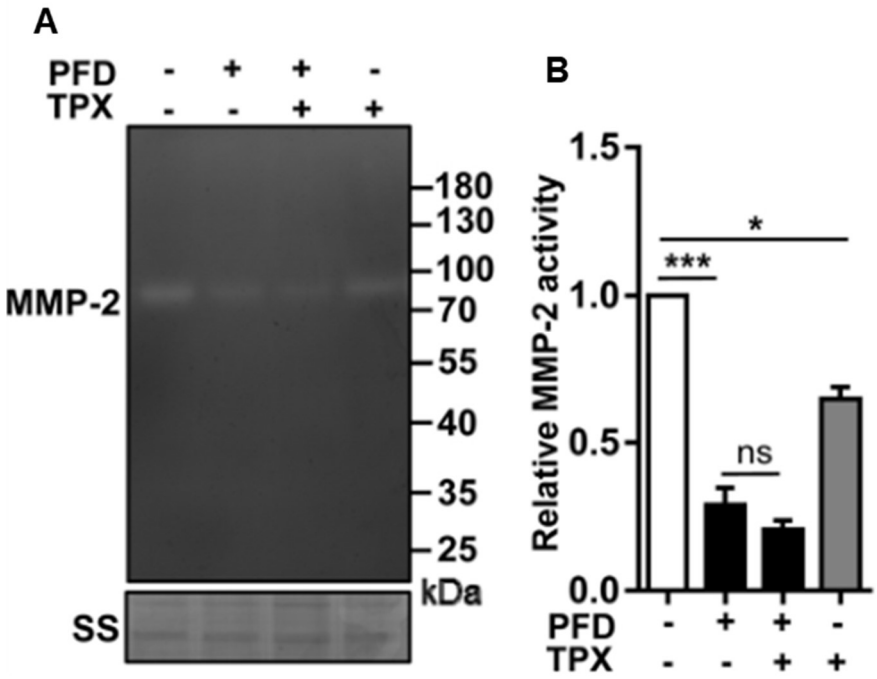

Figure 4.13 Tiplaxtinin does not affect the pirfenidone-triggered reduction of MMP-2 activity.

A) Matrix metalloprotease (MMP)-2 activity in cell supernatant after the treatment of A549 cells for 24 h with 0.8 mg/ml pirfenidone (PFD) and/or 10 µM tiplaxtinin (TPX) as assessed by gelatinase zymography. Silver staining (SS) of a SDS-PAGE was used as a loading control. n = 3. B) The size of the lysis zones (shown in A) was determined. The control was set up as 1. *p ≤ 0.05, ***p ≤ 0.001, ns, not significant. n=5.

Interestingly a combined treatment of A549 cells with PFD and TPX restored uPA activity to the level of the untreated cells, thus supporting the hypothesis that PAI-1 is one of the PFD targets (Fig. 4.14 A,B).

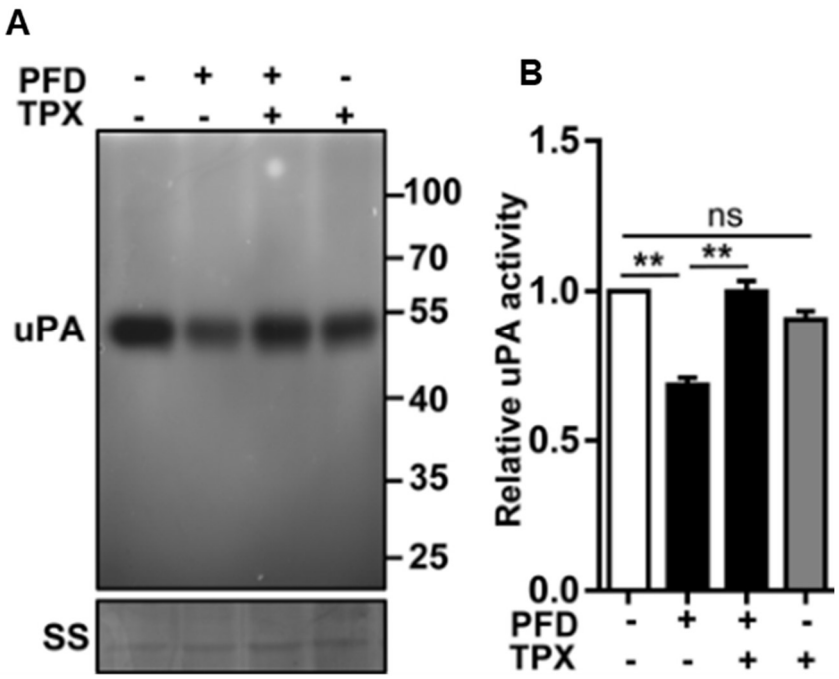

Figure 4.14 Tiplaxtinin reverses the pirfenidone-triggered reduction in uPA activity.

A) Activity of urokinase-type plasminogen activator (uPA) in cell supernatant following the exposure of A549 cells for 24 h with 0.8 mg/ml pirfenidone (PFD) and/or 10 µM tiplaxtinin (TPX) as determined by casein zymography. Silver staining (SS) of a SDS-PAGE was used as a loading control. B) The size of the lysis zones (shown in A) was determined. The control was set up as 1. **p ≤ 0.01. n=5. ns, not significant.

4.6. Tiplaxtinin reverses the effect of pirfenidone on 2D-cancer cell migration

To determine, whether TPX can reverse the effect of PFD on cancer cell behaviour, we treated A549 cells with PFD alone or in combination with TPX and measured cell proliferation, 2D- and 3D-cell migration as well as colony formation. The addition of TPX did not affect PFD-induced decline in cell proliferation (Fig. 4.15 A), however, it reversed PFD-mediated decrease in 2D-cell migration (Fig. 4.15 B,C). The inhibitory effect of PFD on 2D-cell migration was not observed when A549 cells were pre-treated

with the uPA inhibitor (Inh), thus supporting the pivotal role of the PAI-1-uPA system in the regulation of the bidirectional cancer cell motility (Fig. 4.15 D).

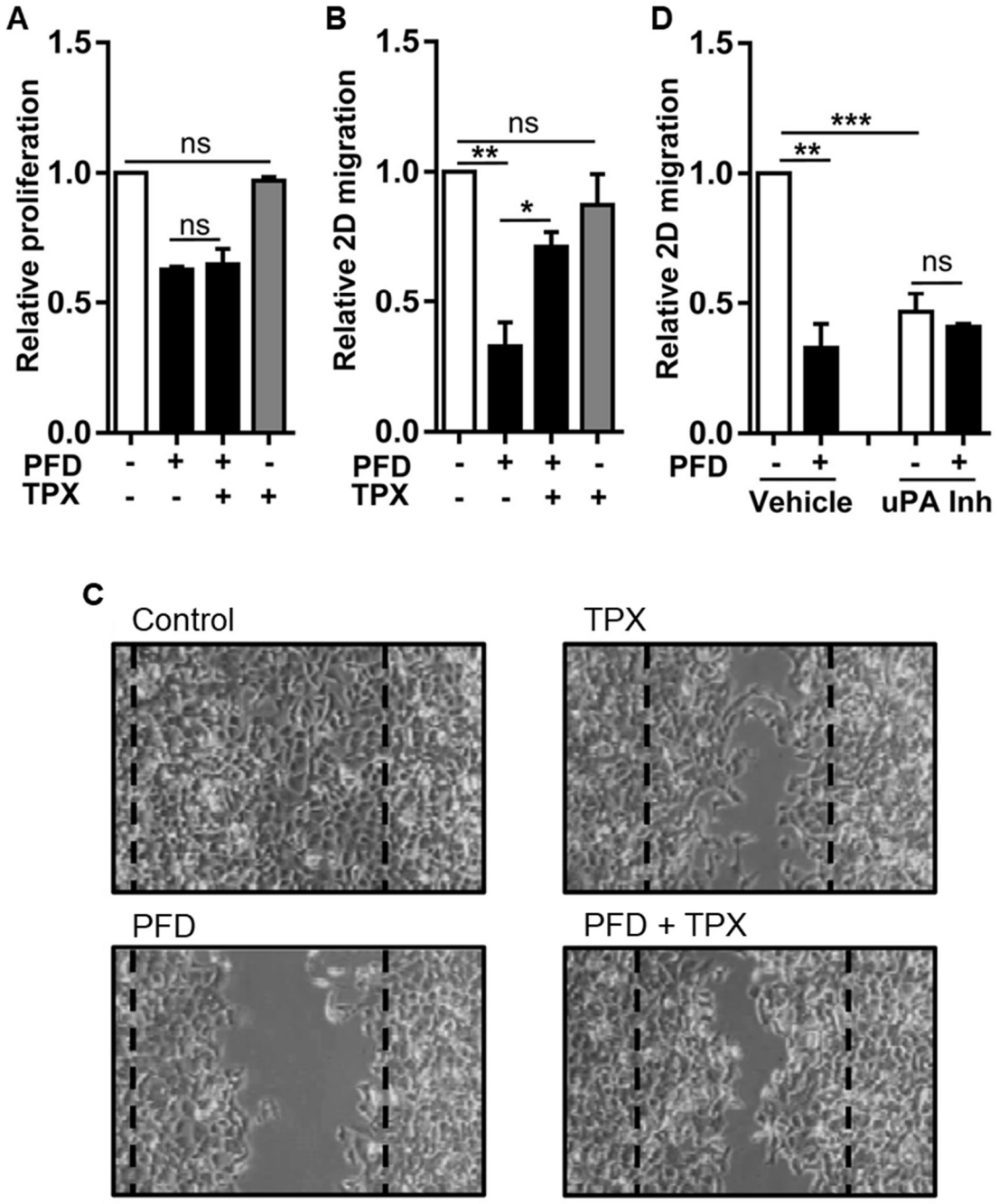

Figure 4.15 Tiplaxtinin reverses the effect of pirfenidone on 2D-migration of A549 cells.

A, B) Relative proliferation (A) and 2D-migration (B) of A549 cells stimulated for 16 h with 0.8 mg/ml pirfenidone (PFD) and/or 10 µM tiplaxtinin (TPX). n=4. C) Representative pictures of A549 cells migrating into a gap 16 h after the application of 0.8 mg/ml PFD and/or 10 µM TPX. D) Relative 2D-migration of A549 cells pretreated with 10 µM urokinase-type plasminogen activator inhibitor (uPA Inh) and then stimulated for 16 h with 0.8 mg/ml PFD *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ns, not significant. n=3.

In contrast, TPX did not restore PFD-mediated decline in 3D-cell migration of cancer cells (Fig. 4.16 A) and did not have any impact on PFD-induced blockage of cancer cell

colony formation (Fig. 4.16 B-D), indicating that the effects on 3D-migration and colony formation are multifactorial and not only mediated through the PAI-1 uPA axis.

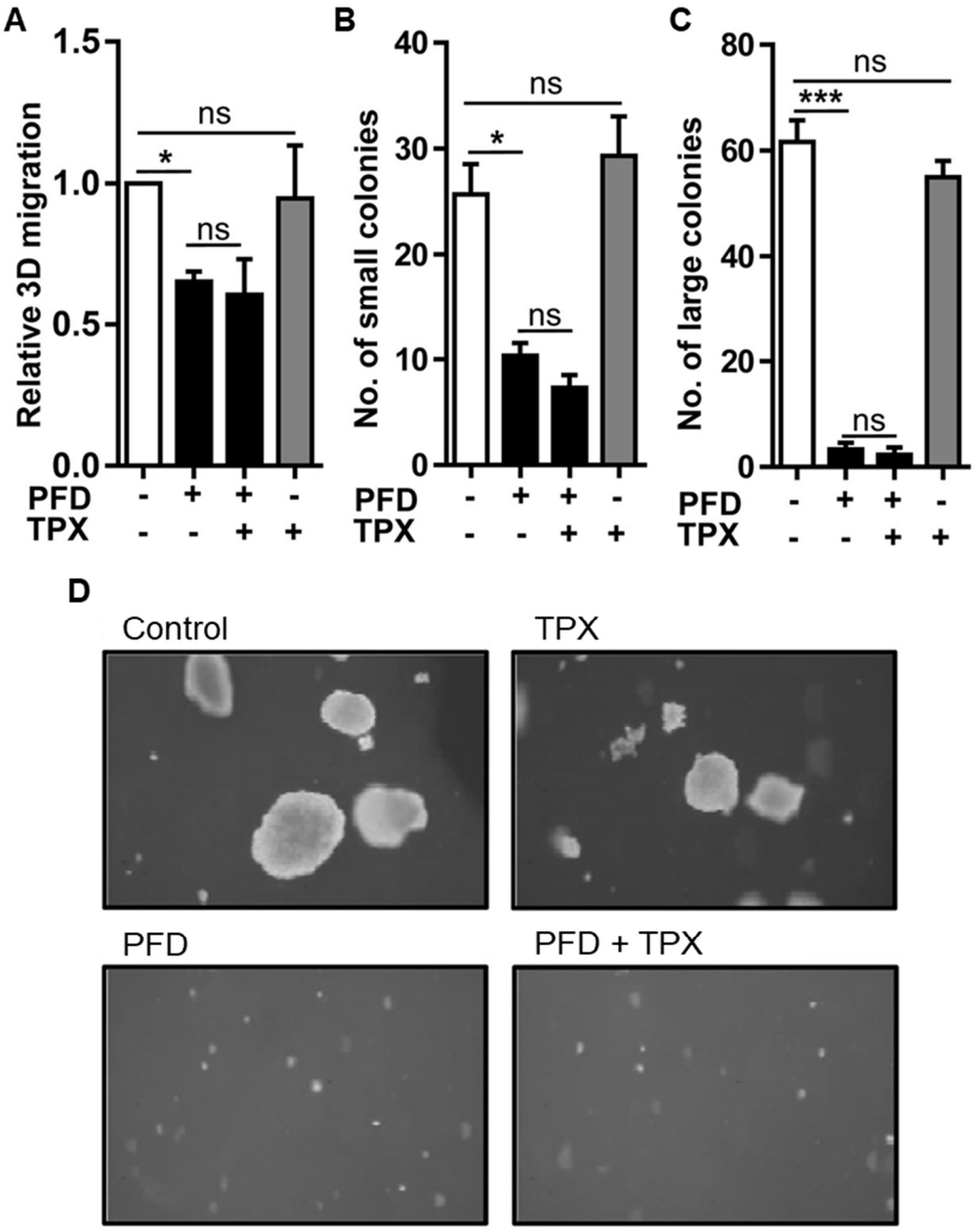

Figure 4.16 Tiplaxtinin does not affect the PFD-triggered inhibition of 3D migration and colony formation of A549 cells.

A) Relative 3D-migration of A549 16 h after the application of 0.8 mg/ml pirfenidone (PFD) and/or 10 µM tiplaxtinin (TPX). n=3. B, C) Numbers of small (B) and large (C) colonies 21 days after stimulation of A549 cells with 0.8 mg/ml PFD and/or 10 µM TPX. n=3. D) Representative pictures of the colonies taken at day 21 after exposure to 0.8 mg/ml PFD and/or 10 µM TPX. *p ≤ 0.05. ***p ≤ 0.001. n=3. ns, not significant.

5. Discussion

Pirfenidone is an orally available drug, which has been approved for the treatment of IPF patients [225]. On a cellular level, PFD has pleiotropic effects, but the anti-fibrotic, anti-oxidant and anti-inflammatory properties of PFD seem to be the most important for the clinical success of the drug [249]. Additionally, PFD possesses strong anti-cancer activities. Multiple studies, which used prostate cancer [250], mesothelioma [234], NSCLC [238] and glioma cell lines [251], observed reduced proliferation and migration after the treatment with PFD. These studies described different modes of action of PFD. Firstly, PFD was found to induce a G0/G1 cell cycle arrest [238, 250]; Secondly, pro-mitogenic and pro-survival pathways, including ERK 1/2, AKT and Survivin, were described to be inhibited by PFD [234, 238]; Thirdly, the transcription, expression and release of TGFβ was reported to be supressed by PFD [251].

In vivo experiments demonstrated that PFD alone decreases tumor growth of NSCLC cells (LLC1) [238] as well as co-transplanted human pancreatic cancer (SUIT-2) and pancreatic stellate cells (PSC) in mice [235]. These results were, however, not recapitulated in nude mice inoculated with a combination of A549 cells and lung cancer-associated fibroblasts [236] and in nude mice, which were implanted with the human MCF10CA1a breast cancer cell line [241]. Interestingly, in all aforementioned studies PFD treatment improved tumor perfusion and induced alterations in the ECM of the tumor microenvironment by reducing the collagen and hyaluronan levels [235, 241, 252]. Both, the tumor perfusion and the composition of the tumor microenvironment, play a critical role in tumor progression and the development of chemotherapy resistance [253] and are targets for new types of cancer therapies [254]. These observations built the hypothesis that PFD could be used in cancer therapy, either alone or in combination with classical chemotherapeutic drugs to improve their efficiency. Indeed, a combined therapy with PFD and chemotherapeutic drugs, like gemcitabine, cisplatin or doxorubicin, inhibited cancer cell proliferation, tumor progression and metastasis formation more efficiently than a monotherapy [235, 236, 241].

The present study demonstrates that PFD reduced proliferation, 2D- and 3D-migration and colony formation of NSCLC (A549) cells. Thereby, it has been shown that the PFD-mediated reduction of the 2D-migration of A549 cells depends on the activity of the uPA-PLA system. On a molecular level PFD modulated the activity of PAI-1, the main inhibitor of the uPA-PLA system, in two different ways: Firstly, PFD interacted directly with PAI-1 and modified its inhibitory potency towards uPA. Secondly, PFD increased PAI-1 expression and secretion. As a result, the pericellular activity of uPA was reduced after PFD treatment.

The impact of PFD on the inhibitory potency of PAI-1 was concentration-dependent and it was observed when PFD was used in the concentration range from nM to low µM. In contrast, in the functional assays PFD was applied in a much higher concentration of 4.2 mM. Therefore, the PFD-triggered changes of the PAI-1 expression were rather responsible for the suppression of the pericellular uPA activity and the impairment of 2D-migration of the cancer cells, than the PFD-mediated effects on the PAI-1 activity. The binding affinity between PFD and PAI-1 was found to be in the low nanomolar range, with a K_d value of 46.2 ± 11.3 nM. There is another small molecular compound named CDE-096, which is a high affinity inactivator of PAI-1 and has a comparable K_d value (K_d of 22.0 ± 6.0 nM) [255]. However, the two K_d values cannot be compared directly as two different methods were used for the determination of the binding affinity (MST vs surface plasmon resonance (SPR)). Interestingly, the binding affinity between PFD and PAI-1 is much higher than between TPX and PAI-1 (K_d of ~ 15 µM; [248]), which to date is the best characterized PAI-1 inhibitor. The theoretical comparison with other PAI-1 inhibitors like PAI-749 [256], TM5007 [257] or TM5725 [258] is even more restricted since only their half maximal inhibition (IC_{50}) values were published.

In contrast to all described PAI-1 inhibitors, the interaction between PFD and the reactive center loop of PAI-1 potentiated the inhibitory activity of PAI-1 towards uPA rather than inhibiting it. Although a conclusive explanation for this effect cannot be deduced from this study, one may speculate that PFD may inhibit the conversion of active PAI-1 into latent PAI-1 by stabilizing the active conformation of the protein. Another explanation may be that PFD alters the conformation of PAI-1, especially in the protease binding site in a way that the association of PAI-1 with a protease is facilitated. The latter explanation is supported by a different migration rate of PAI-1 on a native-PAGE in the samples treated with PFD as compared to the samples exposed to TGFβ. However, it remains difficult to predict the biological meaning of the PFD-mediated effects on the PAI-1 activity as multiple interactions between PAI-1 and other molecules affect the activity of PAI-1 *in vivo*. The most important molecule of this type is the glycoprotein vitronectin (VN), which has a somatomedin B domain [259] that can bind to the active PAI-1 near the reactive center loop (between αhelix E, strand 1A and αhelix F) [147, 260]. After binding to VN, the transition of active PAI-1 into latent PAI-1 is slowed down [261]. As a result, the effects of PAI-1 in fibrinolysis and cell migration can be modulated by binding to VN [147]. Other PAI-1 binding proteins, especially for pericellular PAI-1, are the cell surface receptors uPAR and LRP1. The binding of PAI-1 to one of these receptors can either lead to the activation of intracellular pathways [203, 205] or the internalization and degradation of PAI-1 [262]. The most important

intracellular effect of PAI-1 is the LRP1-dependent activation of the Jak/Stat signalling pathway, which stimulates cell migration and motility [203, 205].

Plasminogen activator inhibitor-1 functions as an important regulator of the pericellular proteolytic activity through the inhibition of uPA activity [263, 264] and thereby the reduction in the PLA formation. Plasmin can degrade ECM [265] and basal membrane components [161], but more importantly, it plays a key role in the activation of a complex proteolytic network. Further components of this network are matrix metalloproteinases like MMP-2, MMP-3 and MMP-9, cysteine proteases like cathepsin B and C and serine proteases like kallikrein 2 and 4, elastase and furin [review [66]]. The PLA-mediated activation of the network in close proximity to the cell surface leads to a focalized proteolysis of ECM components, especially collagen and fibronectin [60] and the disruption of cell-ECM contacts [266, 267]. Altogether, PLA can remodel the ECM and modify the tumor microenvironment leading to detachment of cancer cells from their surrounding tissue and formation of tissue gaps for the tumor cells to move into [62]. It has been shown in multiple studies that these effects of PLA are of particular relevance for tumor cell migration, invasion and metastasis formation [268-270]. Additionally, PAI-1 can control the cell adhesion and migration independent of its function as PA-inhibitor. There are numerous studies, which show direct effects of PAI-1 on the cell surface, especially through interfering with the binding between uPAR and VN [271, 272] and between integrins (e.g. integrin $\alpha_v\beta_3$) and VN [246, 273]. All of these interactions are known for their pro-adhesive and pro-migratory effects in various types of cancer cells [274-278]. Since all these anti-migratory effects of PAI-1 are well-known and PFD is modulating the activity of PAI-1, it is possible that the effects of PFD on cancer cell migration may be mediated by PAI-1. The results of this study are in line with this theory and show, that TPX, a PAI-1 inhibitor, can reverse the effects of PFD on 2D-migration of A549 cells. However, it is surprising, that TPX did not influence the impact of PFD on 3D-migration and colony formation of cancer cells, although PAI-1 is involved in these processes. One possible reason could be the complexity of the molecular mechanisms during cell penetration into a matrix. There are different types of cancer cell invasion and migration mechanisms described and all depend on complex interactions between multiple components, involving integrins, ECM constituents, cytoskeletal proteins, proteases, and growth factors [279]. Due to this multifactorial regulation of cell invasion, the impact of one factor on the whole system is minor and a changed activity of one factor can be compensated by the other members of the system. Although such compensatory mechanisms have not been described for PAI-1 inhibitors they are known for MMP inhibitors [280-282]. Another possible explanation could be the pleiotropic effects of PFD, which are better described in fibrotic diseases

[249, 283]. Therefore, it cannot be ruled out, that PFD interacts with other members of the network, which also regulate cancer cell invasion and colony formation.

Another aspect of the study is the evaluation of the effects of PFD on the proliferation of cancer cells. Uncontrolled proliferation of cancer cells is a key characteristic in all types of cancer [284] and increased proliferation correlates with a worse clinical outcome in all stages of cancer [285, 286]. The high proliferation rate in cancer cells is caused by a dysregulated cell cycle and disrupted apoptosis [33, 287]. The most important regulators for the cell cycle are cyclins and CDKs, which in turn are regulated by CDK inhibitors (CKI). There are two distinct families of CKIs: the inhibitor of CDK 4 (INK4) family and the CDK interacting protein (Cip/Kip) family [288]. Apoptosis is regulated by the activity of caspase-3, which can be activated by an intrinsic and an extrinsic pathway [289, 290]. Earlier studies have described that active PAI-1 can increase cell proliferation by the inhibition of apoptosis in prostate cancer cells and in healthy cells [291]. However, it has also been shown that PFD deregulates the expression of multiple proteins involved in cell survival, including p21 [250], p38γ [245], caspase-3 [292], β-catenin [293] and Survivin [238]. Due to these multiple targets of PFD, which are closely linked with the regulation of cell survival, it is unlikely that PFD exerts its anti-proliferative effects on A549 cells through the regulation of PAI-1 expression and activity alone. This assumption is supported by the findings showing that TPX does not interfere with the PFD-mediated effects on proliferation of A549 cells. Additionally, this study shows that PFD also represses the proliferation of invasive breast cancer cell lines (MDA-MB-231 and SK-BR3) and a melanoma cell line (MDA-MB-435), indicating that PFD interacts with inherent regulators of cell proliferation. However, no further experiments adressing underlying mechanisms have been performed in this study. Interestingly, the proliferation of the less invasive and aggressive breast cancer cell line MCF-7 [294] was not affected by PFD. An explanation for this finding could be the central role of hormones, especially estradiol, and their receptors in the regulation of the proliferation of MCF-7 cells [295].

A minor observation of this study is the suppression of the MMP-2 activity in A549 cells after treatment with PFD. This suppression was not reversed by the addition of TPX and is therefore not associated with the effects of PFD on PAI-1. It is known that the PLG/PLA-system is involved in the pro-MMP-2 activation [158]. Consequently PAI-1 can modulate the pro-MMP-2 activation by inhibiting uPA activity and thereby reducing PLA formation [296]. However, there are various other regulators of the MMP-2 activity, most importantly tissue inhibitors of MMPs (TIMPs) [297] as well as members of the proteolytic network like cathepsin B [298] and furin [299, 300]. Different studies showed

that PFD can modulate the expression of TIMPs [283, 301] and furin [251]. Thus providing rational for the observed effect of PFD on the MMP-2 activity in A549 cells.

The main limitation of the study is that most of the experiments were performed on the A549 cell line. Only the impact of PFD on tumor cell proliferation and 2D-migration was also examined on the breast cancer cell lines MDA-MB-231, MCF-7 and SK-BR-3 and the melanoma cell line MDA-MB-435. The A549 cells are lung adenocarcinoma cells, which originate from type-2 alveolar epithelial cells [302]. They are a well-established model of NSCLC [303] and as adenocarcinoma they represent the most frequent histological subtype [304]. However there are various other subtypes of NSCLC, most importantly squamous cell and large cell carcinoma, and even adenocarcinomas can be differentiated into subtypes by histological status [305], gene mutation status [306] and mRNA expression [307, 308]. Therefore a single cell line cannot represent the genetic complexity of all these subtypes. Hence, further studies using other NSCLC cell lines with different migration and invasion patterns as well as primary cancer cells have to be performed to support the translational potential of the findings.

Another limitation is the lack of *in vivo* experiments in this study to confirm the results in more complex conditions. There are some studies, which investigated the effects of PFD on the growth and metastasis formation of implanted cancer cells in mouse models and described positive effects on the tumor and its microenvironment [235, 236, 241]. Also experiments with NSCLC cells, which were implanted in a murine mouse model, showed a reduced tumour growth and improved tumor infiltration by immune cells after PFD treatment [238]. However, the effects of the PFD treatment on PAI-1 expression or the effects of PAI-1 inhibitors on the tumour cell growth and invasion was not examined in any of these studies. Thus, the effects of PAI-1 inhibitors like TPX on cancer cell growth in different mouse models [247, 309] as well as the effects of PFD in PAI-1 deficient mouse models [310] could be investigated in further studies.

In summary, this study describes the modulation of PAI-1 expression and activity as a novel mode of PFD action in cancer cells. Thus, these findings provide a molecular mechanism for previous observations demonstrating the ability of PFD to modify the ECM of the tumor microenvironment. Therefore this study warrants the idea to use PFD as a supportive drug in cancer therapy in conjunction with current radio-, chemo- and immunotherapies, which was brought up by some authors [235, 238, 241, 311]. In addition, this study raises awareness of possible adverse effects of the PFD application in prothrombogenic diseases including specific types of cancer [312], cardiovascular diseases [313, 314], chronic lung conditions like asthma and chronic obstructive lung disease (COPD) [315-317] and glomerulonephritis [318]. Further studies in more

complex cancer models are necessary to reveal the complexity of direct and indirect actions of PFD in cancer and to evaluate the efficacy of PFD in cancer treatment.

6. Summary

The orally available synthetic drug pirfenidone (PFD) was approved for the treatment of mild to moderate idiopathic pulmonary fibrosis (IPF) in Europe in 2010. In addition to its use in IPF, further research in the last decade revealed anti-proliferative and anti-migrative effects of PFD in various cancer models. PFD was found to induce modifications in the tumor microenvironment and the composition of the extracellular matrix (ECM). However, the molecular mechanism behind the anti-tumorigenic effects of PFD remained elusive. All these observations raised the question, whether PFD could be used in cancer therapy, either in monotherapy or as addition to the pre-existing chemotherapeutic regimes.

Following this idea, the present study evaluates the effects of PFD on different cancer cell lines. The investigations aim at determining possible molecular mechanisms underlying the effects of PFD on cancer cells with a focus on the pericellular proteolytic activity. This study demonstrates that PFD reduces proliferation and 2D-migration of cancer cell lines originating from non-small cell lung cancer (NSCLC), breast cancer and melanoma. Further investigations on the NSCLC cells showed reduced 3D-migration and colony formation after the application of PFD. On a molecular level, it has been seen that PFD modulates the activity of plasminogen activator inhibitor-1 (PAI-1) in two different ways. On one hand, PFD directly interacts with PAI-1 (K_d of 46.2 ± 11.3 nM) and thereby affects its activity. On the other hand, PFD increases the expression of PAI-1. Consequently, the activity of the urokinase-type plasminogen activator (uPA) is reduced. Additionally a reduced activity of matrix metalloproteinase (MMP) 2 after PFD treatment was observed. Finally, this study reports that the PAI-1 inhibitor tiplaxtinin (TPX) reverses the effects of PFD on 2D-migration of NSCLC cells indicating that this effect of PFD depends on the activity of PAI-1 and the uPA system. In contrast, the PFD-induced changes in cancer cell proliferation, 3D-migration and colony formation were not reversed by TPX and are therefore at least in part PAI-1 independent. On a molecular level, TPX reversed the effects of PFD on uPA activity, but not on MMP-2 activity.

In conclusion, this study shows the interaction between PFD and PAI-1 as a novel mode of action of PFD, which may modulate the architecture of the ECM within the tumor stroma and regulate the migration of tumor cells. These investigations draw attention to the possible effects of PFD on pericellular proteolysis in cancer.

7. Zusammenfassung

Das Medikament Pirfenidon (PFD) erhielt 2010 in Europa die Zulassung zur Therapie der milden bis moderaten idiopathischen pulmonalen Fibrose (IPF). Neben seinem Einsatz bei der IPF zeigten Forschungsergebnisse in den letzten Jahren auch anti-proliferative und anti-migratorische Effekte von PFD in verschiedenen Krebsmodellen. Dabei wurde festgestellt, dass PFD das Tumormikromilieu und die Zusammensetzung der extrazellulären Matrix (ECM) beeinflusst, wobei die molekularen Mechanismen hinter diesen Antitumoraktivitäten von PFD weitestgehend unklar sind. Diese Beobachtungen werfen die Frage auf, ob PFD Verwendung in der Krebstherapie finden könnte, sei es als Monotherapie oder als additives Medikament zu klassischen Chemotherapiekonzenpten.

Auf dieser Idee aufbauend untersucht diese Studie die Effekte von PFD auf verschiedenen Krebszelllinen. Die Untersuchungen zielen darauf ab, mögliche molekulare Mechanismen von PFD in Krebszellen zu bestimmen, wobei der Fokus auf der Regulation der perizellulären Proteolyse liegt. Dabei zeigt diese Studie, dass PFD sowohl die Proliferation als auch die 2D-Migration von Krebszelllinien aus nicht-kleinzelligen Bronchialkarzinomen (NSCLC), Brustkrebs und malignen Melanomen reduziert. Weitere Untersuchungen der NSCLC Zellen zeigten zudem eine verringerte 3D-Migration und Koloniebildung nach Behandlung mit PFD. Auf der Proteinebene konnte zwei Mechanismen nachgewiesen werden über welche PFD die Aktivität des Plasminogenaktivatorinhibitor 1 (PAI-1) moduliert. Erstens interagiert PFD direkt mit PAI-1 (K_d von 46.2 ± 11.3 nM) und beeinflusst dadurch dessen Aktivität. Zweitens erhöht PFD die Expression von PAI-1. Durch die modifizierte Aktivität von PAI-1 wird die Aktivität des Urokinase-Typ Plasminogen Aktivator (uPA) inhibiert. Zudem konnte eine reduzierte Aktivität der Matrix Metallopreoteinase (MMP) 2 nach der Behandlung mit PFD nachgewiesen werden. Zuletzt zeigt diese Studie, dass die Effekte von PFD auf die 2D-Migration von NSCLC Zellen durch den PAI-1 Inhibitor Tiplaxtinin (TPX) aufgehoben werden können. Dadurch wird nachgewiesen, dass diese zellulären Effekte von PFD über die Aktivität von PAI-1 und uPA vermittelt werden. Im Gegensatz dazu werden die Effekte von PFD auf Proliferation, 3D-Migration und Koloniebildung der Krebszellen durch TPX nicht beeinflusst, sodass diese vermutlich nicht primär über PAI-1 vermittelt werden. Bei der Proteinaktivität konnte TPX die Auswirkungen von PFD auf die Aktivität von uPA umkehren, nicht aber die Effekte von PFD auf die MMP-2 Aktivität.

Zusammenfassend bringt diese Studie die Interaktion von PFD und PAI-1 als einen neuen Wirkmechanismus von PFD auf. Dieser scheint eine Rolle in der Modulation der Struktur der extrazellulären Matrix und der Regulation der Migration von Krebszellen zu

spielen. Diese Beobachtungen zeigen damit möglich Effekte von PFD auf die perizelluläre Proteolyse in Krebserkrankungen.

8. List of abbreviations

AP-1	Activator protein 1
CDK	Cyclin dependent kinase
CYP	Cytochrome P450
DMEM	Dulbecco's modified Eagle's medium
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
FCS	Fetal calf serum
FGF	Fibroblast growth factor
HIF	Hypoxia induced factor
IPF	Idiopathic pulmonary fibrosis
JAK	Janus kinase
KLK	Kallikrein
LDH	Lactate dehydrogenase
LRP-1	Low density lipoprotein receptor-related protein-1
MLCK	Myosin light chain kinase
MMP	Matrix metalloproteinase
MST	Microscale thermophoresis
NF-κB	Nuclear factor-κB
NSCLC	Non-small cell lung cancer
PAI	Plasminogen activator inhibitor
PAR	Protease activated receptor

PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PFD	Pirfenidone
PKB/AKT	Protein kinase B
PLA	Plasmin
PLG	Plasminogen
PVDF	Polyvinylidenfluorid
qPCR	Real-time polymerase chain reaction
ras	Rat sarcoma
RB	Retinoblastoma
RCL	Reactive centre loop
RHO	Ras-homologue
RT	Reverse transcription
scuPA	Single chain urokinase-type plasminogen activator
SDS	Sodium dodecyl sulfate
SERPIN	Serine protease inhibitor
STAT	Signal transducer and activator of transcription
tcuPA	Two-chain urokinase-type plasminogen activator
TEMED	Tetramethylethylenediamine
TGFβ	Transforming growth factor beta
TIMP	Tissue inhibitor of matrix metalloproteinases
TNFα	Tumor necrosis factor alpha
tPA	Tissue-type plasminogen activator

TPX	Tiplaxtinin
uPA	Urokinase-type plasminogen activator
uPAR	Urokinase-type plasminogen activator receptor
VEGF	Vascular endothelial growth factor
VN	Vitronectin
WT	Wild type

9. List of figures and tables

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10. References

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Datum

Unterschrift

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