New Therapeutic Strategies for the Treatment of Experimental Pulmonary Hypertension: Role of the Epidermal Growth Factor

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INDEX OF FIGURES

INDEX OF TABLES.

I. INTRODUCTION

I.1. Pulmonary arterial hypertension (PAH)

Definition, classification, epidemiology. Pulmonary arterial hypertension (PAH) represents a severe condition associated with a poor prognosis, a high rate of mortality and limited survival[145]. Clinically defined as an elevation of mean pulmonary arterial pressure to levels higher than 25 mmHg at rest and/or 30 mmHg during exercise[25;145], PAH is characterized by a progressive increase in pulmonary vascular resistance, that leads to a gradual rise in pulmonary arterial pressure, to right ventricular failure and ultimately death[74;145]. PAH is included in the classification accepted in Dana Point in 2008, as one of the subcategories of pulmonary hypertension (PH) (see Table 1)[163] and comprises a) the idiopathic (formerly known as "primary") form- IPAH (PAH without established etiology), b) the familial form (FPAH) and c) pulmonary hypertension associated with a number of other conditions, such as collagen vascular disease, congenital systemic to pulmonary shunts, portal hypertension or HIV infection. Furthermore, pulmonary veno-occlusive disease (PVOD), pulmonary capillary haemangiomatosis (PCH) and persistent pulmonary hypertension of the newborn (PPHN) are at present included in the group of pulmonary arterial hypertension.

IPAH affects twice as many women as men, most often in the third and forth decades of life, with a mean age of diagnosis of 36 years. The disease is rarely diagnosed and treated from the onset of the clinical manifestation, which has as a consequence the rapid progression of the disease and a medial survival interval of 2-8 years from the moment of diagnosis[145]. The familial form of the disease accounts for approximately 6% of the total cases of PAH[149], is characterized by an autosomal dominant inheritance and recent investigations have connected it to mutations of genes encoding the TGF-β receptor family members BMPRII (responsible of at least 70% of FPAH cases and also of 10-20% of IPAH cases) and activin receptor-like kinase 1 (ALK-1)[99;120]. Secondary PAH, on the other hand, can be induced by the ingestion of several appetite suppressants like aminorex fumarate or dexfenfluramine, by the ingestion of toxic rapeseed oil (as these factors have been incriminated in several epidemics of PAH in the 1960's)[1;56;92]or by a number of diseases that predispose to PAH[174].

Table 1. Classification of pulmonary hypertension (Dana Point, 2008) (after Simonneau et al., 2009 [163])

1. Pulmonary arterial hypertension (PAH)

- 1.1. Idiopathic PAH
- 1.2. Heritable
  - 1.2.1. BMPR2
  - 1.2.2. ALK1, endoglin (with or without hereditary hemorrhagic telangiectasia)
  - 1.2.3. Unknown
- 1.3. Drug- and toxin- induced
- 1.4. Associated with
  - 1.4.1. Connective tissue disorders
  - 1.4.2. HIV infection
  - 1.4.3. Portal hypertension
  - 1.4.4. Congenital heart diseses
  - 1.4.5. Schistosomiasis
  - 1.4.6. Chronic hemolytic anemia
- 1.5. Persistent pulmonary hypertension of the newborn

1'. Pulmonary veno-occlusive (PVOD) and/or pulmonary capillary hemangiomatosis (PCH)

2. Pulmonary hypertension owing to left heart disease

- 2.1. Systolic dysfunction
- 2.2. Diastolic dysfunction
- 2.3. Vascular disease

3. Pulmonary hypertension owing to lung diseases and/or hypoxia

- 3.1. Chronic obstructive pulmonary disease
- 3.2. Interstitial lung disease
- 3.3. Other pulmonary diseases with mixed restrictive and obstructive pattern
- 3.4. Sleep-disordered breathing
- 3.5. Alveolar hypoventilation disorders
- 3.6. Chronic exposure to high altitude
- 3.7. Developmental abnormalities

4. Chronic thromboembolic pulmonary hypertension (CTEPH)

5. Pulmonary hypertension with unclear multifactorial mechanisms

- 5.1. Hematologic disorders: myeloproliferative disorders, splenectomy
- 5.2. Systemic disorders: sarcoidosis, pulmonary Langerhans cell histiocytosis: lymphangioleiomyomatosis, neurofibromatosis, vasculitis
- 5.3. Metabolic disorders: glycogen storage disease, Gaucher disease, thyroid disorders
- 5.4. Others: tumoral obstruction, fibrosing mediastinitis, chronic renal failure on dialysis

Diagnosis. Most patients in early phases of PAH present with nonspecific symptoms like fatigue and dyspnea on exertion, which delays the diagnosis many times by months or years. Fatigue, chest pain, syncope, peripheral edema or abdominal distension are manifestations of the already advanced disease, reflecting the inability of the heart to adjust to the necessary increase in cardiac output during activity and the progressive right heart failure[145].

Transthoracic echocardiography is an important non-invasive diagnostic test that can be used in all PAH patients and can give important information regarding the dimensions of the chambers of the heart, the estimated systolic pulmonary arterial pressure, possible intracardiac shunts or defects leading to PAH[145]. The diagnosis suggested by echocardiography must be confirmed by right heart catheterization, which remains the main tool for the diagnosis of PAH[115;145]. Other non-invasive tests include the 6-min-walk test (which correlates inversely with the functional class severity), chest radiographs (which can show enlarged pulmonary arteries and right heart dilation), electrocardiograms (which can show right axis deviation and right ventricular hypertrophy) and ventilation-perfusion testing (especially for the detection of chronic thromboembolism) or computer tomography scanning of the chest[25;115;145].

Morphological changes. In addition to vasoconstriction, the disease is associated with complex and progressive morphological changes involving all layers of the vessel wall[74]. This process affects mainly small pulmonary arteries and arterioles, in which the neomuscularization of peripheral normally nonmuscularized vessels, the development of neointima and plexiform lesions, adventitial proliferation, as well as in situ thrombosis represent main histopathologic features[74;120].

Although the exact initiating events of PAH remain unclear, at present it is believed that endothelium injury (caused by shear stress, hypoxia, inflammation, drugs or toxins) is the first step in the progression of PAH[120]. The damaging factor can induce, especially on a background of genetic susceptibility, an imbalance between endothelium-derived vasodilator (prostacyclin, nitric oxide) and vasoconstrictor agents (thromboxane, endothelin-1, angiotensin II), with the predominance of vasoconstrictive proliferating agents[74;120]. The consequence is variable vasoconstriction, the intense proliferation of vascular smooth muscle cells (with distal

extension), the formation of neointima, the proliferation and migration of adventitial fibroblasts into the media and ultimately the intima, as well as the increase in the neovascularisation of vasa vasorum[74]. Thus, the increase in pulmonary arterial pressure triggers a vicious cycle by stimulating through endothelial cell injury the deposition of collagen and the proliferation of smooth muscle cells, which further increase the pulmonary vascular resistance[91].

Neointima is a typical histological change of PAH and represents a new layer of myofibroblasts and extracellular matrix localized between the endothelium and lamina elastica interna. Due to its positive staining for α-smooth muscle actin[83], it is supposed that the cells composing it represent mainly a specialized subpopulation of smooth muscle cells. However, the possibility of a fibroblastic or endothelial origin can not be totally ruled out, since fibroblasts can migrate into the medial layer and transform into smooth muscle cells (SMCs)[172] and endothelial cells can also transdifferentiate into smooth muscle cells (endothelial-mesenchymal transdifferentiation) when stimulated with growth factors such as PDGF[52].

Plexiform lesions, formed by the clonal expansion of endothelial cells supported by a stroma of matrix proteins and myofibroblasts represent another hallmark of human PAH (detected in 80% of PAH cases[120]), which could unfortunately not be reproduced in any of the existing animal models[74]. Most often they are localized distal to the area of obliterative thickening, in pre-and intra-acinar pulmonary arteries and at arterial branching points. *Arteritis* associated with fibrinoid necrosis and inflammatory cell infiltration as well as *dilatation lesions* localized distal to plexiform lesions, which can lead to pulmonary haemorrhage and scarring, have also been mentioned[132].

Along with the structural modifications of the vessel wall, the damage of endothelium generates a thrombogenic surface, which triggers a continuous process of *intravascular coagulation*. Normally, the endothelium maintains an equilibrium between thrombosis (induced by activating factor X, the extrinsic pathway of coagulation or by the release of von Willebrand factor and plasminogen activator inhibitor-1 {PAI-1}) and the inhibition of thrombosis and promotion of fibrinolysis (by producing nitric oxide, prostacyclin, thrombomodulin or tissue plasminogen activator {t-PA})[12]. However, marked elevations of circulating PAI-1 and von Willebrand factor as well as reduced soluble thrombomodulin levels were described in patients with severe PAH, suggesting an impaired process of fibrinolysis and increased thrombosis[194].

In addition, inflammatory cells seem to be involved in some forms of human or animal PH, including monocrotaline (MCT)-treated rats, as suggested by the high levels of inflammatory cell infiltrates around plexiform lesions (mainly macrophages, mast cells, T and B lymphocytes). Also, the elevated circulating levels of proinflammatory cytokines IL1 and IL6[40;121], of inflammatory chemokines like macrophage inflammatory protein-1α, RANTES, as well as of P-selectin (a glycoprotein involved in neutrophil adhesion to the endothelium and platelet activation), have been mentioned to play a role in the inflammatory process[6;41;47;155].

Current treatment of PAH. Until the last decade, before the development of new specific therapies, the 1-year survival rate of patients diagnosed with PAH was 68%, the 5-year survival rate was approximately 34% and the common course of the disease was a rapid progression towards right heart failure and death[145].

For a long period of time, the classical therapy of PAH patients included the administration of anticoagulants, calcium-channel blockers and prostacyclins (intravenous epoprostenol)[149]. Anticoagulant therapy with warfarin has been shown to improve survival in patients with IPAH[81], and is also recommended in all patients with thromboembolic PH[25] due to its capacity to prevent in situ thrombosis and pulmonary embolism. Calcium-channel blockers (like nifedipine or diltiazem) can be used only in those patients having a positive response to the vasodilator challenge, which unfortunately represent only 10% of IPAH patients and even less in other forms of PAH[166]. Epoprostenol (a synthetic prostacyclin) is a product that causes vasodilation and inhibits vascular proliferation and platelet aggregation and can improve exercise tolerance, hemodynamic parameters, the quality of life and survival in IPAH patients when administered intravenously[25]. Its short half-life and, as a consequence, the requirement of continuous infusion remain, however, important drawbacks of this therapy.

As a response to the need for products with better availability and easier delivery, longer-acting prostacyclin analogs like treprostinil, iloprost and beraprost have been created and are available for intravenous, inhaled or oral use[25]. All these agents can significantly improve hemodynamic parameters and exercise tolerance and are at present considered as therapy for IPAH patients in functional classes II and III[71].

Based on the potent effect of endothelin as vasoconstrictor and mitogen, oral endothelin-1 receptor A and B antagonists like bosentan, ambrisentan and sitaxsentan have been recently

introduced. At present, due to its effect of improving survival, hemodynamics, exercise capacity and dyspnea, bosentan is recommended in clinical use for patients with stable functional class III or IV[25;145] and the other two agents have also shown promising results by improving the exercise capacity and hemodynamics in 12- to 18- week clinical trials[8;55].

Furthermore, PDE-5 inhibitors like sildenafil and tadalafil increase the effects of locally produced NO, thus representing potent vasodilators. They have proven their efficacy not only in experimental PH but also in human patients, in which they improved the symptoms, hemodynamic parameters, 6-min walk distance and 1-year survival[25].

I.2. Animal models of pulmonary hypertension

In order to gain more knowledge about the pathophysiological mechanisms of pulmonary hypertension, several animal models have been introduced, some of them resembling very closely the human disease. However, none of them is able to fully mimic all characteristic features of human PAH patients.

Pulmonary hypertension can be experimentally induced by ligating ductus arteriosus in fetal lambs[2], by exposing rats to hyperoxia[139], by repeated microembolisations of small pulmonary vessels[161] or by genetically engineering animals (5-HTT overexpressing mice[62], ET-B receptor deficient rats[77], BMPRII +/- mice[11], IL6[170] or Ang-1[39] overexpressing animals). However, the monocrotaline and hypoxia induced models of pulmonary hypertension still remain the most used models, due to their highest similarity to the human condition.

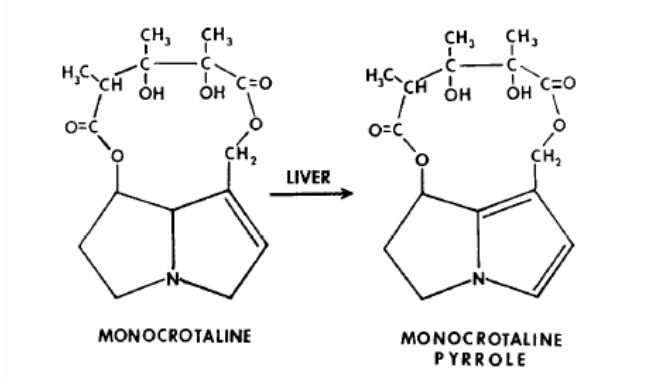
a. ***The hypoxia model*** is based on the observations that the reduction of oxygen concentration leads, after a short exposure, to acute pulmonary vasoconstriction whereas prolonged hypoxia induces medial and adventitial hyperplasia in pulmonary arterial walls[187]. Normo and hypobaric hypoxia have been used to induce PH in rats, mice or neonatal calves and the hypoxic model is considered a very useful tool for studying the human disease due to its high predictability and reproducibility. In rats, it is characterized by the muscularization of small, normally non-muscular arteries in the alveolar wall, the increased thickening of already muscularized pulmonary arteries and by an early and persistent vascular inflammatory response. All these changes lead to a 2-fold increase of mean pulmonary arterial pressure (PAP) in rats

subjected to hypoxia for 2 weeks, as well as to the development of right ventricular hypertrophy. On the other hand, in mice exposed to chronic hypoxia -although elevations in pulmonary artery pressure were observed- the vascular remodeling process was less significant than in rats[173], whereas neonatal calves exposed to chronic hypobaric hypoxia seem to be more sensitive to this stimulus and to develop severe PH with more extensive vascular remodeling than that observed in rats or mice[171]. Unlike in the human disease, however, which does not respond to oxygen therapy, the hypoxia-induced PH is fully reversible in rat and mice after the return to normoxic conditions and is not associated with the development of neointima or plexiform lesion formation.

b. Introduced more than 40 years ago, ***the MCT model*** is based on the complex effects triggered by the administration of a pyrrolizidine alkaloid- monocrotaline (Figure 1) obtained from the seeds of a plant (Crotalaria spectabilis). It is characterized by the rapid development of severe pulmonary disease in the absence of intrinsic heart disease. In humans, MCT is a recognized as a carcinogen and it is suspected to have gastrointestinal, liver, cardiovascular, kidney and respiratory toxicity. Whereas some animal species (e.g. mice) are resistant to this toxic agent and do not develop PH in time, others (rats, turkeys, pigs and sheep) react to this compound and present lung damage[42;104]. In rats, after the typical administration as a single injection (60 mg/kg) intraperitoneally or subcutaneously, monocrotaline is metabolized in the liver mainly by the cytochrome P450 to the reactive MCT pyrrole (MCTP), the initial dehydrogenation product of MCT. After a short-term stabilization due to the binding to red blood cells and transportation through the circulation to the lungs, MCTP produces lung vascular endothelium injury, the early event in the development of the disease[129].

In adult rats, the initial injection leads within a few hours to the injury of the vascular endothelium, followed by the extravascular leakage of proteases that act on extracellular matrix components and trigger the early inflammatory response[173]. After the initial events, a phase of increased reactivity to vasoconstrictor agents occurs, followed by a progressive thickening of the pulmonary arterial medial layer, even after the complete excretion of the alkaloid.

Figure 1. Chemical structure of monocrotaline and its metabolite (after Roth R. and Ganey P., 1988 [148])



Studies performed in the MCT model have evidenced at day four a process of endothelial cell injury and fragmentation of the internal elastic lamina, by day eight the extension of muscle into peripheral nonmuscular arteries without elevation in PAP and at day twelve significant elevations in PAP and vascular resistance as well as right ventricular hypertrophy and further progression of structural lung changes[85]. Pulmonary sequestration of platelets in thrombi and moderate thrombocytopenia as well as an early and sustained rise in lung polyamine levels[126], increased collagen synthesis in pulmonary arteries[89], dilated lymphatic vessels, alveolar edema[176] and hypertrophy of pulmonary veins[196] have also been described during the progression of the disease.

Differences between rat strains and even within the same strains have been noted regarding the sensitivity and response to MCT administration. Furthermore, age is another factor influencing the progression of the disease in rats: after the initial manifestations induced by the injection of MCT, adult rats continue to develop progressive vascular changes and pulmonary hypertension, whereas infant rats show spontaneous regression of the disease[85;183].

Although plexiform lesions could not be identified in this model, other characteristics like initial pulmonary vascular endothelial cell injury, development of thromboemboli, perivascular inflammation, necrotising arteritis, neomuscularization of small pulmonary arteries, decrease of peripheral arterioles number and right heart hypertrophy, also present in the course of the human disease, make the MCT model a very valuable tool for the investigation of the inner mechanisms of PH and for the evaluation of efficacy of different regimens.

I.3. The EGF pathway

I.3.1. Overview of the EGF pathway

Epidermal growth factor (EGF) is a growth factor that plays an important role in the regulation of cell growth, proliferation and differentiation. It belongs to the family of EGF-like molecules, together with TGF-α, heparin binding-EGF (HB-EGF), amphiregulin, epiregulin, betacellulin, neuregulins 1,2,3,4 and the pox virus growth factors, which have highly similar structural and functional characteristics[13;21;75]. These EGF-like molecules have the common characteristics of binding with high affinity to the EGF receptor and producing mitogenic responses in EGF-sensitive cells.

Epidermal growth factor. Human epidermal growth factor originates from a large precursor protein of 1207 aminoacids encoded by the gene found in humans on chromosome 4q25-q27, which needs enzymatic trimming before activation[59]. Although in the mouse submaxillary gland the precursor is rapidly processed to the active form of the growth factor, in some cells of the kidney the precursor can accumulate without being processed intracellularly[140]; in these cases, the prepro-EGF can exist as a glycosilated membrane protein and retains the EGF-like biological activities[113].

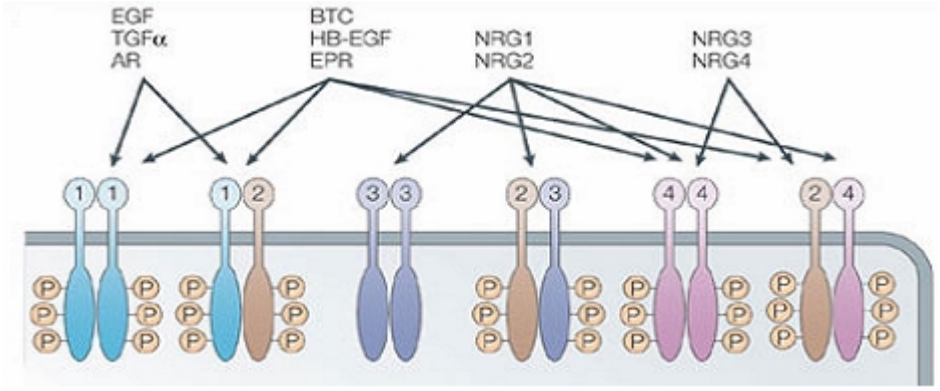
Early studies have shown that epidermal growth factor is a polypeptide of 53 aminoacids, produced in large quantities in the granular convoluted tubule cells from the male mouse submandibular gland, the first organ where it was found and the richest biological source of EGF[60]. In humans, EGF has been found in varying concentrations in milk, saliva, urine, plasma, cerebrospinal fluid and most other body fluids. Although cells from a wide range of organs can produce it, the main source for EGF synthesis and release are the distal tubules of the kidney, the parotid and submaxillary glands, Brunner's glands (submucosal glands located throughout the duodenum), pancreas, pituitary gland, nervous system as well as the thyroid and mammary glands[87;133;181].

Receptors. EGF receptors are specific, high-affinity but low-capacity receptors encoded by genes located on the human chromosomes 7 (7p14-p12)-for EGFR[190], 17 (17q21-q22)-for HER2[34], 12 (12q13)-for HER3[94] and 2 (2q33.3-34)-for HER4[202], respectively**.** Their

presence has been identified throughout the embryonic development in several organs from the human and murine fetuses (brain, placenta, intestine, lung, liver, kidney)[3;118;137], suggesting their role in the organogenesis of mammalians. In adult humans, almost all types of tissues express them and in normal cells their expression ranges from 40.000 to 100.000 copies/cell[20].

The four related EGF receptors, EGFR1 (ErbB1, HER1), HER2/c-neu (ErbB-2), ErbB3 (HER3) and ErbB4 (HER4) belong to the ErbB family of receptors and can be activated not only by EGF, but also by the other EGF-like growth factors, especially TGF-α (Figure 2)[75].

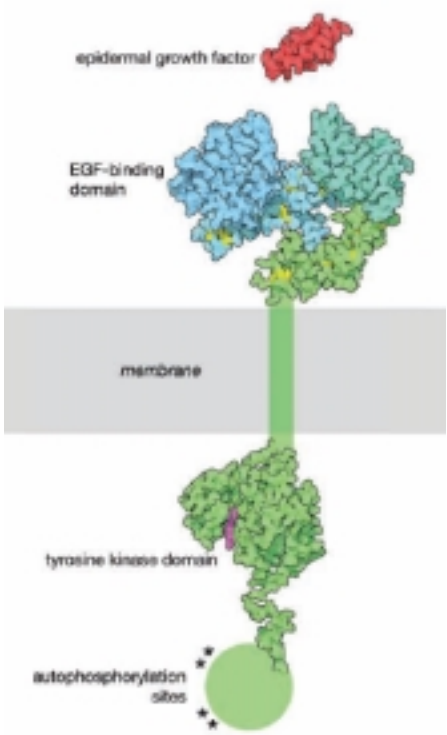
Figure 2. ERBB receptors and ligands (after Hynes N.E. and Lane H.A., 2005 [75]). 1,2,3 and 4 represent HER 1, 2, 3 and 4 respectively.



The receptor is found on the membrane of cells and consists of a single chain with three main parts: the EGF-binding domain (the outer part), the transmembrane domain and the tyrosine-kinase domain (the inner part) (Figure 3). In the absence of the ligand, the EGF receptor folds into an inactive form that cannot dimerize; binding of EGF to the globular parts of the outer domain of the receptor triggers the release of one of the long cysteine-rich sections, allowing the receptor to dimerize with other similar or different types of receptors (homo- or heterodimerization). As a particularity, ErbB2 has even in the absence of a ligand a conformation resembling the ligand-activated state, which makes ligand binding impossible (thus the inability of ErbB2 to bind any ligand) and renders it as the main dimerization partner for the other activated ErbB receptors[123]. Furthermore, HER3 is the only ErbB receptor that does not have an active kinase domain and cannot convey the signal into the cell but it can form

heterodimers with other EGF receptors, the ErbB-2/ErbB-3 heterodimers being the most potent regarding cell proliferation[29].

Figure 3. Structure of the EGF receptor (after Goodsell D., 2003 [58]).

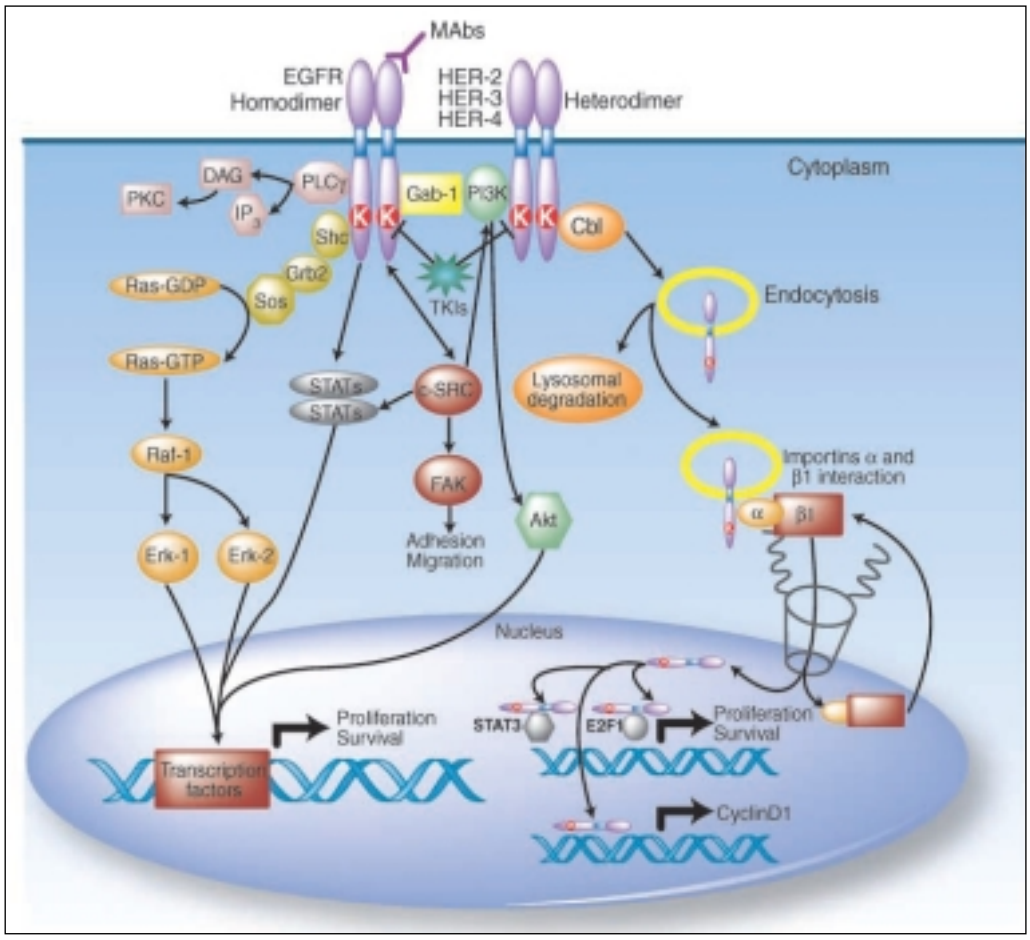


Dimerization brings the two tyrosine kinase domains of the receptor closer to each other, allowing them to add phosphates groups to the tyrosine residues on the neighboring chain (the process of activation of the intrinsic protein tyrosine kinase and tyrosine autophosphorylation). These newly phosphorylated sites, located mainly at the C-terminal tail of the receptor recruit various signaling proteins inside the cell, initiating the signaling cascades that ultimately lead to DNA synthesis and cellular growth[58].

Intracellular pathways. The dimerization and activation of EGF receptors leads to the activation of the Ras-Raf-MAPK pathway (which causes the phosphorylation of the c-Fos transcription factor) and of STAT-1 and STAT-3 transcription factors, both of them ultimately leading to proliferation. The proliferative effects of EGF are also signaled through the PI3K/Akt pathway, which regulates processes like gene expression, cell survival or angiogenesis[69;157]

and through PLCγ, which hydrolyzes PIP2, mobilizes Ca2+ from the intracellular stores (through IP3) and activates PKC (through 1,2-diacylglycerol), leading to NF-KB dependent transcription[80] (Figure 4).

Figure 4. Signaling pathways activated by EGFR (according to Scaltriti M. and Baselga J., 2006[157])



Effects. Due to its important role in the regulation of cell growth, proliferation and differentiation, EGF is a strong mitogen especially, but not exclusively, for epithelial cells. Early studies on EGF function in the intact animal demonstrated its stimulatory effect on epidermal proliferation and its inhibitory effect on gastric acid secretion[21]. Furthermore, it has been shown that EGF also influences the proliferation of fibroblasts[98], human glial cells[117] or extracellular matrix (by regulating the synthesis of fibronectin, collagen or glycosaminoglycans)[66;110;189]. It also plays an important role in the normal function of the

central nervous and endocrine system by modulating the synthesis of a number of hormones and can influence the activity of some types of GABAergic and dopaminergic neurons. The involvement of EGF in the central nervous system (CNS) functions is demonstrated by the inhibitory effect it has on food intake in rats after intracerebroventricular injection[134] as well as by the reduced serum EGF levels in schizophrenic patients[54]. Although sialoadenectomy seems to have no effect on testosterone and FSH levels despite the important decrease of EGF circulating levels, results published by Tsutsumi et al.[186] prove the involvement of EGF in the normal function of reproductive organs in humans. They demonstrate an important decrease of the number of spermatids in the testis and of mature sperm in the epididymis after sialoadenectomy and the correction of these changes by administrating EGF.

Due to the fact that the main function of EGF is the maintenance of mucosal integrity and the acceleration of regeneration of the epithelial cells[125], different topic products containing EGF have already been marketed for wound healing in burn treatment creams. Positive effects have been shown after topical applications of EGF in diabetic foot ulcers or injured corneal tissues. The ability of EGF to decrease gastric acid secretion and to stimulate the proliferation of mucosa cells can explain its gastroprotective role and its efficiency in healing of ulcers, making it a promising new line of therapy for gastroduodenal ulcers.

Role of EGF pathway in organogenesis

Investigation of tissues from midterm human fetuses has proved that immunoreactive EGF is found at almost the same sites as in the adult and has brought light on the time of appearance of EGF in tissues. Placenta, the distal tubules of kidney, the surface epithelium of stomach and the skin of the fetus are the early sites for the immunohistochemical localization of EGF (as early as week 15). The tips of villi, the exocrine glands of the gastrointestinal tract, the proximal and collecting tubules of the kidney and the glands of trachea are the next to present EGF, from weeks 16-17 of gestation[138].

In human lungs, however, throughout the gestational period the expression of EGF appears to follow a developmental pattern: in the early stage of lung development (weeks 7 to 26 of gestation), when the formation of preacinar airways, the extension and branching of the respiratory bronchioles and invasion of capillaries into the lung take place, EGF is highly expressed in the proximal airways (trachea and bronchi). Later on, during the saccular and

alveolar stages of lung development when alveolar units are formed, EGF seems to be mainly localized to alveolar type II cells and to alveolar macrophages[61;150]. This information comes in agreement with studies that have evidenced the role EGF has in promoting the maturation of distal airways in rabbits[22] and lambs[177] and in the acceleration of alveolar type II proliferation in vitro as well as in vivo[61;135].

Moreover, extensive studies on human lungs have shown that from week 11 of gestational age until 1 year of age EGF and its receptors colocalize and they are consistently present in the bronchial epithelium and submucosal glands, bronchiolar and alveolar epithelium as well as in vascular smooth muscle cells[118;175]. Intraalveolar macrophages were also consistently immunostained for EGF and EGF receptor[169;175]. Furthermore, immunohistochemical assays performed in human adults confirmed the distribution observed in fetuses, determining that EGF is found mainly in bronchial epithelial cells and, to a lesser extent, in trachea[87]. Also, the fact that EGF serum concentrations change with age, the highest concentration being seen up to 9 years of age[133] suggests that EGF continues to play a role in the process of cell proliferation even in the postnatal period.

I.3.2. Pathologic involvement of the EGF pathway

The upregulation of the EGF pathway has been shown to be involved in the pathogenesis of many types of malignancies, contributing to their development by stimulating proliferation and inhibiting apoptosis, by inducing angiogenesis and promoting tumor cells motility, adhesion and metastasis[4;68].

a. The **overexpression of EGF receptors** is one of the incriminating factors in the development of human tumors like breast, head and neck, gastric, colorectal, prostate, ovarian or non-small cell lung cancers, being associated with a high metastatic rate, a poor tumor differentiation, resistance to standard therapy, an increased risk of disease recurrence and poor patient prognosis[9;13;192].

EGF receptor type I overexpression can be caused by an increased EGFR gene transcription or by EGFR gene amplification and cells presenting a pattern of EGFR overexpression have more aggressive growth and invasiveness characteristics[199]. Studies

performed on more than 20.000 patients have identified the high levels of EGFR expression as having a strong prognostic value for head and neck carcinoma, ovarian, bladder, cervical and esophageal cancers, a moderate prognostic value for breast, gastric and endometrial tumors and a weak prognostic value for non-small cell lung cancers[119].

The *overexpression of HER2* (mostly due to gene amplification) has been evidenced in several types of neoplasias like breast, ovarian, stomach, pancreatic, colon carcinomas or non small cell lung cancers[156] and it has been shown that it can be an important factor in the oncogenic transformation of normal tissues especially from the mammary gland[63]. HER2 overexpression, especially coexpressed with HER1 and/or HER3, was associated with a very high invasive potential and reduced survival in breast cancer patients[106;123;146] and in some studies, to resistance to anti-estrogen therapy[15].

b. The **degree of activation of the EGF receptors** is, however, as important for carcinogenesis as the level of receptor expression. Factors affecting the activation status (increased levels of receptor ligands, EGFR mutations or heterodimerization of EGFR with other members of this receptor family –for example with HER2) can also lead to increased EGFR signaling.

Mutations of EGF receptors: The loss of 267 aminoacids from the EGFR extracellular domain due to the deletion of exons 2 to 7 of the wild-type EGFR gene is the most common mutation of this receptor (also known as EGFRvIII, del 2-7 EGFR or ΔEGFR), rendering the resulted form of the receptor not downregulable by endocytosis and thus constitutively active[122]. Less frequent mutations like deletions of aminoacids 746-750 or aminoacid substitution have also been described[128] and their presence has been detected not only in malignant cells[162] but also in the normal epithelium of respiratory structures surrounding the tumor, identifying these molecular perturbations as an early event of carcinogenesis, at least in the lung[178]. It has been recently shown that mutations in the kinase domain of EGFR in particular render a better response to TK-inhibitors[100;128], whereas mutations in the extracellular could not be correlated with a response to TK inhibitors[13].

Heterodimerization of EGF receptors: In contrast with the homodimeric forms of EGFR, which signal weakly because they are subjected to receptor downregulation and

degradation after ligand mediated activation[167], heterodimers of EGFR and HER2 are more stable due to their increased affinity for the ligands and ability to be recycled[198]. This explains the poorer survival and higher treatment failure rates in patients with coexpression of EGFR and HER2[16]. Coexpression of EGFR, ErbB2 and ErbB3 also has a negative synergistic effect on patients outcome, independently of the tumor size and lymph node status[197], being often associated with a more aggressive phenotype and a worse prognosis[123].

The involvement of EGF pathway in the development of neoplasias can be explained by the important anti-apoptotic and pro-angiogenic effects it has on cells. EGFR can prevent apoptosis by upregulating the expression of apoptosis inhibitory proteins like c-FLIP capable of inhibiting caspase-8 function[10] or by upregulating the anti-apoptotic molecules of the Bcl-2 family (Bcl-2, Bcl-X_L, Mcl-1)[67;96]. Also, EGF and the activated EGFR can enhance the process of angiogenesis through the upregulation of VEGF[48;142].

I.3.3. EGF receptors inhibitors

The large amount of data regarding the involvement of EGF signaling pathway in the development and progression of neoplasias has raised the interest on designing and testing EGFR inhibitors that could block signaling at different levels of this pathway. Gefitinib (Iressa®), erlotinib (Tarceva®) and lapatinib (Tykerb®) are antagonists that inhibit the intracellular normal function of the EGFRs tyrosine-kinases by competing with ATP for its binding site on the intracellular domain of EGFR. They have already proven antiproliferative effects both in experimental settings and clinical trials and are currently being used in the clinical practice as first or second line therapies.

Gefitinib (Iressa®, originally coded ZD1839, AstraZeneca), an anilinoquinazoline (chemical formula: 4-Quinazolinamine N-(3-chloro-4-fluorophenyl)-7-methyl-6-[3-4-morpholynpropoxy]) (Figure 5) available for oral administration, is a reversible and highly specific inhibitor of EGFR tyrosine-kinase. Gefitinib can be administered both intravenously and orally; after oral administration it is moderately slow absorbed (mean bioavailability of 59%), and peak plasma concentrations are reached after 3-7 hours. After 90% binding to plasma proteins (serum albumin and alpha-1 acid glycoprotein), gefitinib is extensively distributed into

tissues and mainly metabolized by CYP3A4. Having a mean half-life of 41 hours, gefitinib achieves a steady state after 7-10 days, with a 2 to 8 fold accumulation after once daily administration[76].

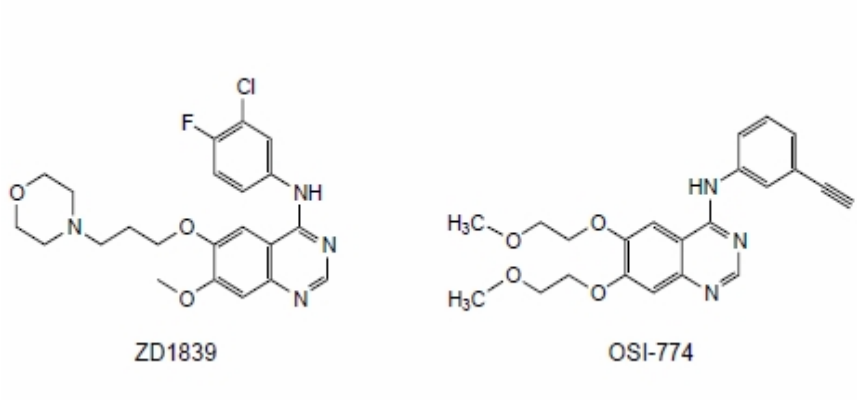
Due to its effect of tumor shrinkage and stabilization, improvement of symptoms as well as to its very good tolerability[53], it has been approved for clinical administration as monotherapy in patients with locally advanced or metastatic non small cells lung cancer (NSCLC) after failure of chemotherapy in more than 30 countries worldwide. However, because it failed to improve survival rates in patients with NSCLC included in a large double-blind, placebo-controlled randomized trial[164], gefitinib use has been restricted only to those patients that have already benefited from it (especially Asian patients, females, non-smokers and patients with adenocarcinomas), which account for approximately 10% of patients with NSCLC[53;95;180]. Due to the fact that it is a selective drug, gefitinib has a superior safety and tolerability profile in comparison with cytotoxic agents; the most common side effects are anorexia, asthenia, nausea, vomiting, mild to moderate diarrhoea, dehydration, skin rash and asymptomatic elevations of liver enzymes. However, although rare (<1% of patients), several severe adverse effects were described after treatment with Iressa: corneal erosion, aberrant eyelash growth, interstitial lung disease (with fatal cases), pancreatitis and hepatitis, myelosuppression and Stevens Johnson syndrome.

In patients in which gefitinib has not proven its efficacy, **Erlotinib** (OSI-774, Tarceva®, Roche), another low-molecular-weight, specific and reversible EGFR tyrosine-kinase specific inhibitor has been administered, with better results. Due to its synergistic and additive effects when associated with chemotherapy agents or radiotherapy and to the survival benefit it has proved in the treatment of lung cancer in phase III trials, erlotinib is currently being used as monotherapy in patients with locally advanced or metastatic NSCLC that have not benefited from gefitinib or chemotherapy and, since 2005, as first line therapy for locally advanced, unresectable or metastatic pancreatic cancer in combination with gemcitabine[43;45]. Several serious adverse effects have been described after the use of erlotinib, including gastrointestinal perforations (with very high mortality), liver function impairment (elevated transaminases and bilirubin), corneal ulcerations or perforations, keratitis, conjunctivitis, skin and subcutaneous tissue disorders (hair and nail changes, bullous, blistering or exfoliative skin disorders, Stevens-

Johnson syndrome). Also, an excess of myocardial infarction/ischaemia and cerebrovascular accidents was observed in the group of patients treated with both Tarceva and gemcitabine in comparison with patients treated with gemcitabine alone[179].

Erlotinib is a quinazolinamine (N-(3-ethynylphenyl)-6,7-bis(2-methoxyethoxy)-4-quinazolinamine) (Figure 5) that is well absorbed after oral therapy (bioavailability of 59%) and reaches peak plasma levels at approximately 4 hours after administration. Following absorbtion, it is highly bound in blood, especially to albumin and alpha-1 acid glycoprotein, metabolized mainly by the hepatic cytochromes CYP3A4/CYP3A5 (80-95%) and eliminated as metabolites mainly via the faeces (>90%). With a half-life of 36.2 hours, erlotinib administered daily at a dose of 150 mg reaches a steady state plasma concentration in approximately 7-8 days[179].

Figure 5. Chemical structures of gefitinib (ZD1839) and erlotinib (OSI-774)

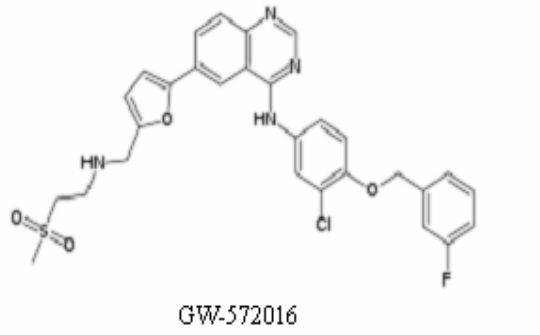


Lapatinib (GW572016, Tykerb®, GlaxoSmithKline) is a low molecular weight, reversible, dual EGFR/HER2 tyrosine kinase inhibitor, which also belongs to the 4-anilinoquinazoline class of kinase inhibitors (chemical formula: N-(3chloro-4-{[(3-fluorophenyl)methyl]oxy}phenyl)-6-[5-({[2(methylsulfonyl)ethyl]amino}methyl)-2-furanyl]-4-quinazolinamine bis(4 methylbenzenesulfonate) monohydrate) (Figure 6). Following oral administration, lapatinib is incompletly absorbed and peak plasma concentrations are achieved after 4 hours from administration. Lapatinib is highly bound (>99%) to albumin and alpha-1

acid glycoprotein, metabolized mainly by CYP3A4 and CYP3A5 (with a half-life of 14.2 hours) and daily treatment allows the achievement of a steady state in approximately 6-7 days[188].

Lapatinib has been used since the moment of its approval by FDA in 2007, in association with capecitabine for the treatment of women with refractory advanced and metastatic breast cancer overexpressing HER2[50] who had progressive disease after treatment with trastuzumab (Herceptin) or other cancer therapies[18]. It is currently investigated as monotherapy or in combination with other regimens. Results from 44 clinical trials indicated an overall favourable toxicity profile of lapatinib with a low frequency of events that are usually reversible[185]. However, treatment with lapatinib was associated in some patients with the decrease of left ventricular ejection fraction, QT prolongation as well as with interstitial lung disease or pneumonitis, palmar-plantar erythrodysesthesia, severe diarrhea and hepatotoxicity, which underline the necessity of increased monitorisation of patients with already impared cardiac, pulmonary or hepatic function.

Figure 6. Chemical structure of lapatinib (GW572016)



GW-572016

I.4. Aim of the study

Using rats in which PH was induced by administrating a single dose of MCT, the present study aimed to:

1. investigate the therapeutic efficacy of continuous chronic EGF signaling pathway inhibition
2. evaluate the therapeutic efficacy of three different EGF receptor inhibitors (gefitinib, erlotinib, lapatinib) in delaying the progression of the disease in a head-to head experiment.

II. MATERIALS AND METHODS

II.A. MATERIALS

II.A.1. Animal experiments

Adult male Sprague- Dawley rats, weighing 300 to 350 g were obtained from Charles River and Harlan Winkelman Laboratories. Animals were housed under controled temperature (approximatelly 22º C) and food and water were provided *ad libitum*. All experiments were performed according to the international guidelines regarding animal experiments.

II.A.2. Substances and reagents

SUBSTANCE (CHEMICAL NAME)	SUBSTANCE (COMMERCIAL NAME OR KIT)	DISTRIBUTION COMPANY
A. *Induction of pulmonary hypertension in Sprague-Dawley rats*		
Monocrotaline	Crotaline ®	*Sigma Aldrich Chemie GmbH, Steinheim, Germany*
Sodium hydroxide 1N (1 mol/l)		*Merck, Darmstadt, Germany*
Chlorhidric acid 1 N (1 mol/l)		*Merck, Darmstadt, Germany*
Enrofloxacine	Baytril 2.5 % ®	*Bayer Vital GmbH, Leverkusen, Germany*
Isoflurane		*Baxter Deutschland GmbH, Unterschleißheim, Germany*
Methylcellulose		*Sigma Aldrich Chemie GmbH, Steinheim, Germany*
B. *Catheterization of the right heart and tissue processing*		
Ventilation gas (50% O_2, 50% N_2)		*Air Liquid (ehem.Messer), Siegen, Germany*
Povidone-iodine solution	Braunoderm ®	*B.Braun Melsungen AG, Melsungen, Germany*
Ketaminhydrochloride 100 mg/ml	Ketavet ®	*Pharmacia GmbH,Berlin,Germany*

Medetomidinhydrochloride 1mg/ml	Domitor ®	*Pfizer GmbH, Berlin,Germany*
Heparine	Heparin-Natrium-25.000-ratiopharm®	*Ratiopharm GmbH,Ulm, Germany*
Physiological saline solution (NaCl 0.9%)		*B.Braun Melsungen AG, Melsungen, Germany*
Distilled water		*B.Braun Melsungen AG, Melsungen, Germany*
Aprotinin	Trasylol ®	*Bayer Vital GmbH, Leverkusen, Germany*
Pentoxifylline 20 mg/ml	Trental ®	*Sanofi Aventis, Frankfurt am Main, Germany*
Formaline	Formaldehyd-Lősung 3.5-3.7%	*Otto Fischar GmbH&Co.KG, Saarbrücken, Germany*

C. ***Histology***

Paraffin embedding medium	Paraplast Plus ®	*Sigma Aldrich Chemie GmbH, Steinheim, Germany*
Sodium chloride		*Carl Roth GmbH&Co, Karlsruhe, Germany*
Potassium chloride		*Carl Roth GmbH&Co, Karlsruhe, Germany*
Dinatriumhydrogenphosphat dihydrat ($Na_2HPO4 \cdot 2H_20$)		*Merck, Darmstadt, Germany*
Kaliumdihydrogenphosphat (KH_2PO4)		*Merck, Darmstadt, Germany*
Roti-Histol (Xylol)	Roti®- Histol	*Carl Roth GmbH, Karlsruhe, Germany*
Ethanol 70%, 96%, 99.6%		*Otto Fischar GmbH&Co.KG, Saarbrücken, Germany*
Isopropylalkohol (99.8%)		*Sigma Aldrich Chemie GmbH, Steinheim, Germany*
Mounting medium	Pertex ®	*Medite GmbH, Burgdorf, Germany*

Specific reagents for medial wall thickness assessment

Resorcin-Fuchsin		*Waldeck GmbH&Co.KG, Münster, Germany*
Nuclear-Fast Red- Alum. Sulpha	Kernechtrot Aluminiumsulfat	*Waldeck GmbH&Co.KG, Münster, Germany*

Specific reagents for muscularization degree assessment

Methanol		*Sigma Aldrich Chemie GmbH, Steinheim, Germany*
Hydrogen peroxide 30%		*Merck, Darmstadt, Germany*
Trypsin	Digest All 2 ®	*Invitrogen Corporation, Camarillo, CA,USA*
Normal Horse Serum		*Alexis Biochemicals, Grünberg, Germany*
Streptavidin-Biotin-Blocking Kit		*Linaris Biologische Produkte GmbH, Wertheim-Bettingen, Germany*
Vectastain ABC Kit		*Linaris Biologische Produkte GmbH, Wertheim-Bettingen, Germany*
Horseradish peroxidase streptavidin	RTU Horseradish peroxidase streptavidin	*Vector Laboratories, Burlingame, CA,USA*
Vector VIP	Vector® VIP substrate kit for peroxidase (SK-4600)	*Vector Laboratories, Burlingame, CA,USA*
DAB Substrat Kit	DAB Substrate Kit for peroxidase (SK-4100)	*Vector Laboratories, Burlingame, CA,USA*
Methylgreen	Methyl Green (H-3402)	*Vector Laboratories, Burlingame, CA,USA*

Specific reagents for proliferation degree assessment

Citrate Buffer		*Zymed Laboratories Inc., Invitrogen, Carlsbad, USA*
Methanol		*Sigma Aldrich Chemie GmbH,*

		Steinheim, Germany
Hydrogen peroxide 30%		*Merck, Darmstadt, Germany*
Proteinase K	Dako Real ™, Proteinase K (40x)	*Dako Denmark A/S, Glostrup, Denmark*
Proteinase K diluent	Dako Real ™ Proteinase K diluent	*Dako Denmark A/S, Glostrup, Denmark*
Peroxidase	ImmPress™ Reagent Kit	*Linaris Biologische Produkte GmbH, Wertheim-Bettingen, Germany*
Nova RED Substrate Kit	Vector ® Nova RED ™ Substrate Kit for peroxidase	*Linaris Biologische Produkte GmbH, Wertheim-Bettingen, Germany*
Hematoxylin	Hematoxylin QS (H-3404)	*Vector Laboratories, Burlingame, CA,USA*

Antibodies

Anti- alpha smooth muscle actin antibody (Monoclonal, mouse anti-human)	Clone 1A4 Sigma	*Sigma Aldrich, Steinheim, Germany*
Anti- von Willebrand factor antibody (Polyclonal, rabbit anti-human)		*Dako Cytomation, Hamburg, Germany*
Anti –PCNA antibody (Rabbit polyclonal IgG)	PCNA antibody (FL-261): sc-7907	*Santa Cruz Biotechnology, Santa Cruz, CA, USA*

II.A.3. Consumables

PRODUCT	PRODUCT (COMMERCIAL NAME OR KIT)	DISTRIBUTION COMPANY

A. Induction of pulmonary hypertension in rats, chronic treatment and catheterization of the right heart

Single use gloves	Nitra-Tex®	*Ansell Ltd, Tamworth, Staffordshire, UK*
Napkins		*Tork, Mannheim, Germany*
Needles: 18G (1.2 mm x 40 mm), 20G (0.9 mm x 40 mm), 26G (0.45mm x	BD Microlance 3®	*Becton Dickinson GmbH, Heidelberg, Germany*

13mm)		
Single use syringes 1ml, 2 ml, 5 ml, 20 ml	Injekt®- F	*B.Braun Melsungen AG, Melsungen, Germany*
Black thread no.16		*Coats GmbH, Kenzingen, Germany*
Medical adhesive bands 2.5 cm/ 9.2 m	3M™ Durapore™ Surgical Tape	*3M Health Care, St.Paul, MN, USA*
Gauze 4x5 cm	Purzellin ®	*Lohmann und Rauscher, Rengsdorf, Germany*
Gauze balls size 6, unsteril		*Fuhrmann Verrbandstoffe GmbH, Munich, Germany*
Surgical instruments		*Fine Science Tools GmbH, Heidelberg, Germany* *Martin Medizintechnik, Tuttlingen, Germany*
Cannula for vein catheter support 22G	Vasocan Braunüle ®	*B.Braun Melsungen AG, Melsungen, Germany*
Instrument for venous catheter insertion	Intradyn ™ Venous Hemostasis Introducer	*B.Braun Melsungen AG, Melsungen, Germany*
Silicone catheter for right heart catheterization		*Custom-made*
Polyethylene cannula for insertion into the carotid artery	Vasofix ® Safety ®, 22G	*B.Braun Melsungen AG, Melsungen, Germany*
Tracheal cannula		*Custom-made from BD Microlance 3® 15 or 20G needles (Becton Dickinson, Germany) shortened to 1.5 cm*
Stopcock for infusion therapy and pressure monitoring	Discofix ® C-3	*B.Braun Melsungen AG, Melsungen, Germany*
Rat restrainer (model 81)		*IITC Life Science Inc., Woodland Hills, CA, USA*
Heating underlay	ThermoLux®	*Witte + Sutor GmbH, Murrhardt, Germany*
Eppendorf tubes (Microtubes 1.5 ml)		*Sarstedt, Nürnbrecht, Germany*
Scalpels	Feather Disposable Scalpel	*Feather Safety Razor Co, LTD, Osaka, Japan*

B. *Histology*

Histological glass slides 25x75x1 mm	SuperFrost UltraPlus®	*R. Langenbrinck, Emmendingen, Germany*
Embedding cassettes		*Leica Microsystems, Nussloch, Germany*
Coverslips 24x 36 mm		*Menzel GmbH&Co.KG, Braunschweig, Germany*
Microtom blades	Microtome blade type A35	*Feather Safety Razor Co Ltd, Osaka, Japan*
Tips for automatic pipettes 200 µl, 1000 µl, 10 µl		*Sarstedt, Nürnbrecht, Germany*
Automatic pipettes 10-100 µl, 1-10 µl	Eppendorf PhysioCare concept	*Eppendorf AG, Hamburg, Germany*

II.A.4. Systems and machines

PRODUCT	PRODUCT (COMMERCIAL NAME)	DISTRIBUTION COMPANY

A. *Induction of pulmonary hypertension in rats, chronic treatment and catheterization of the right heart*

Weighing machine for animals	Sauter RP 3000	*August Sauter GmbH, Albstadt-Ebingen, Bayer Leverkusen*
Balance for substances	Mettler Toledo PB303 Delta Range®	*Mettler Toledo, Switzerland*
Transducers	Combitrans Monitoring Set Mod.II for Arterial Blood Pressure Management	*B.Braun Melsungen AG, Melsungen, Germany*
Ventilation system for rats	SAR- 830/P Ventilator	*IITH Life Science Inc., Woodland Hills, CA, USA*
Computer and screen		
Blood analyzer	Rapidlab™ 348	*Bayer Healthcare, Fernwald, Germany*

Centrifuge	Hettich Mikro 200R	*Hettich-Zentrifugen GmbH & Co. KG, Tuttlingen, Germany*

B. Histology

Tissue dehydrating machine	Leica TP 1050	*Leica Microsystems, Nussloch GmbH, Germany*
Tissue embedding machine	Leica EG 1140 H	*Leica Microsystems, Nussloch GmbH, Germany*
Cooling table	Leica EG 1150 C	*Leica Microsystems, Nussloch GmbH, Germany*
Automated microtom	Leica RM 2165	*Leica Microsystems, Nussloch GmbH, Germany*
Flattening table	Leica HI 1220	*Leica Microsystems, Nussloch GmbH, Germany*
Flattening bath for paraffin sections	Leica HI 1210	*Leica Microsystems, Nussloch GmbH, Germany*
Computer	Q 550 IW	*Leica Microsystems, Nussloch GmbH, Germany*
Software	Q Win V3	*Leica Microsystems, Nussloch GmbH, Germany*
Light microscope	DMLA	*Leica Microsystems, Nussloch GmbH, Germany*
Ice flake machine	Icematic F100 Compact	*Castelmac SPA, Castelfranco, Italy*

II.B. METHODS

II.B.1. Animal experiments: Monocrotaline-induced pulmonary hypertension

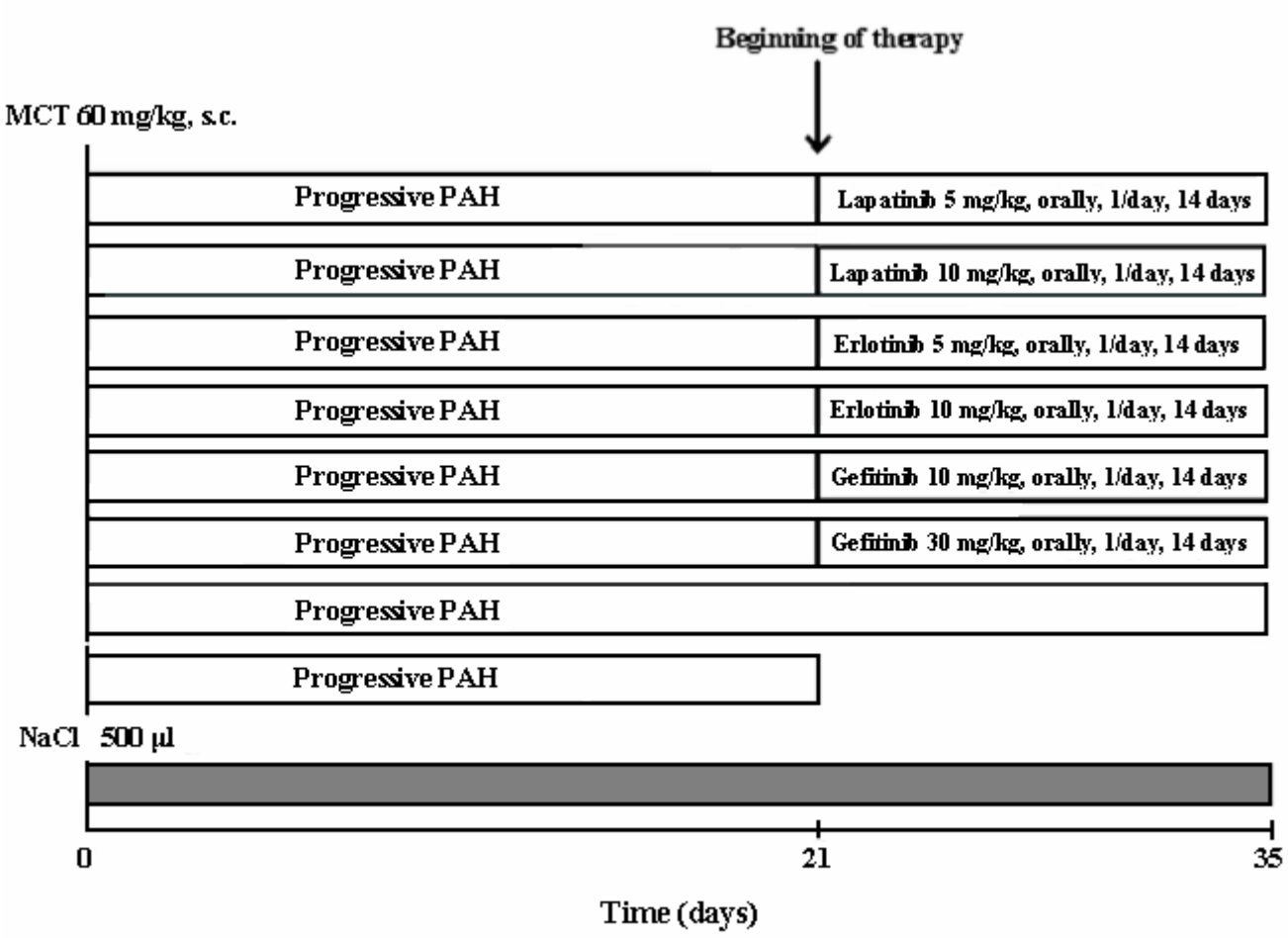
Pulmonary hypertension was induced in adult male Sprague-Dawley rats of 300 to 350 grams in bodyweight (Charles River Laboratories) via subcutaneous administration of monocrotaline (60 mg/kg). Monocrotaline solution was freshly prepared, by dissolving the alkaloid in 1N HCl and 1N NaOH (250 mg alkaloid dissolved in 3 ml 1N HCl and 2 ml 1N NaOH and pH adjusted to 7.4). A single injection of monocrotaline was administered on day 0, after light anesthesia with Isoflurane®. Control rats received 500 µl saline solution subcutaneously under the same conditions. In order to avoid opportunistic infections, rats were administered an antibiotic solution (Baytril® 2.5%) from day 1 to 15 in the drinking water, at a concentration of 2 ml Baytril/ 500 ml water.

II.B.2. Therapy with EGFR inhibitors

Three weeks after the administration of the monocrotaline solution, rats were subjected to daily oral treatment with EGF receptor inhibitors gefitinib (Iressa), erlotinib (Tarceva) and lapatinib (Tykerb). The tablets were crushed and dissolved in 1.5% methylcellulose solution, previously prepared and stored at 4º C. Fresh solutions of the three inhibitors were used at every administration and the necessary volume of solution was calculated every day for each animal according to the rat body weight. Treatment was administered under light anesthesia with Isoflurane® using a rat gavage feeding needle, once per day, from day 21 to 35. The animals were divided into several groups, in order to test two different doses for each inhibitor: 30 and 10 mg/kg bodyweight for gefitinib, 10 and 5 mg/kg bodyweight for erlotinib and lapatinib, respectively (Figure 7). Considering the lack of published articles regarding the efficient range of doses of the investigated EGFR inhibitors in experimental pulmonary hypertension, the doses of gefitinib, erlotinib and lapatinib were selected based on our pilot experiments regarding toxicity and tolerability and on previously published studies in rats with different pathologies[51;64;86;159].

The placebo (MCT 35 days) group received daily 1 ml 1.5% methylcellulose, under the same conditions (orally, once per day, from day 21 to 35).

Figure 7. Experimental plan for chronic administration of EGFR inhibitors gefitinib, erlotinib and lapatinib



II.B.3. Catheterization of the right heart

In order to monitor hemodynamic changes, rats were subjected to right heart catheterization 21 and 35 days after the administration of monocrotaline. Rats were initially anesthetized by intraperitoneal injection with Ketamine/Domitor solution 10:1 (1 µl Ketamine/1 g bodyweight) and placed on a heating pad for maintainance of body temperature in a physiological range. Animals were tracheotomized and artificially ventilated with a mixture of oxygen and nitrogen (1:1), at a constant frequence of 60 breaths per minute, with an inspiratory flow rate of 500 to 600 cc/min. The inspiratory time was 0.5 seconds and the positive end

expiratory pressure (PEEP) was set to 1 cmH_2O. The left carotid artery was isolated and cannulated with a polyethylene cannula connected to a fluid-filled force transducer and the systemic arterial pressure (SAP) was measured throughout the surgical procedure. The right jugular vein was also isolated and cannulated and the right heart was catheterized through the jugular vein, using a home-made silicone catheter connected to a fluid-filled force transducer. The catheter was advanced into the right ventricle under the guidance of pressure tracing. A volume of 1 ml heparin solution (10%, dissolved in saline solution) was administered through the jugular vein, in order to prevent blood coagulation. Using fluid force transducers (calibrated at zero to the hillum level before the beginning of the procedure) and the Labtech Pro (Labtech Notebook Runtime Version 9.02) computer software, records of ventilation pressure, arterial systemic pressure (SAP) and right ventricular systolic pressure (RVSP) were taken for 5 to 10 minutes. Immediately after catheterization, 300 to 400 μl arterial and venous blood samples were prelevated and analyzed for oxygenation and CO_2 levels, arterial and venous pH, haemoglobin and hematocrit levels, using the Rapid lab™ 348 blood analyzer.

Arterial blood samples were also collected for plasma separation using a mixture (1:2) of the anticoagulant pentoxifylline (Trental®) and proteinase inhibitor aprotinin (Trasylol®); blood was centrifuged at room temperature for 10 minutes at 12500 RPM. The supernatant was collected and immediately snap frozen in liquid nitrogen and further used for molecular biology analysis. Cardiac output and index (CI) values were calculated for each rat based on Fick's formula (see Table 2), using haemoglobin, arterial and venous saturation values obtained from the blood samples prelevated at the beginning of the surgery. Pulmonary and systemic vascular resistance indexes (TPR, TSR) were calculated based on right ventricular systolic and systemic arterial pressures measured during the surgery and the cardiac index values obtained as described.

Table 2. Formulas for calculation of cardiac output (CO), cardiac index (CI), total pulmonary vascular resistance index (TPR) and total systemic vascular resistance index (TSR)

CO (ml/min) = 5.46/ ((Hb*arterial saturation*0.0134) – (Hb*venous saturation*0.0134))

CI (ml/min*100 g BW) = Cardiac output/ Bodyweight

TPR (mmHg* 100 gBW *min / ml) = RVSP / CI

TSR (mmHg* 100 gBW *min / ml) = SAP / CI

II.B.4. Tissue processing

The abdomen and thoracic cavity were opened and the left and the right ventricles were incised in order to allow the removal of blood (the right ventricle was incised at 5 mm below the base of pulmonary artery). A cannula connected to a reservoir filled with saline solution was inserted into the pulmonary artery through the right ventricle and the lungs were flushed out of blood at a pressure of 30 cmH_2O above the pulmonary hillum. The right hilum was then tightened with a thin thread, allowing the removal of the right lung which was immediately snap frozen in liquid nitrogen. Plasma and the right lung were then preserved at (-80)º C. The left lung was prepared for histology using a cannula inserted into the pulmonary artery, connected to a reservoir filled with 3.5-3.7% formalin solution. The left lung was perfused at a pressure of 30 cmH_2O above the pulmonary hillum for 5 to 10 minutes, further isolated and stored in 3.5-3.7% formalin solution at 4º C over night and then in phosphate buffer (pH=7.4) until dehydration and histological analysis. The heart was prelevated and the right ventricular wall (RV) was separated from the block of left ventricular wall (LV) plus ventricular septum (S). The samples were dried for 48 hours at room temperature, the dry weights were measured and heart ratio (as an index of right heart hypertrophy) was calculated as RV/ (LV+S).

II.B.5. Histological analysis – medial wall thickness and assessment of the degree of muscularization

After the prelevation of the left lung as described above, sections of aproximately 4 mm in width were obtained and placed in histological cassettes, dehydrated in an automatic dehydration machine and then embedded in paraffin. Sections of 3 μm in diameter were cut from the paraffin blocks using a Leica microtome (Leica RM 2165), were maintained at 37˚C for 24 hours and then used for staining.

For the assessment of the *muscularization degree* of small peripheral pulmonary arteries, the sections were maintained for 20 minutes at 60˚C, deparaffinized in xylol and progressively rehydrated. Endogenous peroxidase was blocked by treating the sections for 20 minutes at room temperature (RT) with a freshly prepared solution of 30% hydrogen peroxide in methanol (1:1) and unspecific background staining due to endogenous streptavidin and biotin was eliminated by

consecutively overlaying the slides with streptavidin blocking solution for 15 minutes (followed by 5 minutes wash in PBS) and biotin blocking solution for 15 minutes at RT (followed by 5 minutes wash in PBS). For the staining of vascular smooth muscle and endothelium, the slides were initially incubated with normal horse serum for 30 minutes (to eliminate non-specific binding caused by immunoglobulin cross-reactivity) and then incubated with a mouse anti α-smooth muscle cells actin primary antibody (at RT) and a rabbit anti-von Willebrand factor primary antibody respectively (at 37° C); both antibodies were diluted 1:900 in 10% BSA and maintained for 30 minutes. Slides were further washed for 4 times (5 minutes each) in PBS and immediately incubated with the corresponding biotinylated anti-mouse and anti-rabbit secondary antibodies, for 30 minutes at RT. Specific substrates were used for the induction of a colour reaction by horseradish peroxidase streptavidin coupled to the secondary antibodies: VIP substrate (maintained for 1.5 minutes at RT) determined the purple/violet colour of the smooth muscle layer and DAB (maintained for 30 seconds at RT) determined the brown colour of the endothelium. Methylgreen (5 minutes, at 60° C) was used as counterstaining for nuclei and slides were then progressively dehydrated, mounted with Pertex mounting medium and left to dry over night.

The sections were subsequently analyzed by light microscopy using a software that distinguishes between the purple and brown colour of the structures and categorizes the vessels into nonmuscularized (smooth muscle covering less than 5% of the vessel wall area), partially muscularized (5 to 75% smooth muscle around the vessel) and fully muscularized (75 to 100% muscle around the vessel). 80 to 100 vessels of 20-50 μm in diameter in each lung were analyzed at 40x magnification, and the percentage of vessels in each muscularization category was calculated by dividing the number of vessels in that category to the total number of vessels counted for each particular lung.

For *wall thickness assessment* the sections were incubated at 58-60° C for 20 minutes, deparaffinized in xylol and progressivly rehydrated in graded alcohols. For staining the elastic fibers the slides were maintaned in Resorcin-Fuchsin over night (16 hours on average) at RT (which determined the blueish-black colour of elastic fibers) and after two times washing with distilled water, Nuclear Fast Red was used as a red nuclear counterstaining for 10 minutes at RT. Excess of dyes was removed by two times immersion in distilled water and progressively

increased ethanol concentrations were used for dehydration of slides before their mount with Pertex mounting medium.

80-100 intraacinar arteries were analyzed at 40x magnification for every lung; the distance between *lamina elastica interna* and *lamina elastica externa* was automatically calculated for every vessel by the Qwin (Leica) software as an average of at least 3 measurements. Arteries were categorized according to their external diameter, as follows: category I included vessels with an external diameter of 20 to 50 μm; category II included vessels with an external diameter of 51 to 90 μm, category III included vessels with an external diameter of 91 to 150 μm whereas vessels with an external diameter of more than 151 μm were considered category IV vessels. Medial wall thickness was automatically calculated as percentage, using the formula:

% MWT= (2*wall thickness/external diameter) *100.

II.B.6. Histological analysis – Assessment of the level of proliferation

In order to assess proliferation in the pulmonary vessel walls from rats injected with MCT and treated with EGF receptor inhibitors, tissue sections were incubated at 58-60° C, deparaffinized in xylol and rehydrated using ethanols with decreasing concentration degrees and distilled water. In order to enhance the antigen retrieval process, the slides were incubated with citrate buffer (pH=6; diluted 1:20 in distilled water) in water bath for 20 minutes, allowed to cool in the buffer for 30 minutes at RT and then washed with distilled water for 5 minutes. The slides were further incubated with a solution of hydrogen peroxide 30% in methanol (1:1) for 20 minutes in order to block endogenous peroxidases and washed in distilled water and PBS (two times 5 minutes each). Further on, they were treated with proteinase K (a second antigen retrieval solution) for 15 minutes at RT, washed in distilled water and PBS (3 times, for 5 minutes each) and incubated in 10% BSA for one hour.

For the specific staining of proliferating cell nuclear antigen, the slides were pretreated with normal horse serum for 20 minutes (to block non-specific bindings of the antibody to the tissue) and then incubated overnight at 4° C (in order to allow a better penetration of the antibody into the paraffin sections) with a primary rabbit anti-PCNA antibody (diluted 1:100 in 10% BSA). The excess of primary antibody was removed by washing the slides with PBS every

20 minutes for 2h. Peroxide-conjugated anti-rabbit IgGs were used as secondary antibody. In order to visualize the proliferating nuclei, the slides were further treated with NovaRED substrate for 5 minutes at RT (which determined the red/brown colour) and the colour reaction was terminated by immersing the slides in tap water for 5 minutes. Hematoxylin QS (30-40 seconds) was used for nuclear counterstaining. The excess of dye was removed by consecutive washing with tap water, PBS buffer and distilled water. The slides were prepared for final mounting with Pertex by progressive dehydration.

The Qwin (Leica) software was used to select vessels with an external diameter of 20 to 50 μm and positive and negative cells from 40 to 80 vessels in each lung were counted. The index of *in situ* proliferation was calculated using the formula:

IOP (%)=Number of PCNA-positive cells in the vessel/ Number of all cells in the vessel

and all variations in the treatment groups were calculated using as reference value (considered 100%) the mean value of IOP from healthy rats.

II.B.7. Bodyweight changes and distress score assessment

Rats from all groups were weighed and monitored and the average bodyweight and distress score were calculated every day from day 21 to 35. In order to determine the gain/loss of weight, the average bodyweight on day 22 was considered as reference value for comparison with the average bodyweight values of every day. Average relative weight change was calculated as percentage of change in weight at day 35 compared with the weight at the beginning of treatment.

During treatment animals were also given a grade from 0 to 4 for several parameters (appearance, bodyweight changes, clinical signs, natural and provoked behaviour) and the total score per day was calculated as a sum of all grades given for the analyzed parameters (see Table 3). A total score of 0 to 4 was considered to indicate a normal animal, a score between 5 and 9 was considered to indicate a suffering animal and possible use of analgesics, whereas a score higher than 10 indicated a severely sick animal, in which termination should be taken into account. Average relative distress score change was calculated as percentage of change in distress score at day 35 compared with the distress score at the beginning of treatment.

Table 3. Distress scoring assessment

Animal identification		Score
Appearance	Normal	0
	General lack of grooming, dull, yellowing coat	1
	Coat staring, ocular/nasal discharge	2
	Pinched features, dehydrated, pale eyes	3
Bodyweight changes	Normal (stagnation is already a sign of illness)	1
	Bodyweight loss <5%	2
	Bodyweight loss 5-10%	3
	Bodyweight loss 10-15%	4
Clinical signs	Normal respiratory rate and pattern	0
	Slight changes, increased rate	1
	Dyspnea, tachypnes with abdominal breathing	2
	Marked abdominal breathing with cyanosis	3
Natural behaviour	Normal	0
	Minor changes	1
	Less alert	2
	Isolation or very still	3
Provoked behaviour	Normal	0
	Minor depression or exaggerated response	1
	Moderate change	2
	Reacts on handling violently/vocalisation or very still	3

II.B.8. Data analysis

Data are given as means ± standard error mean (SEM). Statistical comparisons were made using one-way ANOVA followed by a Student Newman Keuls *post-hoc* test. A value of $P< 0.05$ was considered to be statistically significant.

III. RESULTS

EGF receptor inhibition as treatment of experimental PAH

III.1. Effects of EGFR inhibition on hemodynamic parameters in monocrotaline rats

Right ventricular systolic pressure (RVSP) was significantly elevated after 21 days from the administration of MCT (39.7 ± 5.4 mmHg compared with 25.5 ± 1.1 mmHg in healthy rats) and further increased until day 35 (73.6 ± 1.5 mmHg). Chronic treatment with the EGFR inhibitors from day 21 to 35 resulted in a significant decrease of RVSP. Administration of all three EGFR antagonists reduced RVSP in a dose-dependent manner and gefitinib proved to be the most effective in delaying the progression of the disease (Figure 8), by reducing RVSP to levels comparable to those seen in the MCT 21 days group (Table 4), at both doses used (41.7 ± 3.8 mmHg and 42.1 ± 4.1 mmHg for gefitinib 30 and 10 mg/kg, respectively). Erlotinib and lapatinib also significantly reduced RVSP in comparison with the placebo rats, displaying comparable effects at the same dose (47.7 ± 4.5 mmHg for erlotinib 10 mg/kg versus 49.5 ± 5.1 mmHg for lapatinib 10 mg/kg and 54 ± 5.0 mmHg for erlotinib 5 mg/kg versus 52.7 ± 6.8 mmHg for lapatinib 5 mg/kg) (Figure 8). A tendency towards decrease of the systemic arterial pressure was noticed 35 days after the injection of monocrotaline, although it was not significant (Figure 9).

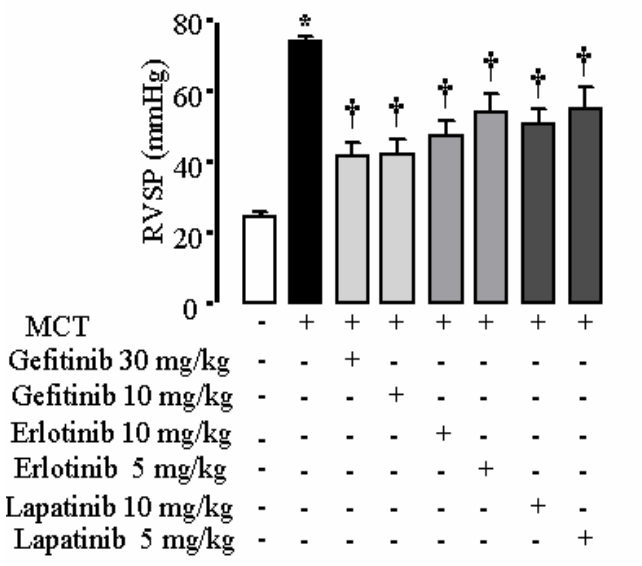


Figure 8. Effects of EGFR inhibitors (gefitinib, erlotinib and lapatinib) on right ventricular systolic pressure in MCT-injected rats. Rats were randomly divided in two treatment groups for each inhibitor and drugs were administered daily, by oral gavage, from day 21 to 35 after the injection of MCT. Two doses were used for every inhibitor (30 and 10 mg/kg for gefitinib, 10 and 5 mg/kg for erlotinib and lapatinib, respectively). An invasive technique for measuring hemodynamic parameters was used for assessing right ventricular systolic pressure (RVSP) (n=10-15). Each bar represents mean ± SEM. * $P<0.05$ vs control; † $P<0.05$ vs. MCT at day 35.

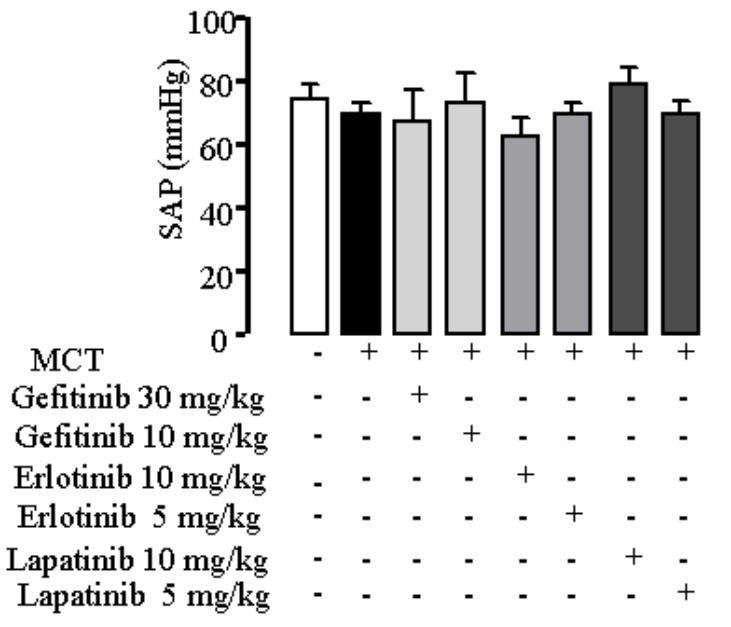


Figure 9. Effects of EGFR inhibitors (gefitinib, erlotinib and lapatinib) on systemic arterial pressure in MCT-injected rats. Rats injected with MCT and treated with EGFR inhibitors gefitinib (30 and 10 mg/kg), erlotinib (10 and 5 mg/kg) and lapatinib (10 and 5 mg/kg) from day 21 to 35 after monocrotaline injection were subjected to invasive techiques for the measurement of hemodynamic parameters and systemic arterial pressure (SAP) was assessed (n=10-15). Each bar represents mean ± SEM. * P<0.05 vs control; † P<0.05 vs. MCT at day 35.

No significant changes in the cardiac index (CI) were noted in the monocrotaline group (26.9 ± 2.9 ml/min*100 g BW in MCT 35 days animals compared to 30.9 ± 1.9 ml/min*100 g BW in healthy rats). Furthermore, no significant changes could be noticed in any of the experimental groups (Figure 10).

The assessment of the oxygenation level showed that thirty five days after MCT administration, arterial oxygenation was significantly decreased (351 ± 38.3 mmHg, in comparison with 506 ± 14.6 mmHg healthy rats) (Figure 11). However, the values of arterial oxygenation in all treated groups were comparable to those observed in placebo rats, showing no significant difference. The doses of the EGFR inhibitors used were selected based on previous publications and on our pilot experiments addressing long-term tolerability (data not shown).

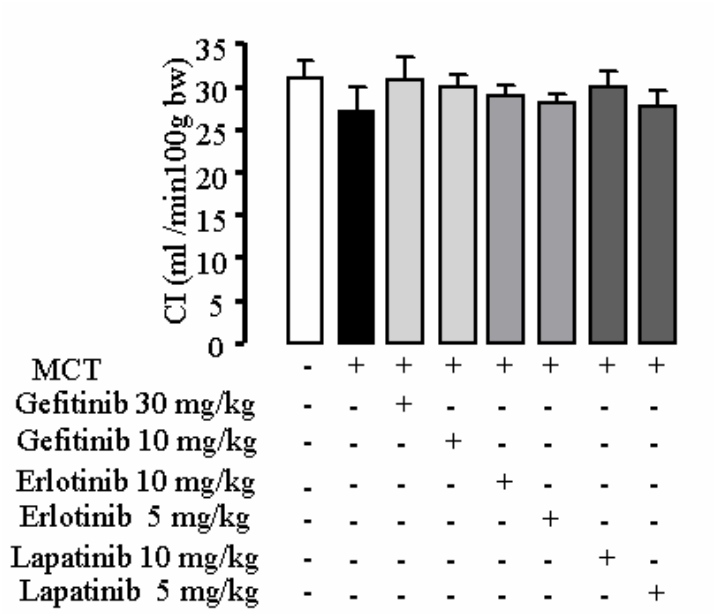


Figure 10. Effect of EGFR inhibition on cardiac index in rats with MCT-induced pulmonary hypertension. Treatment with two doses for each inhibitor tested (30 and 10 mg/kg for gefitinib, 10 and 5 mg/kg for erlotinib and lapatinib, respectively) was administered daily, by oral gavage, from day 21 to 35. Cardiac index was assessed based on Fick's formula and hemodynamic parameters measured during the surgery (n=10-15). Each bar represents mean ± SEM. * P<0.05 vs control; † P<0.05 vs. MCT at day 35.

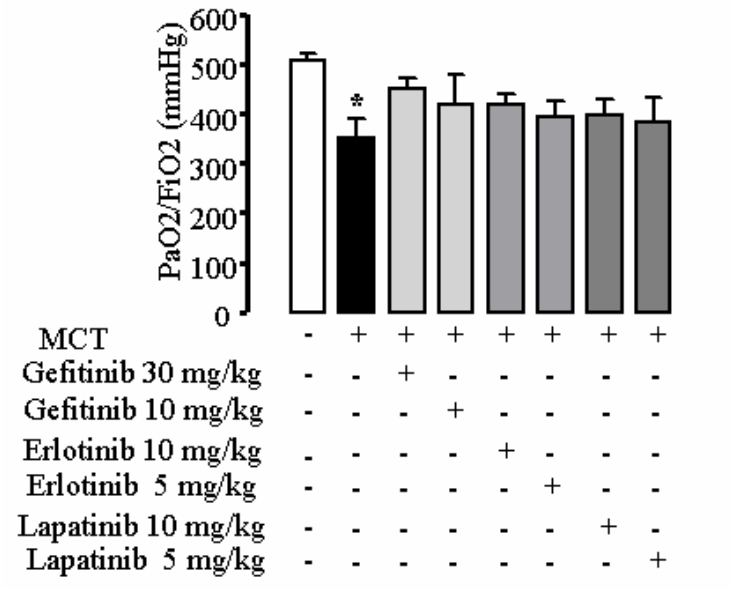


Figure 11. Effect of EGFR inhibition on gas exchange in rats with MCT-induced pulmonary hypertension. Arterial blood samples prelevated during the surgery after right heart catheterization were immediately analyzed and arterial oxygenation index (PaO2/FiO2) (in mmHg) was determined (n=10-15). Each bar represents mean ± SEM. * P<0.05 vs control; † P<0.05 vs. MCT at day 35.

The total pulmonary vascular resistance index (TPR) was significantly increased in MCT-injected rats receiving placebo (2.2 ± 0.1 versus 0.7 ± 0.1 mmHg min/ml 100g BW in healthy rats). Corroborating the RVSP data there was significant decrease in TPR of gefitinib (1.4 ± 0.1 and 1.3 ± 0.1 mmHg min/ml 100g BW respectively for high and low dose) and high dose erlotinib groups (1.5 ± 0.1 mmHg min/ml 100g BW) (Figure 12). However, low dose erlotinib and high as well as low dose lapatinib treatment did not result in a significant reduction of TPR (1.7 ± 0.2, 1.7 ± 0.1 and 1.8 ± 0.1 mmHg min/ml 100g BW respectively).

Also, no significant change among the experimental groups was assessed regarding the systemic vascular resistance index (TSR) (Figure 13).

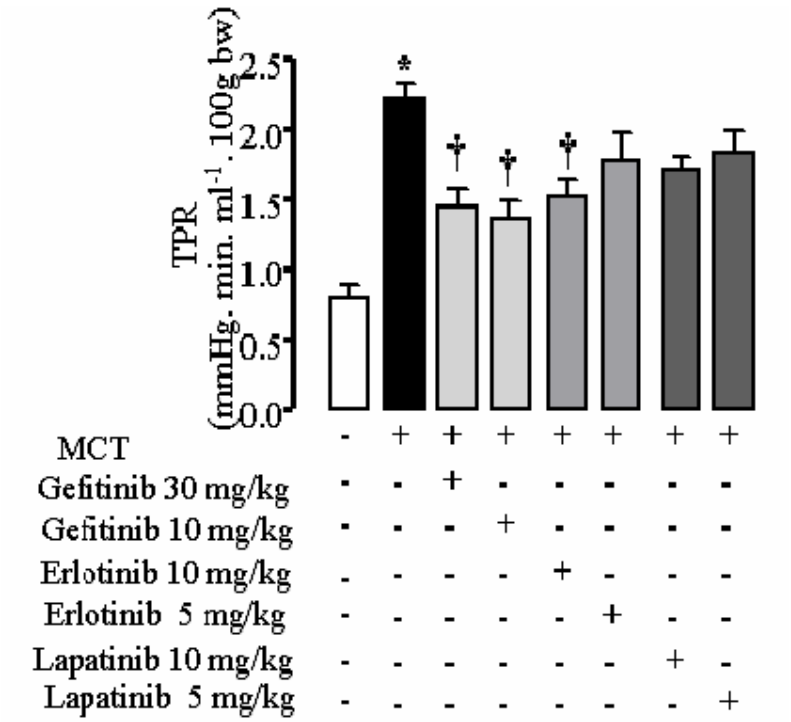


Figure 12. Effect of EGFR inhibition on total pulmonary vascular resistance index in rats with MCT-induced pulmonary hypertension. Rats were treated from day 21 to 35 after MCT injection with EGFR inhibitors. Gefitinib (10 and 30 mg/kg bodyweight), erlotinib (5 and 10 mg/kg) and lapatinib (5 and 10 mg/kg) were administered daily by gavage and hemodynamic measurement was performed at the end of treatment. An equal volume of the vehicle methylcellulose was given to the placebo group. Total pulmonary vascular resistance index (TPR) is given (n=10-15). Each bar represents mean ± SEM.
* P<0.05 vs control; † P<0.05 vs. MCT at day 35.

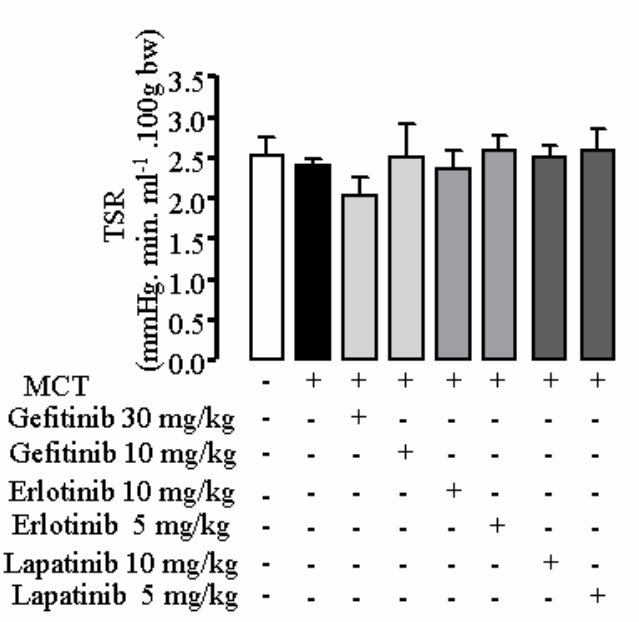


Figure 13. Effect of EGFR inhibition on total systemic resistance index in rats with MCT-induced pulmonary hypertension. Rats were treated with gefitinib (10 and 30 mg/kg bodyweight), erlotinib (5 and 10 mg/kg) and lapatinib (5 and 10 mg/kg) from day 21 to 35 after MCT injection, followed by invasive hemodynamic measurements. Total systemic resistance index (TSR) was evaluated based on hemodynamic parameters determined during the surgery (n=10-15). Each bar represents mean ± SEM.
* P<0.05 vs control; † P<0.05 vs. MCT at day 35.

III.2. Effects of EGFR inhibitors on right heart hypertrophy in monocrotaline rats

The increase in RVSP observed after the administration of MCT was paralleled by a progressive development of right heart hypertrophy assessed by the RV/(LV+S) ratio. Right heart hypertrophy was significantly increased 21 days (0.32 ± 0.02) and 35 days after the administration of MCT (0.6 ± 0.03 versus 0.25 ± 0.01 in healthy rats) (Figure 14, Table 5). The RV/BW ratio (in mg/g) showed the same progressive trend following MCT administration (0.12 ± 0.06 in healthy, 0.18 ± 0.01 in MCT 21 days and 0.3 ± 0.02 in MCT 35 days rats, respectively) (Tables 4, 5). Chronic treatment with gefitinib, erlotinib or lapatinib reduced right heart hypertrophy in a dose-dependent way, gefitinib at both doses demonstrating the most potent effect in this respect (0.37 ± 0.04 and 0.38 ± 0.02 in the case of gefitinib 30 and 10 mg/kg, respectively). A significant effect could be assessed only in the case of the group treated with the

highest dose of erlotinib (0.43 ± 0.01) and no significant reduction of heart ratio could be seen after using lapatinib at both dose regimens (0.5 ± 0.07 and 0.52 ± 0.06 for lapatinib 10 and 5 mg/kg respectively) or erlotinib at the lowest dose (0.48 ± 0.04). The RV/BW ratio also demonstrated a significant reduction of right heart hypertrophy in all treated groups and a tendency towards dose-dependency was noticed (Table 4).

On the other hand, the progressive development of right heart hypertrophy during the course of the disease was not paralleled by a similar increase of (LV+S)/BW ratio (considered as a marker of left heart hypertrophy) in any of the experimental groups we investigated (Table 4).

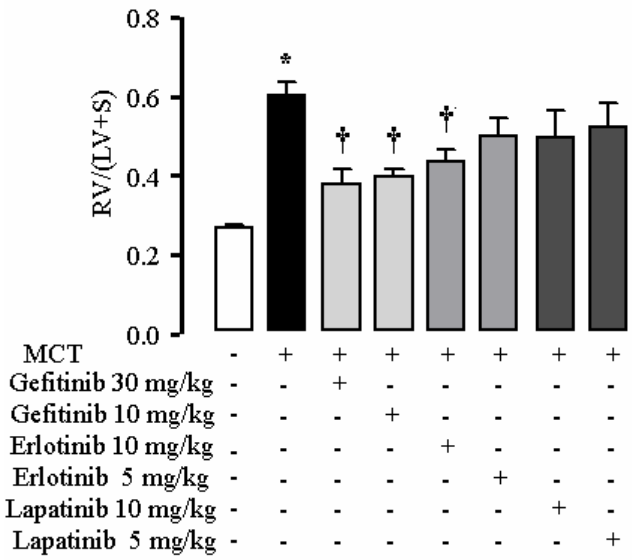


Figure 14. Effect of EGFR inhibition on right heart hypertrophy in rats with MCT-induced pulmonary hypertension. Animals were exposed to MCT and then treated daily, by oral gavage, from day 21 to 35, with two doses for each inhibitor (30 and 10 mg/kg for gefitinib, 10 and 5 mg/kg for erlotinib and lapatinib, respectively). Right to left ventricular plus septum weight ratio (RV/LV+S) of the treated versus placebo groups are given (n=10). Each bar represents mean ± SEM. * P<0.05 vs control; † P<0.05 vs. MCT at day 35.

Table 4. RV/BW and (LV+S)/BW ratios in healthy, MCT 35 days and treated rats (n=10-15)

	RV / BW (mg/g)	**(LV+S) / BW (mg/g)**
Healthy	0.12 ± 0.06	0.47 ± 0.01
MCT 35 days	0.3 ± 0.02 **(*)**	0.51 ± 0.02
Gefitinib 30 mg/kg	0.18 ± 0.01 (†)	0.49 ± 0.01
Gefitinib 10 mg/kg	0.22 ± 0.02 (†)	0.56 ± 0.04
Erlotinib 10 mg/kg	0.2 ± 0.01 (†)	0.49 ± 0.02
Erlotinib 5 mg/kg	0.23 ± 0.02 (†)	0.5 ± 0.02
Lapatinib 10 mg/kg	0.22 ± 0.03 (†)	0.46 ± 0.01
Lapatinib 5 mg/kg	0.22 ± 0.02 (†)	0.44 ± 0.01

***** P<0.05 versus healthy control; † P<0.05 versus MCT 35 days

III.3. Effects of EGFR inhibitors on pulmonary vascular remodeling in monocrotaline rats

We investigated medial wall thickness and the degree of muscularization of small peripheral pulmonary arteries (25 to 50 μm in diameter) as markers of pulmonary vascular remodeling. Injection of MCT resulted in an enhanced pulmonary artery muscularization as evident from the enhanced immunoreactivity for α-SMC actin (Figure 15). Morphometric analysis revealed significantly increased fully muscularized vessels at day 21 (Table 5) and further at day 35 (59 ± 6.1 %) as compared to healthy control animals (2.8 ± 1.1 %) (Figure 16). Similarly, chronic treatment with high and low dose gefitinib and high dose erlotinib significantly reduced the number of fully muscularized vessels (10 ± 3.4 %, 22.5 ± 8.6 % and 17.1 ± 4.2 %, respectively) as compared to the placebo group, whereas the reduction was not significant in the low dose erlotinib (44.4 ± 8.6 %) and both dose groups of lapatinib (38 ± 7.6 % and 35.5 ± 10.0 %) (Figure 16). The increase in fully muscularized vessels was accompanied by a significant decrease of non-muscularized vessels in the placebo group (22.3 ± 5.1 % at day 35) compared to the healthy control (67.7 ± 5.3 %). The non-muscularized vessels in rats treated with high and low dose gefitinib (67.9 ± 6.5 % and 56.6 ± 10.3%) and high dose erlotinib (62.6 ± 8.2 %) were comparable to healthy control. However, a reduced number of non-muscularized vessels was noted in both doses of lapatinib (36.8 ± 9.1 % and 37.8 ± 7.3 %) and low dose erlotinib (30.8 ± 9.9 %) treated groups and it was comparable to that of the placebo group (Figure 16).

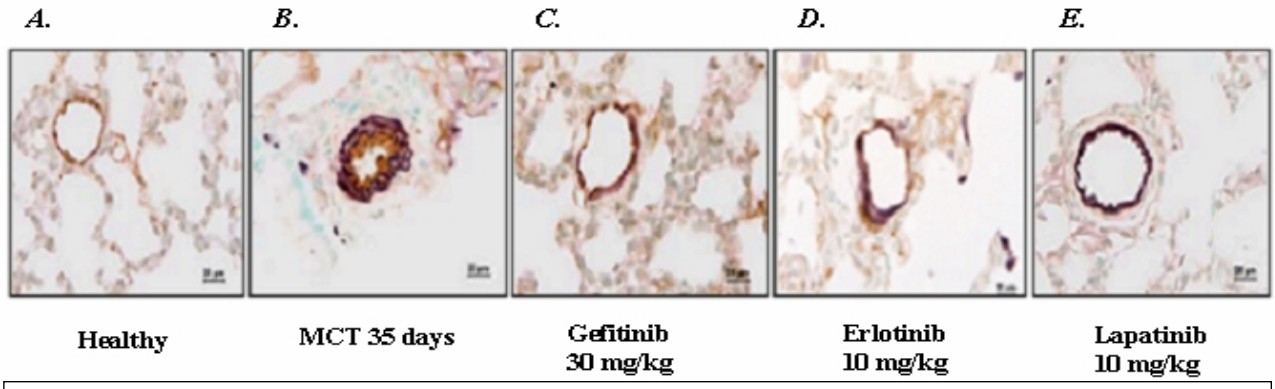


Figure 15. Effect of treatment with EGFR inhibitors on the degree of muscularization of small pulmonary arteries of MCT-injected rats. The rat lung sections were immunostained for von Willebrand factor (*brown*) and α-smooth muscle actin (*purple*). Representative pictures of experimental groups are shown: (A) healthy control, (B) placebo, (C) gefitinib, (D) erlotinib, (E) lapatinib. Pulmonary vascular morphometry was performed on the double immunostained lung sections. A total of 80 to 100 intraacinar vessels were analyzed in each lung. Scale bar: 20 μm.

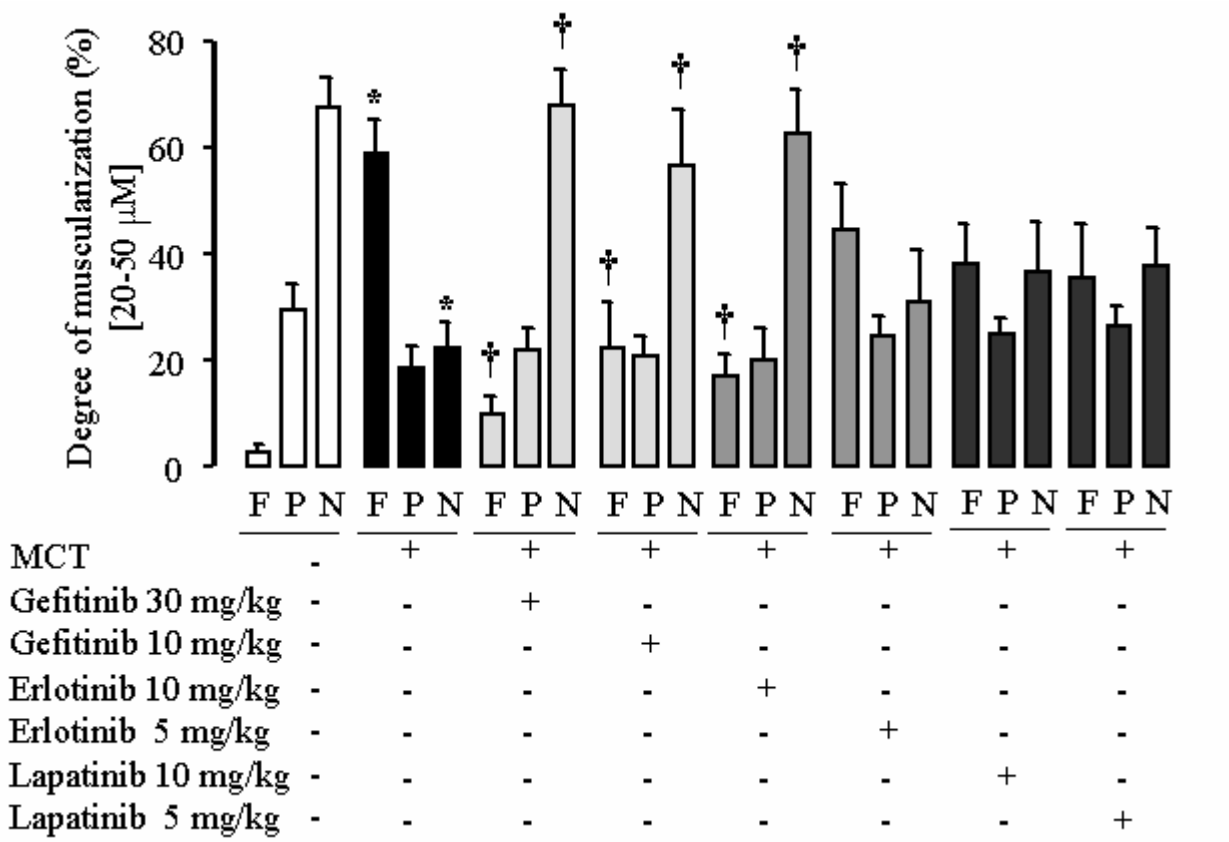


Figure 16. Effect of treatment with gefitinib, erlotinib and lapatinib on the degree of muscularization of small pulmonary arteries of MCT-injected rats. The rat lung sections were immunostained for von Willebrand factor (*brown*) and α-smooth muscle actin (*purple*). Pulmonary vascular morphometry was performed on the double immunostained lung sections. A total of 80 to 100 intraacinar vessels were analyzed in each lung. The degree of muscularization was assessed as percentage of muscle area from the total pulmonary artery cross section (25-50 μm). The proportion of nonmuscularized (N), partially muscularized (P) or fully muscularized pulmonary arteries (F) is given. Results from MCT-injected rats as well as treated groups and healthy control rats are presented. Rats were treated as described in methods (n=8-12). Each bar represents mean ± SEM.

Analysis of the medial wall thickness revealed a significant increase at day 35 (21.1 ± 0.3 %) as compared to healthy control (13.0 ± 0.1 %) (Figures 17, 18). Chronic treatment with the EGFR inhibitors resulted in a significantly reduced medial wall thickness in all investigated groups. Gefitinib (15.1 ± 0.1 and 16.4 ± 0.2 %) and high dose erlotinib (15.9 ± 0.2 %) exhibited

the strongest effect, reducing this parameter to values comparable to MCT 21 days rats (Table 5), whereas the effect was milder in the groups treated with low dose erlotinib (18.0 ± 0.3 %) and lapatinib at both doses (17.8 ± 0.2 and 19.2 ± 0.3 %) (Figure 18).

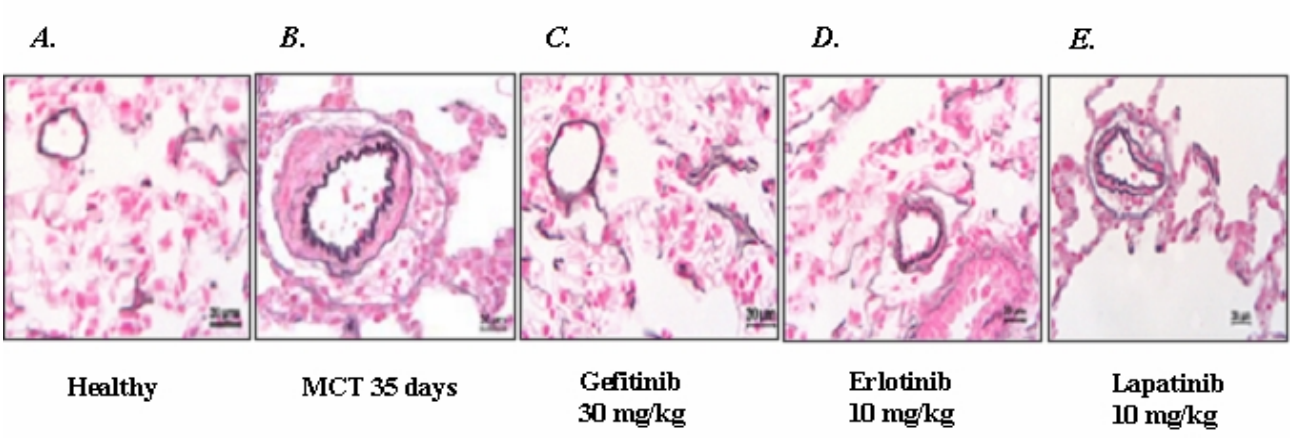


Figure 17. Effect of treatment with EGFR inhibitors on medial wall thickness of small pulmonary arteries of MCT-injected rats. Treatment with gefitinib, erlotinib and lapatinib was administered orally, daily, and two doses were used for every tested drug. The rat lung sections were stained with elastica staining for wall thickness determination. Representative pictures of experimental groups are shown: (A) healthy control, (B) placebo, (C) gefitinib, (D) erlotinib, (E) lapatinib. Scale bar: 20 μm.

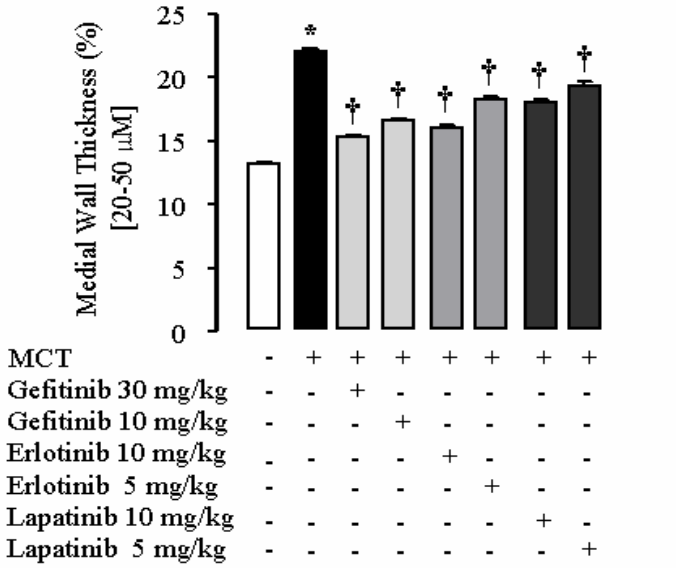


Figure 18. Effect of treatment with gefitinib, erlotinib and lapatinib on medial wall thickness of small pulmonary arteries of MCT-injected rats. Treatment with gefitinib, erlotinib and lapatinib was administered orally, daily, and two doses were used for every tested drug. The rat lung sections were further stained with elastica staining for wall thickness determination. A total of 80 to 100 intraacinar vessels were analyzed in each lung. The medial wall thickness (%) is shown. Results from MCT-injected rats as well as treated groups and healthy control rats are presented (n=8-12). Each bar represents mean ± SEM.
* P<0.05 vs control; † P<0.05 vs. MCT at day 35.

Table 5. RVSP, SAP, RV/(LV+S), RV/BW, (LV+S)/BW, degree of muscularization and medial wall thickness of healthy and monocrotaline-injected rats (day 21) (n=10).

		Healthy	MCT 21 days
RVSP (mmHg)		24.5 ± 1.3	38.4 ±3.4 (*)
SAP (mmHg)		74.3 ± 4.8	66.4 ± 3.9
RV/(LV+S)		0.26 ± 0.01	0.32 ± 0.02 (*)
RV/BW (mg/g)		0.12 ± 0.01	0.18 ± 0.01
(LV+S)/BW (mg/g)		0.47 ± 0.01	0.53 ± 0.04
Degree of muscularization (%)	**Non**	67.7 ± 5.3	39.3 ± 8.4 (*)
	Partial	29.6 ± 4.8	37.1 ± 4.5
	Full	2.8 ± 1.1	23.5 ± 7.0 (*)
Medial wall thickness (%)		13.0 ± 0.14	16.4 ± 0.17 (*)

* $p < 0.05$ versus healthy control

III.4. Effects of EGFR inhibitors on proliferation levels in monocrotaline rats

Following the injection of MCT, we observed an enhanced immunoreactivity for PCNA due to an increased number of proliferating cells within the walls of small pulmonary arteries (Figure 20). The index of in situ proliferation was significantly higher 21 days after the administration of MCT (more than 6 fold versus healthy controls) and further increased until 35 days (more than 8 times than in healthy rats) (Figure 19). Treatment with all EGFR antagonists reduced the index of proliferation to lower levels than those seen in the MCT 21 days rats. Although not statistically significant, the efficacy of all three used drugs was similar when the highest dose was employed for each of them, with gefitinib demonstrating a slightly higher efficacy in inhibiting this parameter (Figure 19).

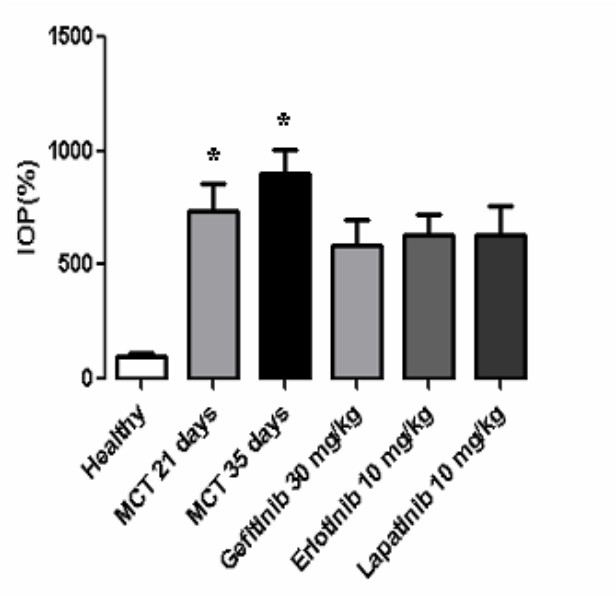


Figure 19. Effect of treatment with gefitinib, erlotinib and lapatinib on proliferation levels within small pulmonary arteries walls. The rat lung sections were stained for proliferating cell nuclear antigen (PCNA) (*red-brown*). Positive and negative cells from 40 to 80 vessels in each lung were counted and the index of in situ proliferation (A) was calculated as percentage of PCNA-positive cells in the vessel from all cells in the vessel. Results from MCT-injected rats as well as treated groups and healthy control rats are presented. Rats were treated as described in methods (n=5). Each bar represents mean ± SEM.
* P<0.05 vs control; † P<0.05 vs. MCT at day 35.

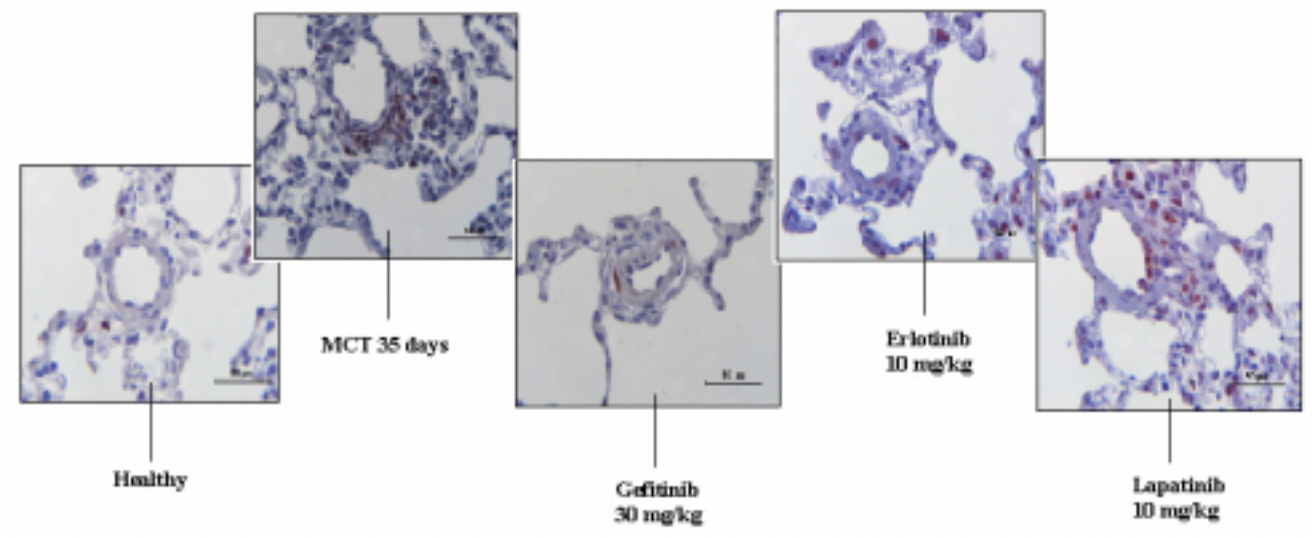


Figure 20. Effect of treatment with EGFR inhibitors on proliferation within small pulmonary arteries walls. Treatment with gefitinib, erlotinib and lapatinib was administered orally, daily, and two doses were used for every tested drug. The rat lung sections were stained for proliferating cells. Representative pictures of experimental groups are shown: (A) healthy control, (B) placebo, (C) gefitinib, (D) erlotinib, (E) lapatinib. Scale bar: 50 μm.

III.5. Effects of EGFR inhibitors on body weight changes and distress score in monocrotaline rats

As compared to healthy animals, placebo rats lost weight during the 2-weeks surveillance period (Figure 21). In contrast, rats treated with gefitinib and erlotinib at both doses had increased bodyweights at day 35 as compared to the weights measured at the beginning of the therapy. Gefitinib demonstrated a similar effect at both doses (more than 2% increase) whereas erlotinib at the lowest dose had a better effect (2.46%) than the higher dose (0.86%). In the lapatinib treated rats we observed that at the end of the 35 days surveillance period animals had no increase in their body weight in comparison with the moment when therapy was initiated and lapatinib rats treated with the highest dose had a worse outcome (0.34% weight loss). Also, an abrupt descending trend was noticed in both lapatinib treated groups during the first week of treatment, followed by a gradual recovery during the second week of treatment.

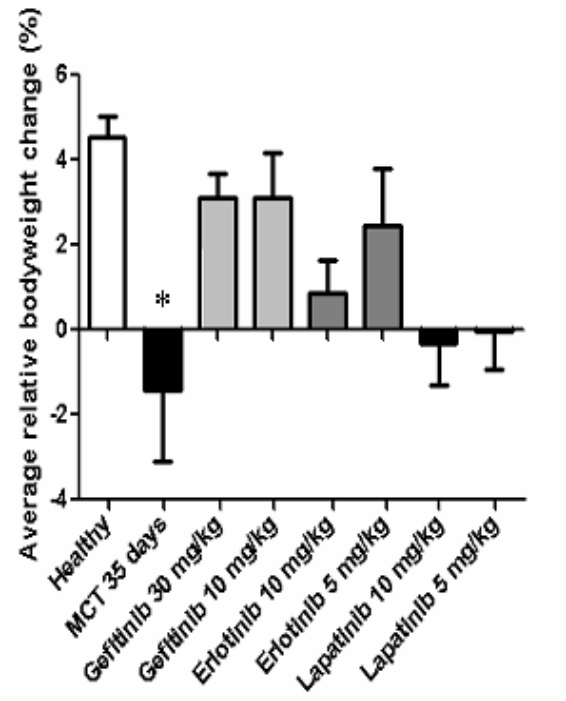


Figure 21. Effect of treatment with gefitinib, erlotinib and lapatinib on body weight. Healthy, placebo as well as treated rats were monitored and body weight changes were recorded daily from the onset of treatment to the end (n=10). Average relative bodyweight change during the surveillance period was calculated as percentage in weight at day 35 compared with the weight at the beginning of treatment.
Each bar represents mean ± SEM. * P<0.05 vs control; † P<0.05 vs. MCT at day 35.

Furthermore, several physical parameters as general appearance, respiratory pattern, natural and provoked behaviour were used to calculate a distress score. Placebo challenged rats had a worsening health state with obvious changes in the respiratory pattern (from increased respiratory rate to marked abdominal breathing with cyanosis), the general appearance (dehydration, yellowing coat, nasal discharge) and natural behaviour (isolation, lack of alertness), which resulted in a high distress score at the end of day 35 (Figure 22). Gefitinib was well tolerated as shown by the low distress score in this group, comparable to that seen in healthy rats, and by the complete lack of development of pathological respiratory signs. Similarly, erlotinib treated rats had a low distress score at the end of day 35 (comparable to that assessed in the gefitinib group) but overt signs of respiratory disease were observed during the treatment period (dyspnea or abdominal breathing). Lapatinib-treated rats, on the other hand, showed no significant improvement of their condition during treatment, presenting frequent pathological changes of the respiratory pattern, side effects like facial edema and diarrhea as well as a high distress score at the end of day 35.

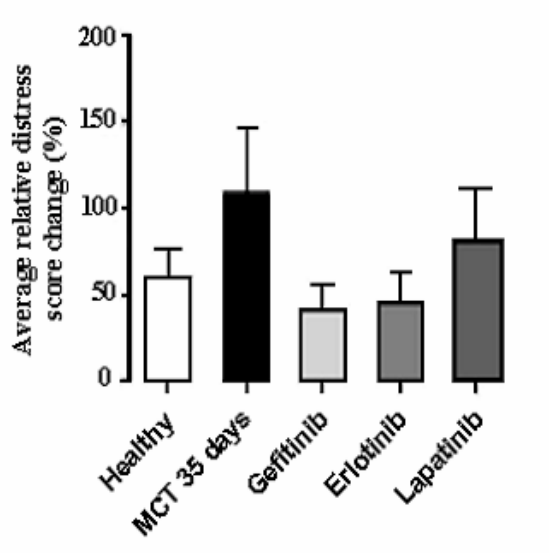


Figure 22. Effect of treatment with gefitinib, erlotinib and lapatinib on the overall state of health. Control and treated rats were monitored daily and a distress score assessing a set of physical parameters, including respiratory changes, was calculated (n=10). Each bar represents mean ± SEM. * P<0.05 vs control; † P<0.05 vs. MCT at day 35.

Gefitinib also proved to be the best tolerated drug, inducing no mortality in the treated rats, as compared to erlotinib, which was associated with a mortality of 8% during treatment and the placebo-challenged MCT injected rats (20%) (Figure 23). Lapatinib treated rats, on the other hand, had the highest mortality rate among the treatment groups, with 87% survival at the end of day 35.

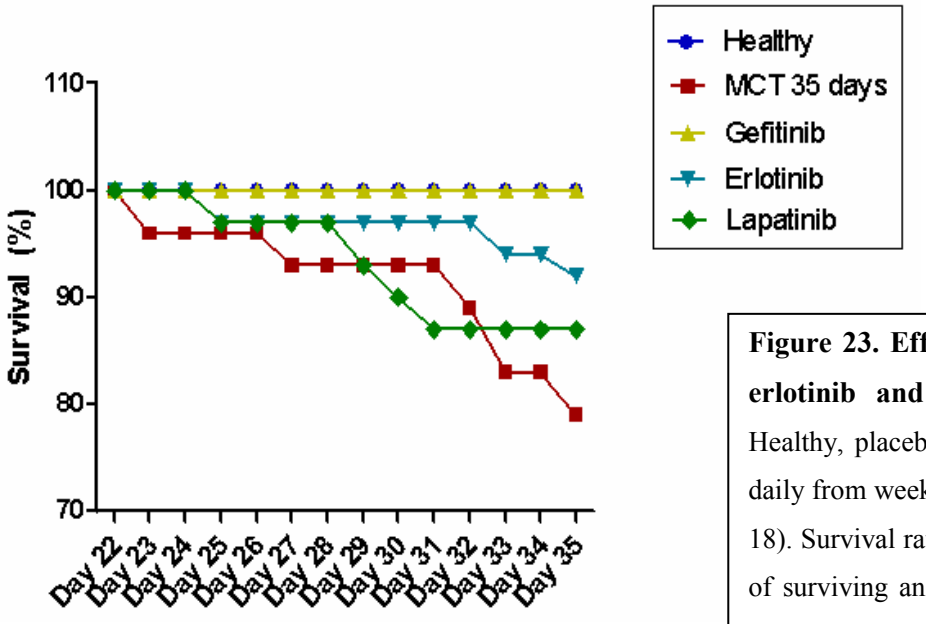


Figure 23. Effect of treatment with gefitinib, erlotinib and lapatinib on mortality rate. Healthy, placebo and treated rats were monitored daily from week 3 to 5 after MCT injection (n=10-18). Survival rate was calculated as the percentage of surviving animals relative to the alive animals on day 21, the first day of treatment. Survival rates of all experimental groups are shown. Each point on the graph represents the day after monocrotaline injection.

IV. DISCUSSION

The pathological mechanisms of PAH are now known to be closely correlated with the involvement of several factors having vasoactive or mitogenic properties, among which the family of growth factors seems to play a significant role. Various growth factors like PDGF[74;158] or VEGF[26;154] have lately emerged as important players in the development of the disease and others like EGF[107], insulin-like growth factor-1 (IGF-1)[5;38] or basic fibroblast growth factor (bFGF)[193] also seem to be involved in the development of the remodeling process taking place in the hypertensive lung. They act as potent mitogens and chemoattractants for smooth muscle cells, fibroblasts and endothelial cells, which results in increased cell proliferation, resistance to apoptosis or migration of pulmonary vascular cells and finally in the establishment of overt pulmonary disease. The progress made in the understanding of PAH pathogenesis has also opened new paths for the therapeutic approach of this condition and novel compounds like endothelin receptor antagonists, nonparenteral prostanoids, phosphodiesterase 5 inhibitors, activators of soluble guanilate cyclase or tyrosine kinase inhibitors have been suggested to be promising new agents in the PAH field[127]. However, search for novel targets and more effective anti-remodeling therapy have remained highly desirable.

Recently, several original publications have demonstrated that Imatinib, by inhibiting PDGF signaling, can reverse experimental PAH in two independent models[158]. These preclinical results have later been translated into the clinic and the first case reports of patients with PAH treated with Imatinib have demonstrated improved hemodynamics and 6-minute walk distance[57;131;168]. Imatinib was designed to target the ATP-binding site of RTKs and has been approved for therapy against chronic myelogenous leukemia and malignant gastrointestinal stromal tumors, thus providing an example of transferred knowledge from cancer treatment. Furthermore, recent studies have demonstrated that PKI166, an EGFR inhibitor, could mediate VSMC apoptosis and improve the survival of MCT-injected rats[107].

IV. 1. EGF signaling in pulmonary hypertension

Recent experiments performed on animal models of PAH (mice overexpressing TGFα and MCT-treated rats) have demonstrated the involvement of the EGF pathway in the pathogenesis of experimental PAH. Le Cras et al. [97] were able to induce the hemodynamic and histologic characteristics of severe pulmonary vascular disease by overexpressing TGFα in mice.

The induced expression of a mutated form of EGFR in the same type of transgenic mice prevented the development of the disease and improved the main parameters that are pathologically affected during the course of the disease.

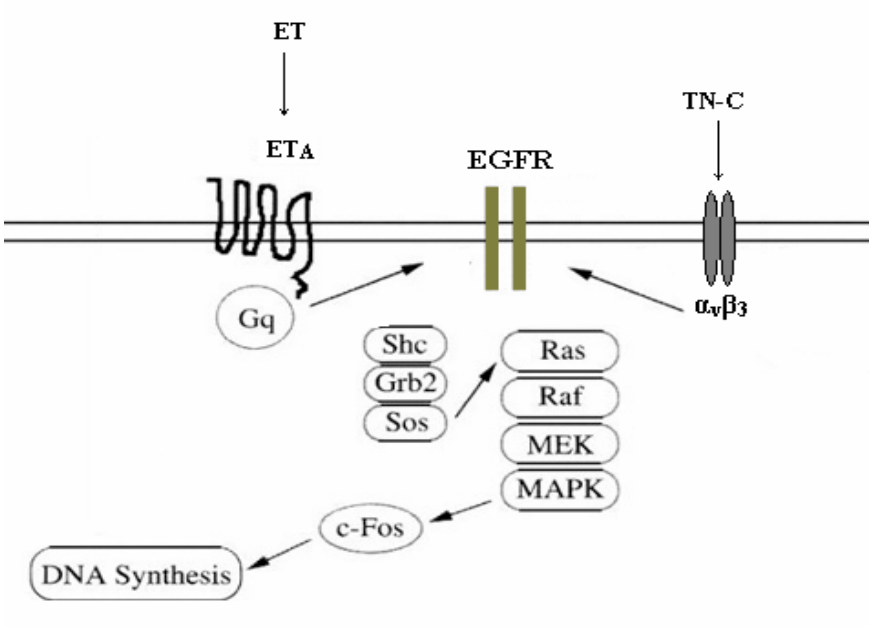
The hypothesis of the involvement of EGFR signaling pathway in the pathogenesis of pulmonary arterial hypertension through the stimulation of pulmonary vascular SMC was supported by experiments performed in rats with baloon-injured carotid artery. In these rats the application of a blocking EGFR antibody before and after the injury led to the attenuation of intimal hyperplasia and to the decrease of SMC proliferation in arterial vessels[24]. Also, *in vitro* experiments performed on cultured vascular SMCs prelevated from pulmonary hypertensive fawn-hooded rats or human pulmonary arteries demonstrated an increased proliferation in response to the EGF exposure[79;85;160]. The hypoxic stimulus has an additive effect, by inducing increases in EGFR expression[7]. It also enhances the mitogenic effects of growth factors like bFGF, EGF and PDGF through the induction of nuclear HIF-1α expression and the stimulation of the DNA synthesis in PASMC[101;160].

In human PAH, however, direct evidences are scarce. The involvement of the EGF pathway in the development of the disease was mainly demonstrated by the histologic assessment of increased expression of EGF in the medial, adventitial and neointimal layers of pulmonary arteries, which accompanied the progression of the pulmonary vascular disease in children[83]. Recently, however, experiments performed by our group, investigating the pulmonary expression of HERs and their ligands in patients with IPAH[37] have revealed no significant alteration of the ligands (betacellulin, amphiregulin and HB-EGF) or of their receptors (HER 1-4) in the lung tissue from these patients. Also, no significant up-regulation of the HER1 protein was observed, suggesting altogether that the EGF signaling pathway may not be an indispensable player at least in the late stage of the disease progression. However, as the lung tissues obtained from patients undergoing lung transplantation may have end-stage pathological alterations, a possibility for EGF signaling contributing to the early disease progression could not be entirely ruled out.

Several factors have been shown to interact with the EGF pathway and converge towards VSMC and fibroblast proliferation, endothelial thickening and ultimately PAH development. The EGF-induced proliferation and migration of SMCs is critically dependent on tenascin-C

(TN-C), as has been shown by the necessity of exogenous TN-C addition on type I collagen gels when EGF-dependent SMC proliferation was attempted. In contrast, other growth factors-induced SMC proliferation (e.g. bFGF) takes place even in the absence of TN-C[85]. TN-C is a component of the extracellular matrix involved in tissue remodeling[85], which promotes the accumulation of EGF receptors in clusters and the redistribution of the actin cytoskeleton at the cell periphery[84]. The interaction with the EGFR signaling pathway is due to its binding to the $\alpha_v\beta_3$ integrins, which form with growth factor receptor signaling components a specialized focal adhesion complex on the actin-based cytoskeletal scaffold[108;136]. Thus, the promotion of the clustering and phoshorylation of receptor tyrosine kinases including the ones of EGF receptors takes place (Figure 27). The interaction between the $\alpha_v\beta_3$ integrins and TN-C is also essential for the EGF-dependent SMC proliferation, as demonstrated by the inhibitory effect of an anti-$\alpha_v\beta_3$ integrin monoclonal antibody on SMC growth in response to EGF[84].

Figure 24. Proposed mechanism of interaction between tenascin-C (TN-C), endothelin (ET) and the EGF pathway (adapted after Iwasaki H. et al., 1998[78]) (Gq=phospholipase C- coupled Gq protein, ET-A= endothelin receptor type A). The black arrows symbolize the induction of intracellular signaling pathways.



The induction of TN-C transcription in pulmonary hypertension has been previously linked to the increased activity of elastase[102;200], which can induce the activation of matrix metalloproteinases. Consequently, the degradation of the extracellular matrix, the release of growth factors in active form and the transcription of TN-C take place, which can trigger the activation of the EGF pathway[107]. Moreover, angiotensin II, an important mediator of VSMCs growth, has been shown to induce, via reactive oxygen species (ROS) like H_2O_2, the phosphorylation of the EGF receptor[191]. Furthermore, the transactivation of EGFR has been connected to the process of thrombin and endothelin signaling leading to SMC migration[78;88] (Figure 27).

IV.2. EGF receptor inhibition in experimental PAH

Results obtained in Sprague-Dawley rats with MCT-induced pulmonary vascular disease confirmed the participation of the EGF pathway in the pathogenesis of PH through the prevention of the development of the disease with EGFR-tyrosine kinase inhibitors. One of the inhibitors used- AG1478, a thyrophostin described in 1989 as a potent and selective EGFR-TK inhibitor, was able to induce, via prevention of EGFR phosphorylation, growth arrest and apoptosis in PASMC cell culture and organ cultures of hypertrophied rat pulmonary arteries[107;116]. Also, PKI166, another EGFR inhibitor, could reduce PAP, right heart hypertrophy, medial wall thickness and the degree of muscularization of small pulmonary arteries. Also, it increased the survival rate of MCT challenged rats when administered daily or three times a week and the maintainance of positive results could be assessed even 4 weeks after the cessation of therapy[107]. However, despite the remarkable potency exhibited in animal models and the fact that it is a dual EGFR/HER2 inhibitor, PKI166 failed to pass the early phases of clinical trials[141], which presently renders it not a viable therapeutic alternative in human PAH.

On the other hand, clinically approved EGFR inhibitors, like gefitinib (Iressa®), erlotinib (Tarceva®) and lapatinib (Tykerb®), which have already demonstrated their efficacy in human patients with NSCLC[30;32], pancreatic[45] and breast cancer[152] are available. Moreover, unlike other RTK antagonists (Sunitinib) or monoclonal antibodies against HER2 (Trastuzumab), which have been described to possess cardiotoxicity[27;103], the above mentioned

drugs have been recently included in the category of low cardiotoxicity receptor TK inhibitors[49]. Also, these EGFR antagonists are orally active (unlike agents as Trastuzumab, which require intravenous administration and can induce allergic reactions), are associated in most cases with mild adverse effects (mostly cutaneous rash and diarrhea) and do not induce the typical cytotoxic reactions affecting patients treated with chemotherapy[28]. These characteristics make them an attractive alternative to current available therapies. These findings provided us with a good rationale to further investigate the potential therapeutic application of clinically approved EGFR inhibitors in PAH.

Considering the previous evidence suggesting an EGFR signaling-mediated development of PAH as well as the availability of cancer-effective EGFR targeted therapy, the aims of the present study were to 1). evaluate the efficacy of chronic EGF receptor inhibition on the progression of MCT-induced PAH and 2). to investigate and compare the *in vivo* effects of three different clinically available EGF receptor inhibitors, in particular gefitinib, erlotinib and lapatinib, in a head-to-head experiment. We therefore used a well–established animal model of PAH (the MCT model) and focused on the study of hemodynamic and pulmonary vascular remodeling-related parameters as well as on the consequences of the above mentioned EGFR antagonists on the general health status and mortality rate. Our results demonstrate 1). a partial and dose-dependent efficacy of EGFR antagonists in improving the hemodynamic and histologic parameters associated with the disease, with gefitinib having the most prominent effect at both doses used. We also show 2). a superior tolerability profile of gefitinib over the other two tested inhibitors regarding the impact of therapy on the organism as a whole, as reflected by the bodyweight changes, lower distress score and survival advantage.

PAH is a progressive disease caused by the thickening of precapillary arteries, which leads to a gradual increase in pulmonary vascular resistance, pulmonary arterial pressure, right ventricle afterload and finally right heart failure[74]. In rats a single injection of the pyrazollidine alkaloid MCT induces, after 21 days, a significant increase of RVSP, right heart hypertrophy and degree of muscularization[143;147], which further progress within the next weeks. In this context, we investigated the effect of chronic daily EGFR inhibition on the progression of the disease in a well established animal model of PAH.

The results of our experiments demonstrated that daily administration of EGFR TK inhibitors in MCT rats for two weeks starting at day 22 (when the disease is completely

established) led to a reduction of pulmonary arterial pressure in all experimental groups, regardless of the used dosage and showed a tendency towards dose-dependency in the case of all three EGFR inhibitors. Considering the lack of detectable vasodilatory effect of any of the investigated inhibitors, as reflected by SAP and TSR, the significant effect on pulmonary arterial pressure is most likely the consequence of the anti-remodeling effect of the drugs employed in this study on pulmonary arteries. This conclusion is supported by our data showing the significant and potent effect these inhibitors have in reducing the proliferation level within the vessel walls, medial wall thickness and the neomuscularization process, which is considered to be one of the main factor involved in the development of the disease. The reduction of muscularization resulted in a consistent delay in pulmonary vascular resistance (TPR) increase and, as a consequence, in pulmonary arterial pressure, which was prominent in the case of gefitinib at both doses and in the high-dose erlotinib group. Our results are not entirely surprising considering the already proven effects gefitinib, erlotinib and lapatinib have on cellular proliferation, as they are drugs with antiproliferative, apoptosis-inducive effects in different cancer cell lines and mice with xenografted human tumors[72;151;165]. In addition, the cardiac index was comparable among the experimental groups, suggesting that the cardiac output was not affected by the treatment and that the EGFR inhibitors do not have cardiac-specific toxicity at least at the doses and duration tested in our study. This is in line with the recent report categorizing these EGFR inhibitors as receptor tyrosine kinase inhibitors with low cardiotoxicity[49]. Furthermore, right heart hypertrophy, which is a constant finding during the course of pulmonary hypertension both in the MCT model and human patients[25;173] was significantly reduced in comparison with placebo-treated rats, as demonstrated by parameters like RV/(LV+S) ratio and right ventricle to bodyweight ratio (RV/BW). Both of these parameters have been shown to progressively increase after MCT administration[82;158]. Our data confirm the previously mentioned results published by Merklinger et al.[107] showing the potent *in vivo* effect of daily administration of the antiproliferative and selective EGFR/HER2 inhibitor PKI166. Used under similar conditions to those employed in our study, PKI166 was able to improve the hemodynamic and histologic parameters associated with pulmonary hypertension, including right heart hypertrophy.

Treatment of animals with MCT leads not only to the development of pulmonary hypertension, but also to a progressive deterioration of the overall physical health status, as assessed by the significant decrease of body weight[82] and a high mortality rate[35;184]. Also, pathological changes in the general appearance, behaviour[46] and respiratory pattern[17;147] have been described during the course of the disease. Furthermore, human patients with severe heart failure often present with significant weight loss leading to cachexia, due to anorexia, nausea and vomiting, to the impairment of intestinal absorption or to the increased work performed by the respiratory muscles[65]. Furthermore, cardiac failure is often associated with a poorer quality of life, closely correlated with the NYHA functional class[70;144] and with a high mortality rate, especially in the first months after diagnosis[36]. In this context, we were further interested to study the effect of these three EGFR inhibitors not only in relation with the pulmonary vascular changes but also on the overall health status of MCT injected rats, with emphasis on bodyweight changes and the distress level.

The majority of the studies investigating the consequence of gefitinib and erlotinib as monotherapy or in combination with other drugs on bodyweight changes failed to show any significant change of the net bodyweight and only a few reported a reduction of animals bodyweight at the end of the treatment[105]. In contrast, we could observe a physical improvement, as shown by the tendency of increase in weight in both gefitinib groups and in the lower dose erlotinib group. On the other hand, administration of erlotinib at the highest dose used as well as lapatinib at both doses had as a consequence an initial decrease of bodyweight, especially in the first week of treatment, which was followed by a gradual recovery. The negative impact of lapatinib on animals bodyweight suggests, in correlation with a high distress score, the high toxicity level of this drug and comes in agreement with studies performed in animal models[44;111]. The decrease in bodyweight was also a frequently described adverse effect in human patients treated for cancer with this drug[114]. Moreover, the presence of respiratory pathological signs and the higher mortality rate in the lapatinib treated animals in comparison with gefitinib and erlotinib treated rats point out a rather negative outcome in this group and support the conclusion that, among the EGFR inhibitors we tested, lapatinib has the worst tolerability profile. Gefitinib, on the other hand, due to the 100% survival rate and distress

score values comparable to healthy levels, was the best tolerated drug when used at the dose of 30 mg/kg.

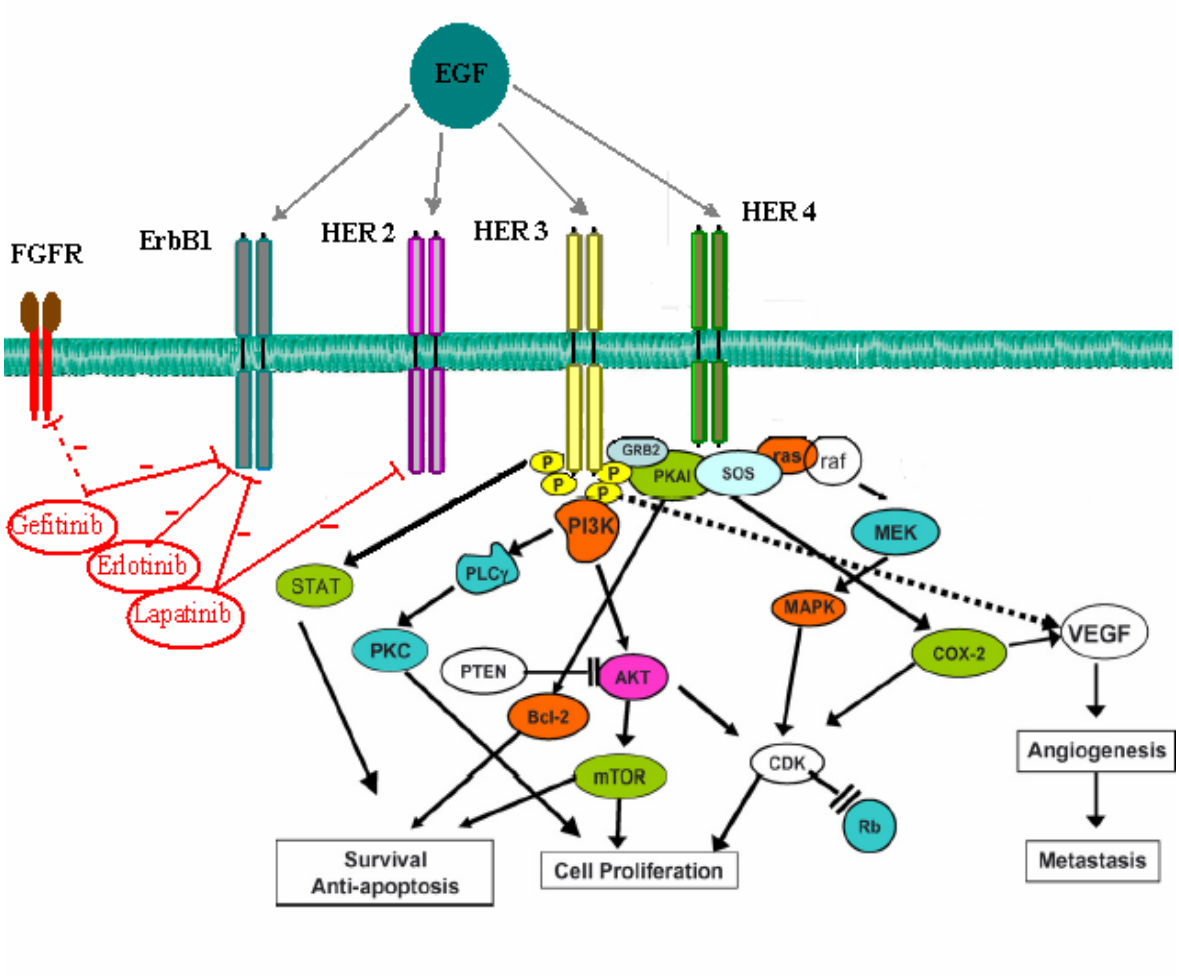
The molecular analysis performed in our group[37] on whole lungs prelevated from MCT challenged rats detected an upregulation of the transcripts for EGF and TGFα, the ligands for EGFR1, as well as a significant upregulation of ErbB1, ErbB2 and ErbB3 but not of ErbB4 transcripts in the lungs from MCT-injected rats as compared with healthy rats. Surprisingly however, the EGFR1 protein level was comparable to that found in the healthy rat lungs. This observation is in agreement with Northcott and colleagues, who found a 4.2-fold increase in mRNA but no difference in the EGFR protein in aorta from deoxycorticosterone acetate-salt hypertensive rats as compared with that from normotensive sham rats[124]. Despite the absence of significant upregulation of EGFR proteins in the MCT model, treatment with gefitinib and erlotinib resulted in beneficial effects, which come in support to the *in vitro* experiments performed in our group[37]. Gefitinib, erlotinib and lapatinib significantly inhibited the EGF-induced proliferation of primary rat PASMCs with comparable efficacy. In contrast, despite its dual EGFR/ErbB2 inhibitory effect and potent *in vitro* effect, lapatinib did not succeed in providing a therapeutic benefit of significantly attenuating the right heart hypertrophy and pulmonary vascular remodeling in the MCT model. Although surprising, the lack of efficacy of lapatinib is however not uncommon, as previous studies in the cancer field have shown that lapatinib activity is not dependent on the EGFR expression level in HER2-overexpressing breast cancer cells and that its clinical efficacy is limited only to the treatment of ErbB2 overexpressing breast cancer[88;112;201].

The therapeutic efficacy of gefitinib and erlotinib may be attributable to their inhibition of EGFR-induced pulmonary vascular SMC proliferation as also observed *in vitro* in cell culture studies performed in our group, and may be explained by the enhanced EGFR signaling, which is possible through mechanisms such as different combinations of receptor dimerization[195] or the crosstalk with other receptor types[88;130;153;182]. Also, although it is classically considered a specific inhibitor of EGFR1, for which it has a 100-fold greater selectivity over other tyrosine kinases in the cell[64], the remarkable efficacy of gefitinib in the MCT model can also be the consequence of its inhibitory activity on numerous other intracellular transmembrane tyrosine kinases[31] (see Figure 28). Indeed, our findings, which are suggestive of the discrepancy in the

efficacy of the EGFR antagonists in inhibiting PASMC proliferation *in vitro* and *in vivo* may be attributable to the presence of other growth factors that are involved in the complex *in vivo* environment and can also play an independent role in the development of the disease, in contrast to the proliferation of PASMC *in vitro* induced by EGF alone. Currently however, we do not have data that could precisely explain the discrepancy in the efficacy of the EGFR inhibitors in MCT-injected rats and this warrants additional studies in the future.

Experiments performed in our group[37] on a mouse model of hypoxia-induced pulmonary hypertension demonstrated, in contrast to the significant response to gefitinib and erlotinib seen in the MCT model, the lack of efficacy of any of the three EGFR inhibitors in reverting the disease, as neither of them could reduce the hemodynamic parameters or delay the remodeling process. The presence of redundant tyrosine kinase receptors and the constitutive activation of downstream mediators[14;19], which are suggested mechanisms for the development of failure or resistance to anti-EGFR treatment in cancer, can represent a possible explanation. Chakravarti et al. have previously demonstrated that the redundant signaling through insulin growth factor receptor (IGF1R), even in the presence of EGFR inhibition, maintains the activation of critical pathways for survival of glioblastoma cells[23]. Furthermore, vascular cells including PASMCs proliferate in response to hypoxia and this proliferative response involves the action of growth factors such as PDGF, FGF and EGF[33;73;90;160] suggesting that multiple growth factors activate their receptor TKs leading to vascular SMC proliferation and survival. The redundancy of growth factors and RTK signaling having overlapping functions and activity during chronic hypoxia may explain the failure of the EGFR antagonists to yield beneficial results in chronically hypoxic mice.

Figure 25. Proposed mechanism of action for EGFR inhibitors gefitinib, erlotinib and lapatinib (adapted after Bianco R. et al., 2007[13]). The black arrows symbolize the induction of intracellular signaling pathways and the red lines indicate the inhibitory effect of EGFR inhibitors in the cell (continuous lines for demonstrated events, dashed lines for postulated mechanisms).



IV.3. Clinical perspective

To our knowledge, the present study is the first to assess and compare in a head-to-head experiment the efficacy of three clinically approved, cancer treatment effective EGFR inhibitors in experimental PAH. Also, it represents a translational research study focusing on the role of EGFR signaling inhibition in a well accepted animal model of pulmonary hypertension.

In this study we proved the therapeutic efficacy of the EGFR antagonists gefitinib and erlotinib in improving not only the hemodynamic and histological features of pulmonary hypertension, but also the symptoms of the disease in MCT rats, when chronic treatment was initiated after the complete establishment of the disease. Interestingly, another EGFR1/ErbB2 inhibitor, lapatinib, failed to demonstrate a significant efficacy in reverting pulmonary hypertension in the same animal model, when used daily at the highest tolerable dose. Also, the complete lack of efficacy of any of these inhibitors in the hypoxia-induced PH in mice demonstrates the partial therapeutic effect of EGFR inhibition depending on the animal model, the inhibitors and doses used. Furthermore, it suggests the fact that these two animal models do not have a common pathomechanism regarding the involvement of the EGF pathway.

Therefore, since neither the monocrotaline nor the hypoxic model can fully mimic the human condition, the investigation of these inhibitors in other animal models of PH as well as a complete study on the survival benefits and maintainance of effects in time would be helpful in a preliminary experimental phase and could help confirming the relevance of these findings to clinical PAH.

V. SUMMARY

Pulmonary arterial hypertension (PAH) is a severe condition associated, if left untreated, with a poor prognosis and a high mortality rate. Pulmonary vascular remodeling, the main cause and the key pathological feature of PAH, subsequently causes the progressive increase of pulmonary vascular resistance and pulmonary arterial pressure, leading to right ventricular failure and death. In the development of the cellular events leading to lung arterial remodeling, like increased proliferation, resistance to apoptosis and migration of pulmonary vascular cells, the involvement of growth factors such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) has been suggested.

Recent studies showed that inhibition of EGFR by the dual EGFR/HER2 inhibitor PKI166, a test compound, could mediate vascular SMC apoptosis and improve survival in monocrotaline (MCT)-treated rats, suggesting the possible therapeutic effect of EGF pathway blockage. In the light of these results, our study has aimed to investigate in the MCT model of PAH the therapeutic efficacy of chronic administration of three EGFR inhibitors which have the advantage of being already approved for use in the clinical practice for cancer patients. Our results have shown that in rats with established PH, gefitinib and erlotinib dose-dependently reduced RVSP and right heart hypertrophy, and, in addition, improved the medial wall thickness and muscularization levels of small pulmonary arteries. Furthermore, our results demonstrate a positive average bodyweight change, an improvement of the distress score and an increase of survival after two weeks of treatment in both gefitinib and erlotinib groups, with gefitinib having the most potent effect. Surprisingly, in contrast with gefitinib and erlotinib, which are EGFR1 antagonists and exhibited a partial effect, lapatinib, a dual EGFR/HER2 inhibitor, provided the least significant therapeutic benefit, as demonstrated by the development of vascular remodeling, right heart hypertrophy and by the negative impact on bodyweight, distress score and survival in treated rats.

To our knowledge, this is the first study to investigate the role of EGFR signaling inhibition using three clinically approved small-molecule inhibitors in an established animal model of PH, in a head-to-head experiment. Our findings may represent the basis for further investigation and for the development of new therapeutic directions, with potential direct clinical application in the field of the human PAH treatment.

VI. ZUSAMMENFASSUNG

Die pulmonale Hypertonie ist eine Erkrankung der Lungengefäße, die durch Erhöhung der Nachlast zu einer Rechtsherzhypertrophie führt. Der strukturelle Umbau der Pulmonalarterien betrifft alle Schichten der Gefäßwand. In der Intima kommt es zur Proliferation von Endothelzellen und zu einer Ausbildung einer Neointima. In der Media weisen glatte Muskelzellen eine Resistenz auf den natürlichen Zelltod (Apoptose) auf und sind durch erhöhte Migartion und Proliferation charakterisiert. In der Adventita werden Fibroblasten zur Kollagenbildung aktiviert. Auf all diesen Ebenen wird die Beteiligung von Wachstumsfaktoren wie Platelet-Derived Growth Factor (PDGF) oder Epidermal Growth Factor (EGF) vermutet, die über nachgeschaltete Signalwege Proliferation, Migration und Apoptoseresistenz vermitteln.

Es konnte gezeigt werden, dass die Hemmung des EGF Rezeptors durch die Testsubstanz PKI166, einem dualen EGFR/HER2-Hemmer, den strukturellen Gefäßwandumbau der Pulmonalarterien verbessert und das Überleben pulmonal hypertensiver Monocrotalin (MCT) Ratten erhöht. Es lässt sich somit ein möglicher therapeutischer Einsatz der EGF-Blocker ableiten. Angesichts dieser Resultate hat unsere Studie darauf abgezielt, im MCT-Modell der pulmonalen Hypertonie die therapeutischen Effekte von drei bereits klinisch zugelassenen EGFR-Hemmern zu untersuchen, um eine schnelle Translation in die Klinik zu erreichen. Wir konnten in dem MCT Modell der PH eine dosisabhängige Senkung des rechtsventrikulären Drucks und der Rechtsherzhypertrophie durch die EGFR1 Hemmstoffe Gefitinib und Erlotinib zeigen. Histologisch wurde eine Abnahme der mittleren Gefäßwandstärke sowie eine Reduktion der Muskularisierung von kleinen Lungenarterien gezeigt. Auch die Proliferation von Gefäßzellen (im wesentlichen in der Media und Adventitia lokalisiert) konnte gesenkt werden. Im Gegensatz dazu zeigte der duale EGFR/HER2 Hemmstoff Lapatinib keine Effektivität in diesem klinisch relevanten Tiermodell der PH, was eventuell auf die Hemmeigenschaften dieser Substanz zurück zu führen ist.

Da diese Substanzen bereits zur Therapie verschiedener Krebserkrankungen zugelassen sind, könnte eine weitere klinische Entwicklung von EGFR Hemmstoffen erfolgen.

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VIII. ABBREVIATIONS AND ACRONYMS

5-HTT	5-hydroxytryptamine (serotonin) transporter
ALK-1	Activin receptor-like kinase type 1
Ang-1	Angiopoietin-1
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
Bcl-2 family	B-cell lymphoma family of proteins involved in apoptosis
bFGF	Basic fibroblast growth factor
BMPR II	Bone morphogenetic protein receptor type II
BSA	Bovine serum albumin
BW	Bodyweight
Ca2+	Calcium ion
c-FLIP	Cellular FLICE-like inhibitory protein (a negative regulator of death receptor-induced apoptosis)
c-Fos	A cellular proto-oncogene belonging to the immediate early gene family of transcription factors
CI	Cardiac index
cmH2O	Centimeters of water (1 cmH_2O= the pressure exerted by a column of water of 1 cm in height at 4 °C)
CNS	Central nervous system
CO	Cardiac output
CO2	Carbon dioxide
CTEPH	Chronic thromboembolic pulmonary hypertension
C-terminal end	Carboxy-terminal end
CYP3A4, CYP3A5	Cytochromes P450 3A4 and 3A5, respectively
DAB	Diaminobenzidine
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
EGFR1 (ErbB1, HER1), EGFR2 (ErbB2, HER2/c-neu), EGFR3 (ErbB3, HER3), EGFR4 (ErbB4, HER4)	Epidermal growth factor receptors 1,2,3 and 4
ErbB	Erythroblastic Leukemia Viral Oncogene Homolog
ET	Endothelin
ET-A, B	Endothelin receptors A,B
FDA	Food and Drug Administration

FiO_2	Fraction of inspired oxygen in a gas mixture.
FPAH	Familial pulmonary arterial hypertension
FSH	Follicle-stimulating hormone
g	Gram (unit of mass)
GABA	Gamma-aminobutyric acid
Gq	Phospholipase C- coupled Gq protein
H2O2	Hydrogen peroxide
Hb	Hemoglobin
HB-EGF	Heparin-binding EGF-like growth factor
HCl	Hydrogen chloride
HIF-1α	Hypoxia-inducible transcription factor 1 alpha
HIV	Human immunodeficiency virus
IGF	Insulin-like growth factor
IGF1R	Insulin-like growth factor 1 receptor
IgG	Immunoglobulin G
IL-1, 6	Interleukin 1,6
IOP	Index of proliferation
IP3	Inositol trisphosphate or inositol 1,4,5-trisphosphate
IPAH	Idiopathic pulmonary arterial hypertension
LV	Left ventricle wall
MAPK	Mitogen-activated protein (MAP) kinases
Mcl-1	Induced myeloid leukemia cell differentiation protein
MCT	Monocrotaline
MCT-P	Monocrotaline pyrrole
min	Minute (unit of measurement of time)
mmHg	Millimeter of mercury (a unit of pressure)
mRNA	Messenger RNA
MWT	Medial wall thickness
N2	Nitrogen gas
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NF-KB	Nuclear Factor-KappaB (nuclear factor kappa-light-chain-enhancer of activated B cells)- transcription factor
NO	Nitric oxide
NSCLC	Non-small cell lung carcinoma
NYHA	The New York Heart Association Functional Classification
O2	Oxygen gas
PAH	Pulmonary arterial hypertension

PAI-1	Plasminogen activator inhibitor-1
PaO2	Partial pressure of oxygen in arterial blood
PAP	Pulmonary arterial pressure
PASMC	Pulmonary artery smooth muscle cells
PBS	Phosphate buffered saline
PCH	Pulmonary capillary hemangiomatosis
PCNA	Proliferating cell nuclear antigen
PDE-5 inhibitors	Phosphodiesterase type 5 inhibitors
PDGF (A,B,C,D)	Platelet derived growth factor (A,B,C,D)
PEEP	Positive end-expiratory pressure
pH	A measure of the acidity or basicity of a solution
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase-C
PLCγ	Phospholipase C gamma
PPHN	Persistent pulmonary hypertension of the newborn
PVOD	Pulmonary veno-occlusive disease
QT interval	A measure of the time between the start of the Q wave and the end of the T wave in the heart's electrical cycle
RANTES	Regulated upon activation, normal T-cell expressed and secreted (a member of the interleukin-8 superfamily of cytokines)
ROS	Reactive oxygen species
RPM	Rotations per minute
RT	Room temperature
RTK	Receptor tyrosine kinase
RV	Right ventricle wall
RVSP	Right ventricular systolic pressure
S	Interventricular septum
SAP	Systemic arterial pressure
SEM	Standard error mean
SMCs	Smooth muscle cells
STAT- 1, 3	Signal Transducers and Activator of Transcription –1,3 (members of the Signal Transducers and Activators of Transcription family of transcription factors)
TGF α, TGF ß	Transforming growth factor –alpha and beta
TK	Tyrosine kinase
TK-I	Tyrosine kinase inhibitors
TN-C	Tenascin-C

t-PA	Tissue plasminogen activator
TPR	Total pulmonary vascular resistance index
TSR	Total systemic vascular resistance index
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cells
α-actin	Alpha-actin (muscle protein localized in myofibrils)
µl	A microliter (one millionth of a liter)
µm	A micrometer (one millionth of a metre)

IX. DECLARATION

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

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…….. So many of our dreams at first seem impossible, then they seem improbable; when we summon the will, they soon become inevitable ……

Christopher Reeve

Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

The curriculum vitae was removed from the electronic version of the paper.