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Institut für Experimentelle Pneumologie der Ludwigs-Maximilians-Universität München
Ehemaliger Direktor: Prof. Dr. Oliver Eickelberg


# Identification of novel compounds for Wnt/beta-catenin induced lung repair in COPD

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## Preface

This thesis is part of a 12 month MD program at the Comprehensive Pneumology Center of the Helmholtz Zentrum München and was implemented under the supervision and mentorship of Prof. Dr. Dr Melanie Königshoff (Ludwigs-Maximillian University Munich/UC Denver) and Dr. Darcy E. Wagner (Lund University). The goal of my work was to investigate the effect of chemical compounds that had been preliminary revealed as Wnt/beta-catenin activators by a High Throughput Screen in 3T3 cells and to elucidate whether these compounds have the potential to attenuate emphysema and induce lung repair by activating Wnt signalling. Throughout my research, my role was to focus on in vitro experiments such as drug validation, drug toxicity determination, functional experiments and literature research.

## Acknowledgements

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Monica Campillos for taking the time and explaining the bioinformatics approach to me.

*„Knowledge is limited.*

*Imagination encircles the world."*

*– Albert Einstein-*

## Summary

When Herakles had to fight against Hydra with the nine heads, the heads would regrow immediately whenever he tried to cut it off making the fight almost impossible to win. Only burning the necks out with fire would kill the monster eventually. Not only in Greek mythology but also in today's animal kingdom, animals like axolotl, zebrafish and hydra, have been shown to have marvelous regenerative potential which humans do not exhibit.

Ever since, regeneration and wound repair has been a fascinating field with the question whether it can be induced pharmacologically by modulating developmental pathways like the Wnt/ß catenin pathway that is responsible for cell fate and tissue differentiation.

One exemplifying disease model for chronic injury and wounding addressed in this thesis is COPD, chronic obstructive pulmonary disease. It is currently considered to be the 4$^{th}$ leading cause of death worldwide and there is no curable treatment available.

COPD is mainly caused by cigarette smoking and is characterized by emphysema and chronic bronchitis which worsens progressively over time, destroying more and more lung tissue. As it had been previously observed that a GSK3 Inhibitor and Wnt/ß catenin activator, Lithium Chloride, could attenuate emphysema (Kneidinger et al., 2011; Uhl et al., 2015), the next step was to find other compounds that could likewise activate Wnt/ß catenin. Thus, we hypothesized that Wnt/ß catenin is a crucial regulator for inducing regeneration in emphysema.

To conquer this problem, a high-throughput screen of 30.000 unique compounds had been previously conducted to identify potential new Wnt/ß catenin inducing compounds based on a TCF/LEF luciferase reporter assay in stably transfected 3T3 cells. Combined with the HitPick software as an in silico method to determine possible targets associated with Wnt signaling, a list of 5 FDA approved drugs was generated for further examination and secondary assays.

As a first approach and thus the main objective of this thesis, the 5 FDA drugs were validated as Wnt/ß catenin activators and we examined in vitro drug toxicity and their ability to induce wound closure. Active Beta Catenin Protein expression was also investigated as well as further literature research to better understand the potential mechanisms of the drugs and their therapeutic potential. Focusing on FDA drugs could enable a faster translation into the clinic and clinical trials. Though having the great advantage of modern technology and robots making screenings possible in a high throughput manner, drug development and discovery for Wnt/ß catenin modulating drugs remains a challenge that will be later illuminated and discussed.

## Zusammenfassung

Als Herakles gegen Hydra mit den 9 Köpfen antreten musste erschien der Kampf so gut wie aussichtlos, denn die Köpfe wuchsen unmittelbar nach sobald er sie zu köpfen versuchte. Nur das Ausbrennen der Hälse mithilfe des Feuers konnte dieses Monster schlussendlich besiegen. Nicht nur in der griechischen Mythologie, auch im heutigen Tierreich gibt es Tiere wie Axolotl, Zebrafische und Hydra-Süßwasserpolypen, die im gegensatz zum Menschen sagenhafte regenerative Fähigkeiten besitzen.

Seit dem übt das Feld der Regeneration und Wundheilung eine unaussprechliche Faszination aus, mit der Frage, ob es möglich sei pharmakologisch in diesen Prozess einzugreigfen und Entwicklungssignalwege wie den Wnt/ ß Catenin Signalweg, welcher für Zelldifferenzierung und Stammzell Determinierung verantwortlich ist, zu modulieren.

Als beispielhaftes Krankheitsmodell für chronische Verletzung und gestörte Wundheilung soll im Rahmen dieser Dissertation die chronisch obstruktive Lungenerkrankung (COPD)dienen. Heute ist COPD die viert häufigste Todesursache weltweit und es gibt es immer noch keine kurativen Behandlungsmöglichkeiten.

Hauptsächlich durch Zigarettenrauchen verursacht, ist die COPD vor allem durch das Emphysem und chronische Bronchitis gekennzeichnet. Der Zustand der COPD Patienten verschlechtert sich zunehmend nach Diagnosestellung und es liegt zunehmend ein zerstörtes Lungengewebe vor. Beobachtungen haben ergeben, das Lithium, ein bekannter GSK3 Inhibitor und demnach ein Wnt/ß catenin activator, den emphysematösen Zustand abschwächen kann (Kneidinger et al., 2011; Uhl et al., 2015).

Als nachfolgenden Schritt wurde es nun zum Ziel gesetzt andere Wnt/ß catenin Aktivatoren zu finden, die ebenfalls das Emphysem abschwächen können. Diese würde also mit der Hypothese einhergehen, dass der Wnt/ß catenin Signalweg an der Regeneration des Emphysems maßgeblich als regulatives Mittel beteiligt ist.

Um dieses Problem anzugehen, wurde vorausgehend ein Screening im Hochdurchsatzverfahren (30.000 Compounds) durchgeführt basierend auf einem TCF/LEF Luciferase Reporter System in transfektierten 3T3 Zellen, welches Wnt/ß catenin aktivierende chemische Verbindungen aufdeckt. In Kombination mit der HitPick Software als in-silico Methode, um Wnt assoziierte Angriffspunkte zu finden, wurde eine Liste mit 5 FDA zugelassenen Medikamenten generiert, die weiter untersucht werden sollten.

Im ersten Ansatz und damit Gegenstand dieser Dissertation wurden diese 5 FDA Medikamente als Wnt/ß catenin Aktivatoren validiert und in Hinblick auf in vitro Toxizität, Wundheilungskapazität, Aktives Beta Catenin Protein Exprimierung sowie weitere Literatur Recherche untersucht. Der Fokus auf bereits FDA zugelassene Medikamente ermöglicht den schnellen Übergang in die Klinik und für weitere klinische Studien. Auch wenn die heutige moderne Technologie einen großen Vorteil bietet Medikamente im Hochdurchsatz und Schnellverfahren zu testen, ist die Endeckung sowie Entwicklung Wnt modulierender Medikamente immer noch eine Herausforderung, die später näher beleuchtet wird.

# 1.Introduction

## 1.1 Background

### 1.1.1 The history and discovery of Wnt

According to legend, Prometheus a titan from greek mythology was forever punished by Zeus for the theft of fire to humanity. Chained to a rock in the Caucasus where an eagle would pick on his liver every day, his liver would likewise regenerate over and over again. The ancient Greek perhaps recognized that the liver has a high regenerative potential for an organ. Organ repair and regeneration or the attempt to induce de-novo organogenesis still has a veil of unresolved mystery even after decades of research.

Even more astonishing is the fact that a hint contributing to resolve this puzzle would be later found in a plain fruit-fly: *Drosophila melanogaster.*

In the 1900s, Thomas Hunt Morgan was a dedicated fruit fly scientist researching in his tiny lab at Columbia University in room 613 of Schemerhorn Hall, later to be known as "The Fly Room". A room so tiny, yet so big in its impact being the root and origin of today's developmental biology with tons of fly bottles standing on the desks and a bunch of bananas hanging from the ceiling emanating an air of fruit fermentation.

Throughout his work, Morgan and his colleagues Bridges and Schulz discovered an X-ray induced mutation in D. melanogaster that was named Glazed (Gla), as the eyes of the fly had a glaze like appearance (Morgan, 1936).

In 1975, a few decades later in India, Sharma and Chopra (Sharma, 1973) (Sharma & Chopra, 1976) (Sharma, 2013) were likewise interested in fruit fly mutations and discovered a wingless phenotype of D. melanogaster caused by an x-ray induced single mutation located on the second chromosome, named Wingless (Wg).

Amusingly, no one realized that both of these mutations being discovered independently by Morgan and Sharma with decades in between were actually on the same allele (Brunner, Brunner, Fu, Hafen, & Basler, 1999).

While Sharma's Wingless is a loss of function mutation due to a deletion, Morgan's Glaze mutation was the consequence of a retrotransposon insertion within the same Wg region (Brunner et al., 1999). Similar to the Glaze gain of function mutation, Roel Nusse and Harold Varmus discovered that mice infected with MMTV (Mouse Mamma Tumor Virus) would develop breast cancer (Nusse & Varmus, 1982). The viral DNA would be integrated within a gene they named Integration-1 (Int-1). This gene had already been discovered under a different name in a different organism, the wingless gene and were found to be identical (Rijsewijk et al., 1987). The vertebral homolog Int-1 and Wg were combined to Wg + Int1= Wnt1 finally resulting in a new term (Nusse et al., 1991). The structure of the Wnt protein contained features of secretory proteins and was therefore thought to be part of a cell signaling pathway enabling cells to communicate. Soon the discovery of 18 other Wnt proteins followed, all 19 sharing a similar amino acid sequence and spread over 10 chromosomes (Miller, 2002).

The Wnt field was born.

It is interesting to mention and to emphasize that Wnt is highly conserved across many species from fly to mouse to human. It not only plays a role in carcinogenesis as demonstrated in the MMTV mice, but also has a crucial role in embryonic development.

In the 1980s, about the same time as Nusse and Varmus discovered Int-1 as a proto oncogene, Christiane Nüsslein-Volhard and Eric Wieschaus were later given the Nobel prize of medicine (1995) for conducting the famous screen of embryonic mutations in drosophila (Nusslein-Volhard & Wieschaus, 1980) and thus discovering that Wingless (today Wnt) is a segment polarity gene responsible for the right segmentation and formation of body axis of the embryonic fruit fly.

Within the past 40 years, the research of Wnt and its relation to diseases ranging from cancer, diabetes and degenerative disorders has now expanded tremendously.

Ironically enough, Thomas Morgan was also focusing on regeneration and published a book in 1901 (Morgan, 1901), not knowing that his own fruit fly research and Gaze discovery would serve to initiate todays modern Wnt regeneration research.

### 1.1.2 Wnt and Lung Development

As developmental pathways are often used in the adult organism and re-activated following injury for the purpose of repair, it is therefore crucial to understand embryogenesis and morphogenesis of the lung as this may give insight into adult repair.

During embryogenesis, 3 germ layers develop: endo-,meso- and ectoderm. The lung epithelium develops from the endoderm within the anterior foregut while the lung mesenchyme derives from the lateral plate mesoderm forming connective tissue, fibroblasts, cartilage, smooth muscle and blood vessels.

After 4 weeks of human embryonic development, a small laryngotracheal diverticulum - the lung bud - evaginates anterior from the foregut and is slowly separated by a tracheoesophageal septum. This initial specification of the lung is mainly orchestrated by the transcription factor Nkx2.1that is expressed by the ventral endodermal cells of the foregut.

Nkx2.1 (NK2 homeobox 1), also known as thyroid transcription factor 1 (TTF-1), is a gene encoding for a homeobox protein. As a transcription factor, it binds DNA that regulates morphogenesis during brain, thyroid and lung development. (Boggaram, 2009)

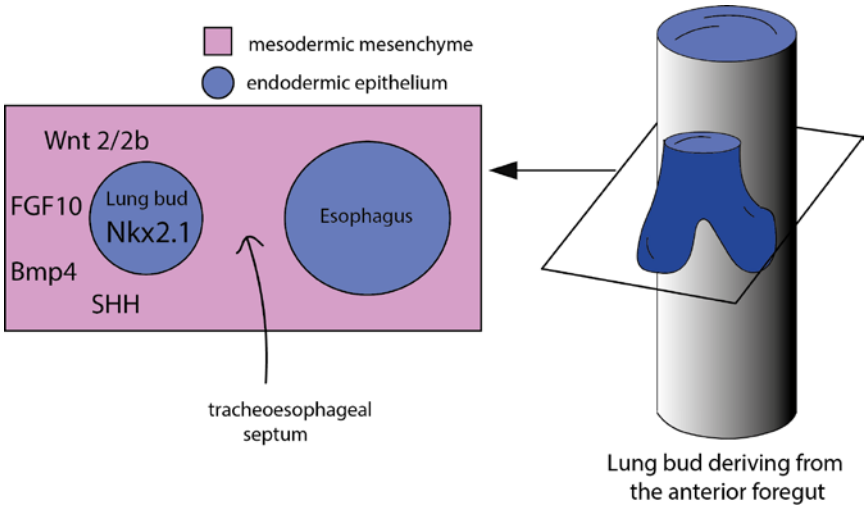



**Figure 1: Lung Development.**

During early lung development the trachea is further separated from the esophagus and two lungs buds form that will subsequently enter different phases of branching morphogenesis: starting off from the pseudoglandular stage (5 - 17 weeks of human pregnancy), then followed by canalicular (16 - 25 weeks of human pregnancy) and saccular stage (24 weeks to late fetal period) to finally end up in the alveolar stage to form a dense tree like branching network which continues to mature postnatally (Warburton et al., 2010).

Many pathways and factors have been identified to be involved throughout the stages of lung development. Wnt, bone morphogenic proteins (BMP), fibroblast growth factor (FGF), transforming growth factor beta (TGF-ß), Notch and sonic hedgehog (SHH) are the most well-characterized to date and a high amount of crosstalk between the pathways has also been identified, indicating the complexity of the fine tuning necessary for proper organogenesis. For reasons of simplicity and due to the topic of the thesis, the focus will hereafter be on Wnt and its role in development. However, it is important to note that the Wnt pathway does not act in isolation and there is much evidence of the necessity of crosstalk with other pathways for proper lung development (Ota, Baarsma, Wagner, Hilgendorff, & Konigshoff, 2016). Failure of one or more events related to Wnt signaling can have a disastrous consequence on organogenesis and lead to failure of the embryo to thrive.

Such a failure can be observed in Wnt2/2b double knockout mouse mutants (Goss et al., 2009) which resulted in complete lung agenesis. Moreover, Nkx2.1 expression was not detectable in the foregut in comparison to wildtype mice, showing that Wnt2/2b is essential for the specification of lung progenitor cells to form the lung bud. In contrast to the lung, other organs like liver, pancreas, kidneys and the gastrointestinal system deriving from the foregut were not affected, hence highlighting the importance of Wnt2/2b specifically for lung development.

The impact of Wnt2/2b for proper lung development has also been shown by one of its upstream regulators, Hox5 (Hrycaj et al., 2015). Hox is homeobox gene that is highly conserved in animals and crucial for regulating morphogenesis and segment formation along the head -tail axis of an embryo. It dictates what genes are turned on by producing transcription factors that determine the fate of a body segment.

There are 13 Hox genes which have been identified in mice and each has two to four paralogous partners (A,B,C to D). Hox 5 was shown to be crucial for lung development as a mesenchymal regulator and part of the Hox5- Wnt 2/2b- Bmp4 signalling axis. To assess the phenotype and exclude the possibility of redundancy, all 3 paralogous Hox 5 genes (A,B and C ) were knocked out (there is no Hox 5 D). The loss of all Hox5 paralogs led to a downregulation of Wnt2/2b in the lung mesenchyme and subsequently to a downregulation of canonical Wnt beta catenin signalling with decreased Axin2 expression and a downregulation of Bmp4 in the distal lung epithelium.

Altogether, the alteration of this Hox5-Wnt2/2b-Bmp4 signalling axis by Hox 5 loss leads to a hypoplastic lung with defects in growth and patterning of the distal branch tips of the Hox5 triple mutant mouse.

By treating the Hox5 triple mutant lung explants with Wnt2/2b enriched media, Bmp4 expression reached normal levels again and branch tips increased nearly as much as control lungs (without Hox5 mutation) within 96 hours, thus compensating the deficiency and rescuing the Hox5 mutant phenotype.

One can thus summarize that for proper lung development Wnt2/2b ligands in the lung mesenchyme are crucial ligands by activating the canonical Wnt beta catenin pathway in the distal lung epithelium, thus keeping the mesenchymal-epithelial crosstalk working. The Hox5 genes are mesenchymal regulators enabling this cross talk. (Hrycaj et al., 2015)

The mesenchyme thus has an essential role for giving developmental signal instructions to the epithelium.

The necessity of mesenchyme-epithelial interactions were classically identified by Rudnick et al. (Rudnick, 1933) where they demonstrated that embryonic chick lungs exposed to chorioallantoic membrane grafts would no longer branch if the mesenchyme was previously removed before *in ovo* cultivation. The same observation was confirmed by others in murine experiments where murine tracheal epithelium denuded from its own mesenchyme was exposed to a murine mesenchymal graft which induced branching.(Alescio & Cassini, 1962; Wessells, 1970)

Alltogether, healthy lung development requires a proper interaction between lung mesenchyme and lung epithelium in which active Wnt beta catenin signalling seems to have an important role.

### 1.1.3 Wnt and Regeneration

In addition to the importance of Wnt signaling and mesenchymal-epithelial crosstalk during embryonic development, there is also evidence of their importance during adult regeneration and repair, as many of these components are recapitulated during wound repair.

Regeneration can be described as a process of recreating cells and tissue that are lost or damaged by injury. The cells involved in tissue regeneration are stem cells and progenitor cells. Stem cells are multipotent cells which can differentiate into one of several different mature cell types, whereas progenitor cells are more restricted and are typically uni or bipotent.

In order for a stem cell to self-renew and produce new cells to replenish the tissue, both extrinsic and intrinsic factors need to be available: first the stem cell needs to be exposed to a signal coming from its environment, the so-called niche, and secondly it needs to be competent to also act as a stem cell with a potency to differentiate into other cell types. Signal molecules like Wnt provides the stem cell with the cues for repair and regeneration. These signals can be secreted in both an autocrine and paracrine fashion, meaning that the stem cell can further produce its own instructions or can receive the signal from adjacent cells of the microenvironment, respectively.

From a therapeutic point of view, recent approaches in medicine have explored the potential of injecting stem cells into humans in order to induce repair. This feasibility is best known for bone marrow transplants, in which hematopoietic stem cells of a donor

can be given to a patient who was exposed to chemotherapy leading to destruction of bone marrow cells thus treating diseases such as lymphoma and leukemia (Raju, 2000).

Alternatively, mesenchymal stem cells (MSC) derived from the bone marrow or from adipose tissue have been explored for chronic lung disease in both pre-clinical models and initial clinical trials (Wagner et al., 2016). MSCs are known to be a heterogenous population of cells but are characterized by their fibroblast-like morphology and cell surface markers. They can be found in various tissues and the different microenvironments seem to dictate their ability to differentiate through the secretion of cytokines and growth factors. MSCs are known to be multipotent, meaning that they can differentiate into a variety of cell lineages such as adipocytes, myocytes, chondroblasts and osteoblasts (Wong et al., 2015). As they have their origin in the stroma, the term mesenchymal stroma cells is nowadays more preferred (Wagner et al., 2016).

It has been proposed that MSCs may be identical with the term pericyte, the perivascular cells that surround small microvessels through the entire body, which would explain their ubiquitous presence in almost every tissue (Birbrair et al., 2015; Caplan, 2008). While mesenchymal stem cells have successfully been used to cure COPD in pre-clinical animal models and have been safely administered to humans with COPD, there was no noticeable improvement in any of the outcomes measured (6 minute walk test, etc.) in a recent clinical trial evaluating the efficacy of using MSCs for COPD treatment (Weiss, Casaburi, Flannery, LeRoux-Williams, & Tashkin, 2013).

While the administration of MSCs appears to be safe and feasible, as of 2018, there is no study which proves the efficacy for inducing tissue regeneration in human COPD patients (Kruk, Heijink, Slebos, Timens, & Ten Hacken, 2018). It thus raises the question of what disease modifying role MSCs have in vivo, how they are recruited to the site of injury, whether pericytes are the in vivo counterparts in the lung and what factors in the microenvironment are essential to modulate them.

It has been observed that canonical Wnt signaling induced differentiation of murine bone marrow derived mMSCs into alveolar epithelial type 2 (AT2) cells in vitro by co-culturing mMSC with murine lung epithelial (MLE-12) cells (A. R. Liu et al., 2013). AT2 cells are the progenitor cells giving rise to type 1 alveolar epithelial cells type 1 (AT1) and are thus important for the reepithelization of injured lung tissue. In addition to the administration of stem cells exogenously, there have also been a number of pre-clinical studies which have examined the potential of activating endogenous stem cell populations for regeneration. Thus, pathways which regulate normal repair and regeneration are of particular interest. One such pathway is the Wnt/beta catenin pathway. In endogenous regeneration approaches, both direct activation of specific pathways as well as regulation of the stem cell niche is important. Even though these cells are considered to be multipotent, they will only differentiate if the right cues are present.

Wnt/ beta catenin signaling is relevant for regenerative medicine as it can control stem cell activity and thus regulates tissue homeostasis. Without proper Wnt/beta catenin signaling tissue homeostasis is highly disturbed (Clevers, Loh, & Nusse, 2014).

One can thus see Wnt signaling as a possible ignitor for inducing repair and regeneration in which certain aspects of developmental stages of the embryonic lung are recapitulated. Canonical Wnt signaling has therefore become an attractive therapeutic target. On a molecular lever, this pathway is highly complex:  In the absence of Wnt ligands, beta catenin (ß-cat) is degraded by the destruction complex, composed of Axin, APC, CK1 and GSK3. GSK3 and CK1 phosphorylate beta -catenin, which is then recognized by ß-Trcp for further ubiquitination. As a ubiquitinated protein, beta-catenin is destroyed by the proteasome and can no longer accumulate in the cytosol. When Wnt ligands are present in the extracellular space, they bind to Frizzled and LRP5/6 receptors, induce receptor phosphorylation thus disrupt the destruction complex by recruiting Axin to the membrane. Beta-catenin is now able to accumulate in the cytoplasm and enters the nucleus to form a complex with TCF/LEF transcription factors and thus induces the transcription of Wnt target genes.

The complexity of the Wnt/beta catenin pathway is also due to crosstalk with several other pathways, such as Hedgehog, in which components like GSK3ß are also an integral part of. This crosstalk can make it a challenge to find targets which can specifically modulate Wnt. So far there is no specific Wnt modulator used in the clinic to induce regeneration that is safe, applicable and shows efficacy at the same time.

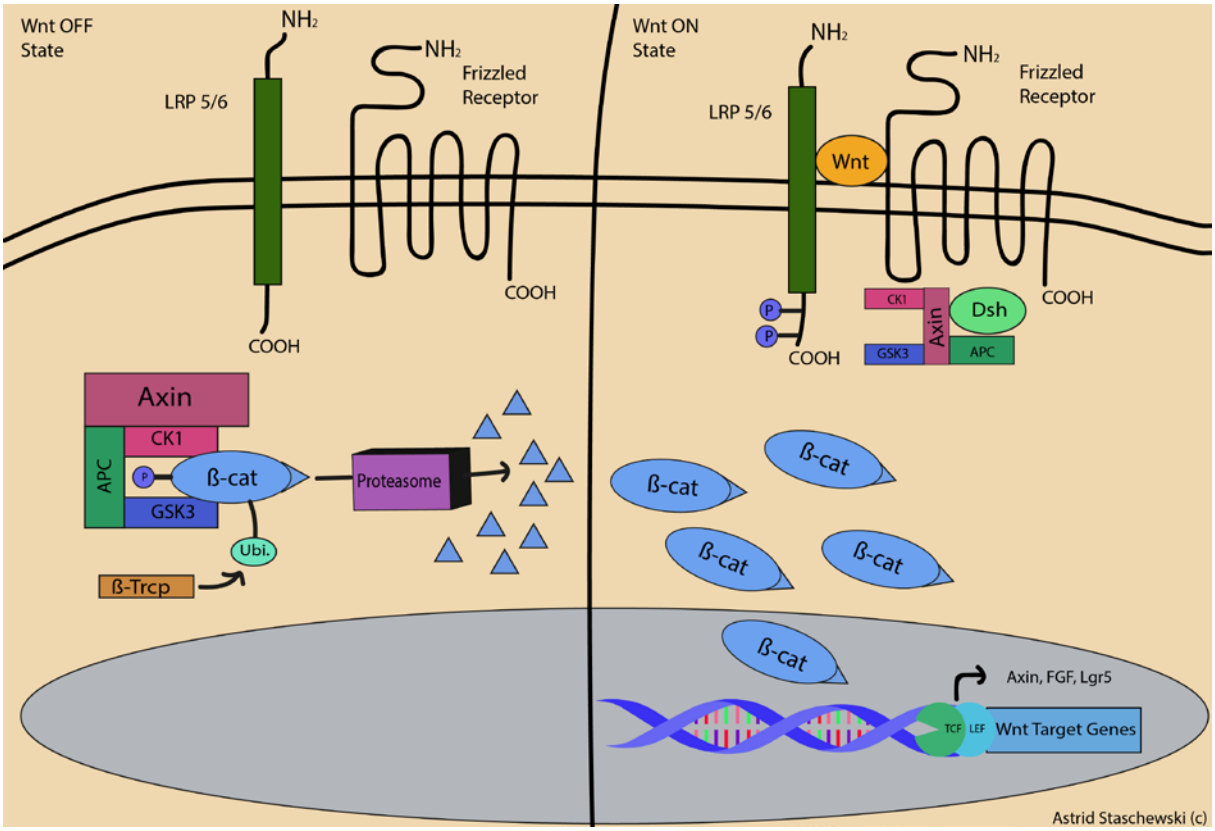



**Figure 2: Canonical Wnt/beta-catenin pathway**

## 1.1.4 COPD

### 1.1.4.1 COPD

While Wnt signaling has long been recognized as playing a critical role in lung development, recent literature has indicated its role in adult regeneration and repair

and a role in chronic lung diseases. In COPD, chronic injury results in a diminished ability for tissue repair. Interestingly, it has been previously shown that Wnt signaling is decreased in COPD patients (Kneidinger et al., 2011) and in particular in the airway epithelium. These observations indicatethat Wnt aberration may play a major role in the pathogenesis of this disease which will be discussed below.

#### 1.1.4.1.1 Definition and Symptoms of COPD, Economics

COPD, currently the fourth leading cause of death worldwide, is a heterogenous disease clinically observed by symptoms like chronic cough, sputum production, dyspnea and periods of acute exacerbations followed by respiratory insufficiency and central cyanosis.

According to the Global Initiative for Chronic Obstructive Lung Disease (GOLD), COPD is defined as *"A common, preventable, and treatable disease that is characterized by persistent respiratory symptoms and airflow limitation that is due to airway and/or alveolar abnormalities usually caused by significant exposure to noxious particles or gases."* (Global Initiative for Chronic Obstructive Lung Disease, 2017)

Due to the high mortality and morbidity COPD is causing within the world population, it has a high economic burden. This is partially caused by high hospitalization rates during exacerbations, treatment costs for maintenance therapy but also by comorbidities. In the US, a study revealed that COPD had health care costs of an estimated $36 billion in 2010 and medication costs of $20.4 billion in 2008.(May & Li, 2015)

#### 1.1.4.1.2 Aetiology of COPD

In terms of the aetiology of COPD, noxious particles like coal dust, biomass smoke, air pollution and, in particular, smoking are known to be the main risk factors. (Buist et al., 2007; Walter, Gottlieb, & O'Connor, 2000; Y. Zhou & Chen, 2013). Despite the fact that tobacco smoking is the main risk factor, non-smokers and never smokers can also be affected as shown from data of the international BOLD Study (Buist et al., 2007). Additionally, individuals who were born premature or with lower birth weights and thus have immature lung growth are more susceptible to early lung infections and show a higher risk of developing COPD in later life. (Barker et al., 1991)

Genetically, alpha 1 antitrypsin deficiency is also associated with a higher risk for COPD susceptibility.(Foreman, Campos, & Celedon, 2012). This genetic disorder is rarely diagnosed, but has a prevalence of 1:4000 in Germany and is thus a rather common disease (Koczulla et al., 2008).

Thus, both environmental as well as genetic conditions can lead to the development of COPD.

#### 1.1.4.1.3 Classification of COPD

The clinical classification of COPD patients is based on lung function tests measuring forced expiratory volume in one second (FEV1) and the forced vital capacity (FVC). According to the GOLD guidelines (Global Initiative for Chronic Obstructive Lung Disease, 2017), an FEV1/FVC ratio of less than 0,7 after using a bronchodilator is an indicator for persistent airway obstruction and limited airflow.

COPD patients who have a FEV1/FVC ratio of less than 0,7 can be classified into four stages A-D from “mild” to “very severe” by comparing FEV1 to predicted values.

The latest 2017 GOLD guidelines also include questionnaires to assess dyspnea and the severity of symptoms: modified Medical Research Council (mMRC) and the COPD assessment test (CAT).

#### 1.1.4.1.4 Current guidelines for COPD treatment

Depending on the classification and severity of symptoms, the recommended course of treatment for COPD patients can be with short or long acting beta agonists and muscarinic antagonists, either as monotreatment or as a combination of both (Global Initiative for Chronic Obstructive Lung Disease, 2017).

In severe cases or during exacerbations, inhaled corticosteroids (ICS) and phosphodiesterase-4-inhibitors can be used as a further escalation therapy.

In cases of hypoxemia, long-term oxygen supplementation is recommended.

As a very last step, a surgical intervention in form of lung transplantation or lung volume reduction surgery to remove the most damaged parts may come into consideration.

The efficacy of lung volume reduction surgery remains controversial and lung transplantation has low survival rates, with a 5-year median survival rate of 50%. Thus, at present, there is no effective, long term treatment option for patients with COPD.

Additionally, there is no treatment or therapy which can prevent the progression of COPD. Thus, the identification of new and better treatment options is urgently needed.

#### 1.1.4.1.5 Pathophysiology of COPD

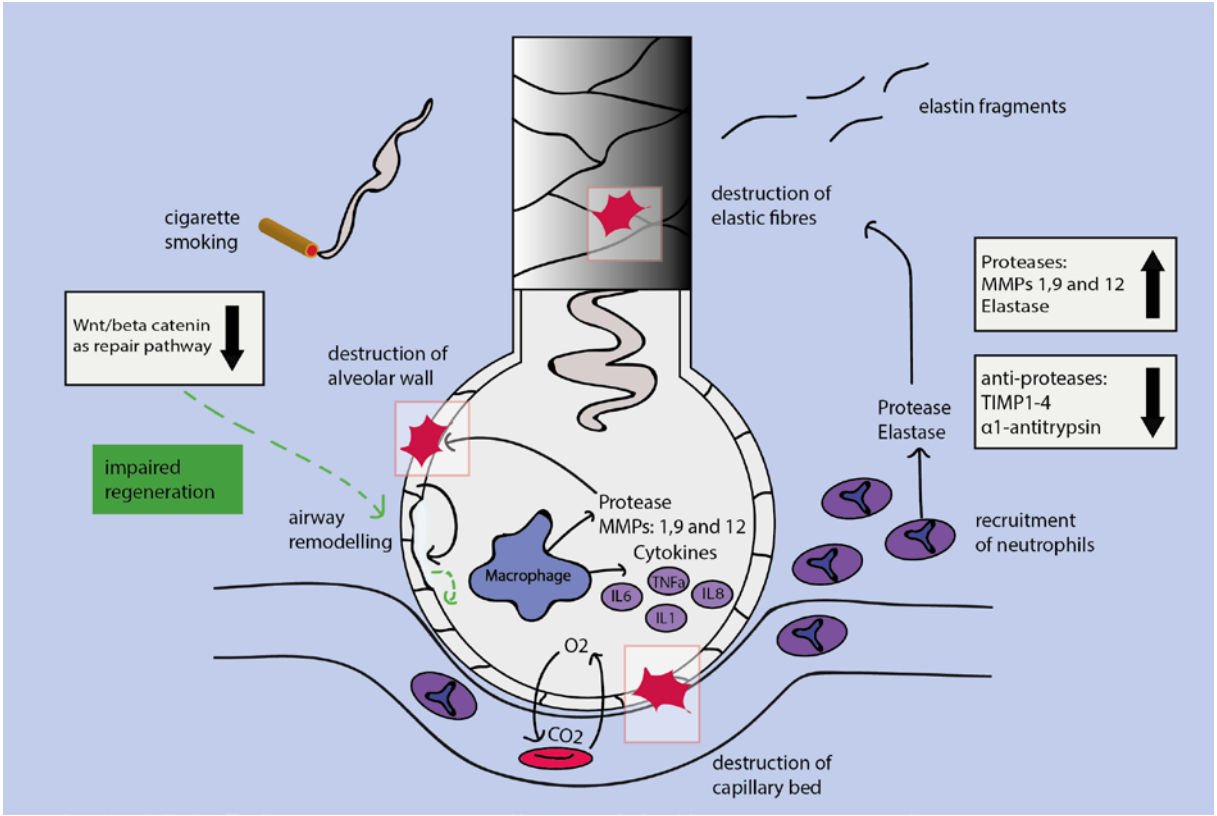



**Figure 3: COPD pathophysiology.**

COPD encompasses a variety of pathological conditions and is mainly characterized by chronic inflammation, chronic bronchitis and emphysema. Emphysema(i.e. loss of distal lung tissue) is driven by a destruction of the alveolar walls, capillary beds, and elastic fibers and is mainly thought to be due to an imbalance of proteases and anti-proteases.

The chronic exposure to external noxes, like those from cigarette smoking, induces oxidative stress and an inflammatory response. Immune cells, such as macrophages and neutrophils, are recruited to the lungs and secrete excessive amounts of proteases like MMP 1, 9 and 12 and neutrophil elastase thus leading to an imbalance of proteases and anti-proteases (Abboud & Vimalanathan, 2008; Churg et al., 2003; Selman et al., 2003; Shapiro et al., 2003). Inflammation also leads to an increase of pro-inflammatory cytokines that will further accelerate the process by recruiting even more neutrophils through chemotaxis. To protect the airways, goblet cells increase in size and number and produce more mucus, smooth muscle cells constrict and the air wall is thickened. This collectively leads to a constriction of the small conducting airways and airflow limitation (Hogg et al., 2004; Hogg & Timens, 2009).

The increased number of proteases in the lung have a devastating effect:
The alveolar wall, the capillary bed and the elastic fibers are destroyed. While it is thought that healthy individuals can regenerate damaged tissue, COPD patients do not have this capability.In COPD patients, regenerative pathways such as Wnt/beta catenin are downregulated and the airway remodeling continues as the ability to repair is now impaired. The disease becomes progressive, compensation is no longer possible and aberrant tissue remodeling takes place to adapt to the chronic inflammation and the toxic inhalative noxes. Thus, tissue destruction becomes irreversible.

The loss of elastic fibers in emphysema decreases the ability of the alveoli to recoil after expiration. The alveoli are remarkably enlarged and the air is trapped since not only recoiling is diminished but also the upper airways are constricted. The destruction of the capillary bed decreases the perfusion and the surface area for proper oxygen and carbon dioxide exchange, thus contributing to hypoxia and hypercapnia.

#### 1.1.4.1.6 WNT and COPD

Although new and improved bronchodilatators have been developed for COPD patients to treat symptoms, the attempts to develop new drugs in particular anti-inflammatory drugs for COPD has been rather disappointing. In the long term, bronchodilatators alone have been shown to not reduce mortality in COPD patients. Hence, finding new drugs that have both anti-inflammatory and regenerative features to slow down the progressiveness of COPD remains a promising area of research (Watz, 2017).

It remains cryptic why repair mechanisms in the emphysema lung are suppressed and regeneration does not fully occur. Investigating developmental pathways that are recapitulated during tissue regeneration might be a novel strategy.

Indeed, it has been shown that Wnt/beta catenin signaling is downregulated in COPD patients as well as in mouse models of emphysema (Baarsma & Konigshoff, 2017). By activating this pathway using a GSK3 Inhibitor, LiCl, emphysema development was attenuated in the elastase-induced emphysema mouse model (Kneidinger et al., 2011).

Consistent with these findings, Uhl et al. could demonstrate that canonical Wnt/beta catenin signaling is activated by LiCl not only in murine but also in human 3D lung tissue cultures (Uhl et al., 2015). The effect in the 3D lung tissues was also observed with CHIR99021 a highly specific GSK3 Inhibitor. According to Uhl and colleagues, both compounds were able to induce induction of active beta catenin (ABC) and gene expression of Axin2 and Nkd1, all indicative markers for Wnt activation and thus supporting the notion that the attenuation was Wnt driven. Moreover, increases in surfactant protein C (Sftpc) and alveolar epithelial markers indicated lung repair (Uhl et al., 2015).

Lithium (in its salt form of Lithium Carbonate, Acetate, Sulfate or Aspartate) is an FDA approved drug currently used for psychiatric conditions like major depression and bipolar disorder. Although it is known to be a GSK3 Inhibitor, the mode of action clinically is not fully understood. Lithium chloride is used *in vitro* but has been previously shown to be toxic in patients (Hanlon, Romaine, & et al., 1949)**.** Clinically, lithium is mainly prescribed in the form of Lithium Carbonate but suffers from low solubility which limits its usage *in vitro*. Furthermore, it has a low therapeutic range and has many side effects when not monitored properly (Curran & Ravindran, 2014; Malhi, Tanious, & Gershon, 2011). Therefore, it is unclear if lithium treatment could be a possible treatment for COPD patients. Moreover, GSK3 is an unspecific target and part of many other pathways which could lead to unwanted effects.

Thus, the identification and translation of novel Wnt/beta-catenin activators which already have FDA approval may provide new treatment options for COPD.

## 2. Hypothesis

### 2.1 Identification of novel Wnt activators that are able to attenuate emphysema

In respect to the results of recent literature and the observation of Lithium's ability to attenuate emphysema, we hypothesized that the activation of the Wnt/ beta-catenin pathway is a promising therapeutic target for inducing lung repair and regeneration in emphysematous tissue.

The aim of this study was to identify new pharmaceutical compounds which are already FDA approved which can both activate the Wnt pathway and attenuate emphysema / COPD. Given that Lithium chloride and other current known Wnt pharmaceutical activators are either toxic or have unspecific targets, there is still a high necessity to find more optimal compounds for clinical Wnt modulation. Lithium in form of Lithium Carbonate and Acetate is currently the only known Wnt activator available in the clinic. It has been used for more than a decade for treating bipolar disorder and major depression. However due to its low therapeutic range and its low solubility it would be challenging to design a nebulized form for direct administration into the lung and thus it is not currently thought to be feasible to treat COPD patients.

## 2.2 Approach to test hypothesis:

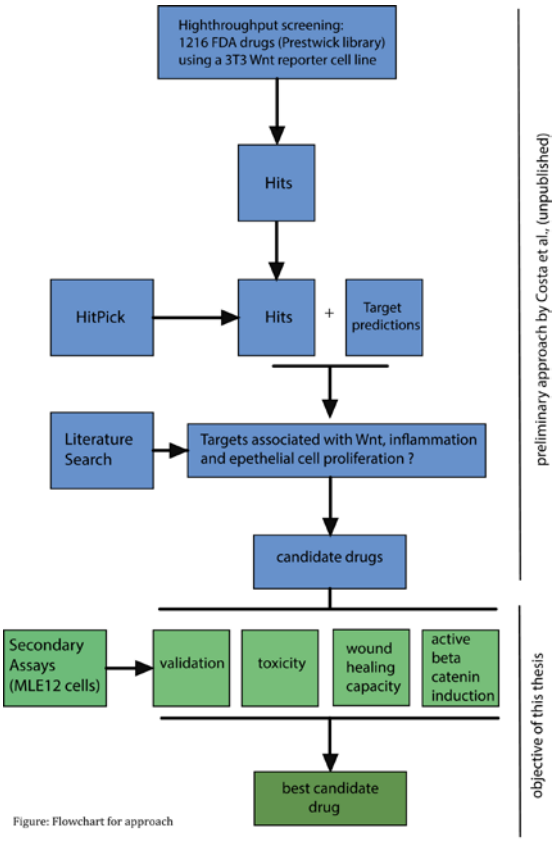



Figure: Flowchart for approach

### 2.2.1High throughput Screening (HTS)

Recently, several studies ihave sought to identify novel Wnt beta catenin signalling modulators in neuropsychiatric disorders using high throughput drug screening approaches. One study was based on a TCF/LEF-luciferase reporter system (Zhao et al., 2012) and identified Wnt modulating compounds in human iPSC derived neural progenitor cells.

Likewise another study used an immortalized mouse hippocampus cell line (HT22) containing a b-catenin-activated luciferase reporter (BAR), to screen FDA compounds for enhancing Wnt activation in the presence of Wnt3a (Biechele et al., 2010). They identified Riluzole and its presumed target GRM1 as a novel regulator for Wnt activation. At that time, Riluzole a drug used for prolonging the survival rate of patients with amyotrophic lateral sclerosis (ALS) was under a clinical trial for treating melanoma and therefore investigated within this context.

In my thesis work, we used a similar approach to find potential compounds to modulate Wnt signaling but for the treatment of emphysematous lung tissue. As Wnt signaling is known to be cell-context dependent, we used an immortalized NIH-3T3 fibroblast containing a TCF/LEF luciferase reporter element as a different initial cell line. This aspect contributes to the novelty of this project as the approach having the main focus on Wnt and emphysema has not been done before.

### 2.2.2 Bioinformatical approach HitPick

A typical drug screen returns around X% of hits. In the current study, a drug screen of 30,000 compounds had been previously conducted (Costa et al. *unpublished*) and yielded 1216 number of novel compounds. Because it is costly to screen each individual hit, we sought to first use a computational approach to narrow down the number of positive hits identified in the HTS and ultimately decrease the amount of time for drug discovery. We applied the HitPick software as an in-silico screening method to filter based on the targets the compounds are predicted to be associated with. We thus generated a list of predicted target compound interactions that enables us to decide which compounds are most promising for *in vitro* secondary assays.

As HitPick alone is not sufficient to determine which targets are relevant for COPD and its pathophysiology, the target prediction of the compounds was complemented with literature research to choose those targets that have associations with Wnt signalling, inflammatory response regulation and epithelial cell proliferation.

### 2.2.3 Secondary Assays

In the third step and now main objective of the thesis, the aim was to validate the hits from the drug screen by using further secondary assays addressing the *in vitro* behavior of the compounds in terms of activation of beta-catenin, toxicity, proliferation and wound healing ability.

# 3. Material and Methods

## 3.1 Lab equipment

The following lab equipment and software were used for the experiments:

**Table 1: Lab equipment**

| Product | Manufacturer |
|---|---|
| -80 °C freezer U570 HEF | New Brunswick; Hamburg, Germany |
| -20 °C freezer MediLine LGex 410 | Liebherr; Biberach, Germany |
| Camera EOS 1000D DSLR | Canon, Tokio, Japan |
| Cell culture work bench Herasafe KS180 | Thermo Fisher Scientific; Darmstadt, Germany |
| Centrifuge MiniSpin plus | Eppendorf; Hamburg, Germany |
| Centrifuge Rotina 420R | Hettich; Tuttlingen, Germany |
| Centrifuge with cooling, Micro200R | Hettich; Tuttlingen, Germany |
| CO2 cell Incubator BBD6620 | Thermo Fisher Scientific; Darmstadt, Germany |
| Dry ice container Forma 8600 Series, 8701 | Thermo Fisher Scientific; Darmstadt, Germany |
| Fridge MediLine LKv 3912 | Liebherr; Biberach, Germany |
| Gel imaging system ChemiDoc XRS+ | Biorad; Hercules, CA, USA |
| Ice machine ZBE 110-35 | Ziegra; Hannover, Germany |
| Microscope Axiovert 40 C | Carl Zeiss, Jena, Germany |
| Plate reader Sunrise | Tecan; Crailsheim, Germany |
| Scale XS400 2S | Mettler Toledo; Gießen, Germany |
| Ultra pure water supply MilliQ Advantage A10 | Merck, Millipore; Darmstadt, Germany |
| Vortex Mixer | IKA; Staufen, Germany |
| Water bath Aqua Line AL 12 | Lauda; Lauda-Königshofen, Germany |

## 3.2 Software in use

**Table 2: Software**

| Software | Producer |
|---|---|
| Adobe Illustrator CC 2017 | Adobe Systems, San Jose, California, USA |
| EndNote X8.1 | Clarivate Analytics, London,UK |
| GraphPad Prism 7.0 | GraphPad Software; La Jolla, CA, USA |
| ImageJ | NIH, Bethesda, Maryland, USA |
| Image Lab Version 5.0 | Biorad; Hercules, CA, USA |
| Magelan Software | Tecan; Crailsheim, Germany |
| Microsoft Office Home & Student 2016 | Microsoft Corporation; Washington, DC, USA |
| Tristar MicroWin 2000 | Berthold Technologies; Bad Wildbach, Germany |

## 3.3 Chemical Reagents

The following reagents and chemicals were used in the experiments described later.

**Table 3: Reagents and Chemicals**

| Reagent | Conc. | Effect | Vehicle | Manufacturer | Product No. |
|---|---|---|---|---|---|
| Amlexanox | 0,5-20µM | | DMSO | Cayman Chemical | Cay14181-500 |
| Ammonium Persulfate | | | | AppliChem | A1142 |
| Bright-Glo Luciferase Assay | | | | Promega | E2620 |
| Bromophenol Blue | | | | AppliChem | A3640 |
| BSA | | | PBS | Sigma Aldrich | A-2058 |
| CHIR99021 | 1-2µM | GSK3 Inhibitor | DMSO | Tocris | 4423 |
| DMEM | | Growth Medium | | Sigma Aldrich | D1145 |
| DMEM/F12 | | Growth Medium | | Life Technologies | 11330032 |
| DMSO | | | | Roth | A994.2 |
| DTT (Dithiotritol) | | | | AppliChem | A2948 |
| FCS | | cell growth | | PAA Laboratories | A15-649 |
| Glo-Lysis Buffer | | cell lysis | | Promega | E266A |
| GlutaMAX | | Supplement | | Life Technologies | 35050061 |
| Glycerol | | | | AppliChem | A3739 |

| | | | | | |
|---|---|---|---|---|---|
| Glycine | 5µM | | DMSO | AppliChem | A1067 |
| IWP2 | 10mM | Wnt/Porcn Inhibitor | H20 | Tocris | 3533 |
| LiCl | | GSK3 Inhibitor | | Sigma Aldrich | 203637 |
| MTT | 1-20µM | | DMSO | Promega | G402A |
| Nabumetone | 10mM | | H20 | Santa Cruz | sc-204813 |
| NaCl | | | | AppliChem | A4661 |
| Penicillin/ Streptomycin | 0,5-20µM | antibiotics | DMSO | Life Technologies | 15070063 |
| Phenazopyridine | | | | Santa Cruz | sc-212544 |
| Poly-L-Lysine | | cell attachement | | Sigma Aldrich | P4832 |
| Rotiphorese | | | | Roth | 3029.1 |
| SDS | | | | Roth | CN30.2 |
| Solubilization Stop Solution | | cell lysis | | Promega | G401A |
| Temed | 1-20µM | | DMSO | AppliChem | A1148 |
| Tiaprofenic Acid | 1-20µM | | DMSO | Sigma Aldrich | T1410900 |
| Tolnaftate | | | | Santa Cruz | sc-237124 |
| Tris | | | PBS | AppliChem | A1379 |
| Triton-X 100 | | cell death | | AppliChem | A1287 |
| Trypsin | | cell detachment | | AppliChem | A3964 |
| Tween-20 | 200ng/ml | | BSA 0,1% | AppliChem | A4974 |
| Wnt3a | | Wnt activation | | R&D Systems | 1324-WN |
| WST1 | | | | Roche | 05 015 944 001 |

## 3.4 Cell Growth and Maintainance

Cells were grown in a CO2 Incubator (5% CO2) at 37°C under humidified conditions.

Every 2-3 days, cells were either split when at confluency or media was changed with PBS in between rinse steps.

| Cell Line | Medium | Manufacturer | Supplementation |
|---|---|---|---|
| MLE12 (ATCC CRL-2110) | DMEM/F12 | Life Technology | 10% FCS , 1% P/S |
| NIH-3T3 (ATCC CRL-1658) | DMEM | Sigma Aldrich | 10% FCS , 1% P/S, 4 mM GlutaMAX |

**Table 4: Cells**

## 3.5 Luciferase Assay: Validation of 5 FDA Drugs

NIH3T3 cells stably expressing luciferase under the control of Wnt responsive promoters (TCF/LEF) were seeded on a poly-l-lysine coated 48 well plate with a cell density of 60.000 cells/well, starved overnight with FCS 0,1% DMEM media for cell synchronization and finally treated for 24 hours in triplicates with the 5 FDA compounds in 4 different concentrations using CHIR99021 1µM, DMSO and unstimulated media as a control. The wellplates were coated with 150µL/well of poly-l-lysine from a 0,01% stock solution for 5min, washed with PBS and then dried in a CO2 incubator for 2 hours.

After treatment, media was discarded and cells were lysed with 65µL/well of Glo-lysis buffer (Promega) for 30min on a shaker, then carefully scraped using a cell scraper and resuspended using a pipette. 25µL from each well was finally transferred onto a 96 well plate and the activity of the reporter system was detected by the Bright Glo luciferase Asssay system (Promega) according to the manufacturer's instruction in a Berthold Tristar LB 941 luminator.

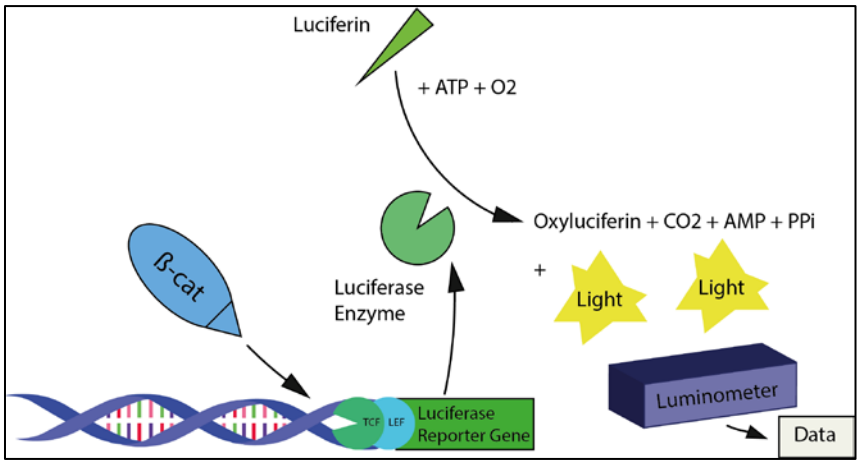



**Figure 5: Luciferase Assay.** To monitor Wnt signaling cells are stably transfected with a TCF/LEF luciferase reporter construct. As Wnt signaling leads to the translocation of ß-catenin into the nucleus, it will form a complex with the transcription factors TCF/LEF to induce the transcription of Wnt target genes and thus the luciferase reporter, encoding for the luciferase enzyme which will catalyze the reaction from Luciferin to Oxyluciferin. The emitted light during this reaction can be detected by a luminometer to draw inferences about the presence and intensity of Wnt signalling.

### 3.6 WST1 Assay 3T3 cells

To test proliferation and cell viability, 3T3 cells were first tested with a WST1 assay.

3T3 TCF/LEF promoter cells were seeded on a 96 well plate with a density of 25.000 cells/well, starved overnight and finally exposed to the 5 FDA compounds for 24 hours.

After 24 hours, 10µl per well of WST-1 reagent (Roche) was added to each well and cells were incubated for 1 hour. The supernatant was removed and transferred into a 96 well plate and absorbance was measured at 450 nm with 650nm as a reference using a TriStar plate reader.

Difference data was quantified and normalized to DMSO treated cells.

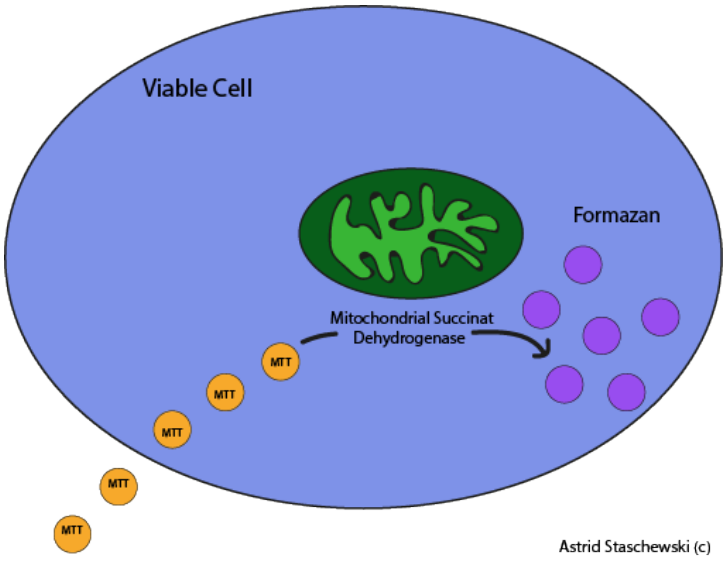
### 3.7 MTT Assay (3T3/MLE12 cells): Determining *in vitro* drug toxicity

MTT assay kit (Promega) was used to test 3T3 and MLE12 cells for cell viability after compound exposure.

3T3 cells were seeded at a density of 25.000cells/well in DMEM media FCS 10% on a Poly L Lysine coated 96 well plate to improve attachment, starved overnight with DMEM Media FCS 0,1% and treated with compounds for 24h. MLE12 cells (an immortalized distal lung epithelial cell line) were seeded with a density of 10.000 cells /well in DMEM/F12 media 10% FCS likewise on an uncoated 96 well plate. 15µL of MTT reagent was added per well and the well plate was incubated in a 37C incubator for 4 hours. Lysis Buffer was added and cells were lysed for 1 hour at room temperature, in a sealed and humidified box to prevent light exposure and evaporation. Lysed cells were mixed with a small pipette tip in each well without resuspension to prevent air bubbles.

Absorbance was measured at 570nm with 700nm as a reference using a TriStar plate reader

Difference data was quantified and normalized to DMSO treated cells.




How the assay works:

**Figure 6: MTT/WST-1 Assay.** The reddish water soluble tetrazolium salt WST1 or MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) is able to enter the mitochondria of a cell. When cells are viable having functional mitochondrial dehydrogenases, the tetrazolium salt will be metabolized into the purple colored Formazan. The higher the amount of viable cells, the more Formazan is produced that can be detected by an ELISA reader thus drawing inferences about the cell viability or toxicity when exposed to a chemical compound.

### 3.8 Scratch Assay: Testing Wound Closure Capacity

MLE12 cells were seeded on a 24 well plate at a density of 0,5 million cells in DMEM/F12 media FCS 10%, starved overnight with DMEM/F12 media FCS 0,1% and treated as duplicates for 72h with each of the compounds in a concentration of 1µM, 5µM and 10µM after a vertical scratch with a 100µL pipette tip was applied on each well.

Pictures were taken after 24 hours, 48 hours and 72 hours using a Canon camera EOS 1000D DSLR to determine the amount of wound closure over time. Images were quantified with ImageJ and the MRI wound healing tool to calculate the area between the scratch borders.

The average area of the duplicates for 72h was divided by the average area of the vehicle at the 72 hour time point. Each compound's wound closure capacity was then compared to DMSO.

$$\frac{\bar{X}(wound\ area\ of\ compound - 72h)}{\bar{X}\ (wound\ area\ of\ vehicle - 72h)} = Wound\ Size\ (\%\ of\ vehicle\ at\ 72h)$$

How the assay works:

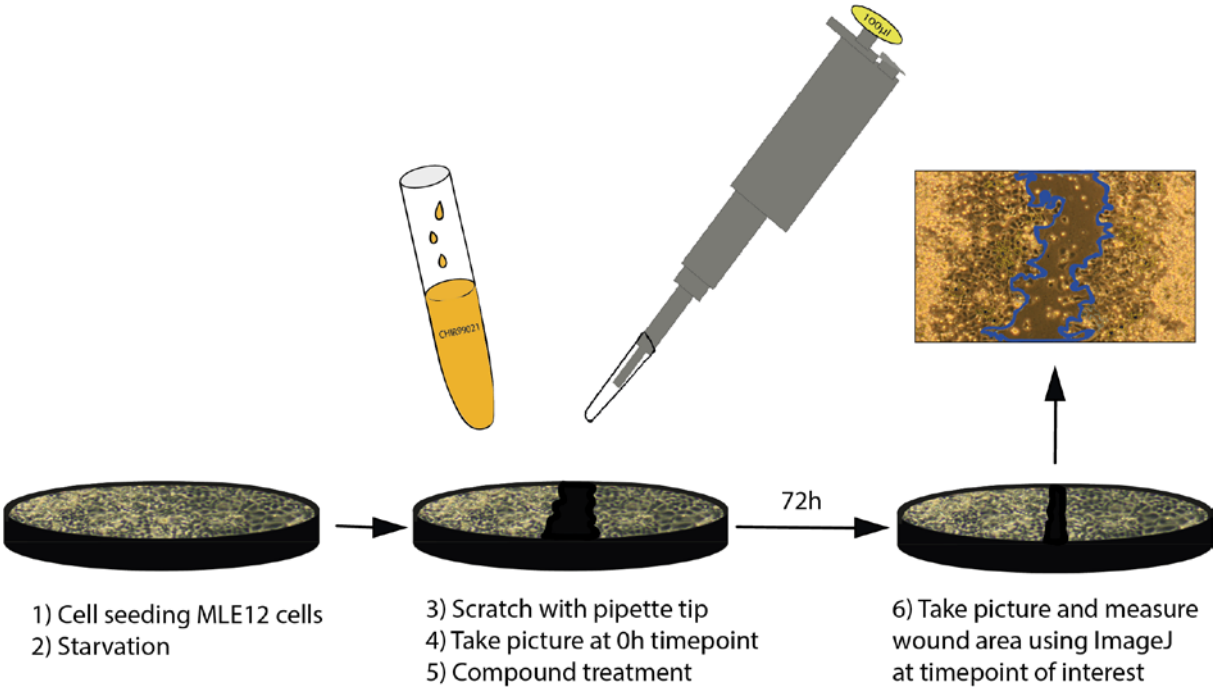



**Figure 7: MLE12 Scratch Assay.**

### 3.9 Western Blot of Active Beta-Catenin

MLE12 cells were seeded on a 6 well plate with a density of 0,5 Million cells/well in DMEM media FCS 10%, starved overnight with DMEM/F12 FCS 0,1% treated with 5 FDA compounds and stopped after 3,6 and 24 hours by washing each well with cold PBS and freezing them upside down at -80°C until further analysis.

T-PER containing proteinase and phosphatase inhibitors was used for protein extraction. The protein concentration was determined with Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

PVDF membranes were used for blotting, anti-active beta catenin clone 8E6 (merckmilipore) as primary antibody and incubation at 4°C overnight, anti-mouse IgG (GE Healthcare) as secondary antibody with 1hour incubation time and ß-actin (abcam) for later quantification. Nonfat Milk 5% (dissolved in TBS-T 0,1%) was used as blocking agent (incubation time 1 hour) and antibody dilution agent. For detection of protein bands Thermo Scientific SuperSignal West Dura was used and imaged by a ChemiDoc XRS+ device (BioRad, Hercules, CA, USA). Membranes were later stripped (Restore PLUS Western Blot Stripping Buffer - Thermo Scientific), blocked with 5% milk and incubated with beta catenin as primary antibody (Cell Signalling) and later anti-rabbit IgG secondary antibody.

**Table 5: Western Blot Material**

| Material | Manufacturer | Product No. |
|---|---|---|
| T-PER | Life Technologies | 78510 |
| PhosphoStop | Roche | 04 906 837 001 |
| Complete Proteinase Inhibitor | Roche | 11 836 153 001 |
| Nonfat dried milk powder | AppliChem | A0830 |
| BSA | Sigma Aldrich | A-2058 |
| SuperSignal Dura | Life Technologies | 34075 |
| SuperSignal Femto | Life Technologies | 34095 |
| Pierce BCA Protein Assay Kit | Life Technologies | 23227 |
| PVDF | Sigma Aldrich | P2938 |
| Stripping Buffer | Life Technologies | 46430 |
| Protein Marker V | VWR PeqLab | 27-2210 |

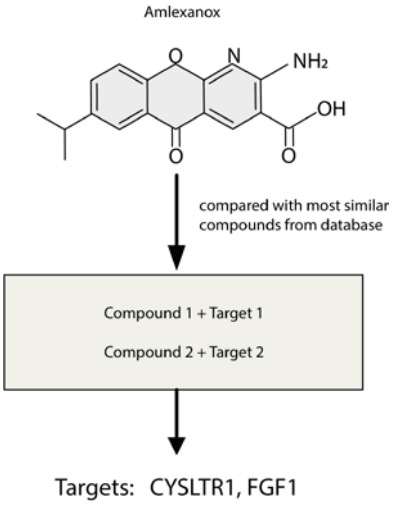
**Table 6: Western Blot Buffers**

| Buffer | Compounds | Concentrations |
|---|---|---|
| Running Buffer | Tris<br>Glycine<br>SDS | 25mM<br>192 mM<br>0,1% |
| Transfer Buffer | Tris<br>Glycine<br>Methanol | 25mM<br>192 mM<br>20% |
| TBS-T p 7.6 | Tris<br>NaCl<br>Tween-20 | 20mM<br>135mM<br>0,1% |
| 4x Laemmli Buffer | Tris<br>SDS<br>Glycerol<br>Bromo<br>-phenol Blue<br>DDT<br>(added before use) | 250mM<br>5%<br>40%<br>0,005%<br><br>10% |

**Table 7: Western Blot Antibodies**

| Antibody | Dilution | Molecular Weight | Host | Manufacturer | Product No. |
|---|---|---|---|---|---|
| <u>Primary Antibodies:</u> | | | | | |
| Anti-ABC ,clone 8E6 | 1:1000 | 92 kDa | Mouse | merckmilipore | 05-665 |
| Anti-ß-catenin | 1:1000 | 92 kDa | Rabbit | Cell Signalling | 9562 |
| ß-actin | 1:50000 | 42 kDa | Mouse | Abcam | Ab20272 |
| <u>Secondary Antibodies:</u> | | | | | |
| HRP conjugated Anti-Rabbit IgG | 1:3000 | | Donkey | GE Healthcare | NA934V |
| HRP conjugated Anti-mouse IgG | 1:5000 | | Sheep | GE Healthcare | NA931V |

## 3.10 Hitpick




As using a high throughput screening will usually end up in a vast amount of data instead of a single hit, the challenge of a HTS remains to narrow hits down and choosing the right compounds for further analysis.

To address this problem, a bioinformatical approach for target prediction was used.

Based on the 2D molecular fingerprint method(1NN similarity searching) the software is able to defragment the chemical compound of interest and compare it with the chemical structure of a compound where a target-compound interaction is said to be known (Cereto-Massague et al., 2015).

**Figure 8: HitPick**

The web server Stitch is hereby used as a reference databank for the known target-compound interactions (Liu, Vogt, Haque, & Campillos, 2013). The more similar the compound of interest is compared to the compound of the stitch database, the more likely it will have the same target.

Using the Laplacian-modified naıve Bayesian target model (Nidhi, Glick, Davies, & Jenkins, 2006) which is based on an algorithm calculating conditional probability, a list of ranked targets are calculated showing the targets of the most similar compound with the highest ranking score.

## 3.11 Statistics

Statistical analysis was performed with GraphPad Prism 7. All values are shown as means with standard deviation. Column statistics with a hypothetical value of one was used for statistical analysis of luciferase assay, WST-1 and MTT assay. For comparison of multiple groups, statistical analysis of scratch assay and immunoblotting was performed with one way ANOVA followed by either Dunett's or Tukey's multiple comparison test. Values were considered as statistically significant when p-value < 0,05.

# 4. Results

## 4.1 Preliminary results: Compound Selection

### 4.1.1 HTS giving 16 positive hits from FDA Prestwick library for Wnt activating compounds in 3T3 cells

Our group previously conducted a high-throughput screen using an NIH 3T3 reporter cell line which stably expresses luciferase under the control of Wnt responsive promoters (TCF/LEF) (Costa et al. *In preparation*). We used the Prestwick library, a library with 1280 FDA approved drugs.

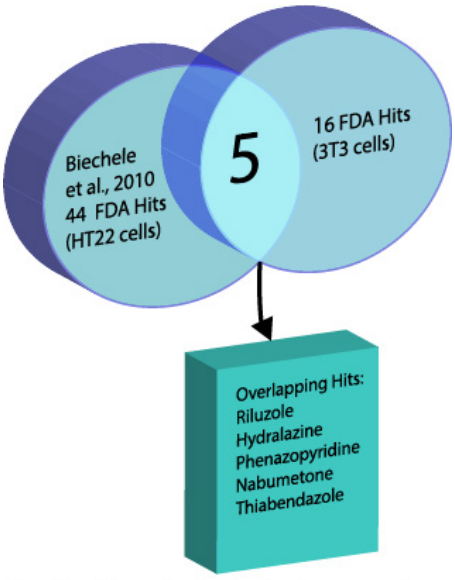


Figure: Venn Diagram showing 5 overlapping compounds between our screen and a screen from a prevoius Wnt study.

To determine whether a compound was a positive hit (i.e. Wnt inducer), the z-score method was applied which reveals how many standard deviations a value is away from the mean. Whenever the z-score was greater than 3 a hit was determined to be positive. Using this method, we identified 16 positive hits in our screen. We next compared the results from the FDA Prestwick Library with the previously mentioned screen from the Moon lab (Biechele et al., 2010) and found that 5 out of 16 FDA compounds were in common between the two independent screens.

**Figure 9: Venn Diagram**

To narrow the 16 hits further down, potential protein targets for each compound were determined with the target prediction tool from the HitPick Software.

These predicted targets were further examined via literature search and we searched for their association with Wnt, inflammatory regulation and epithelial cell proliferation.

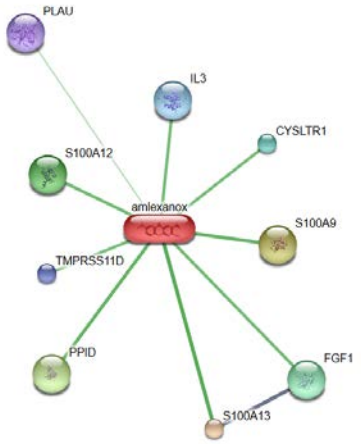
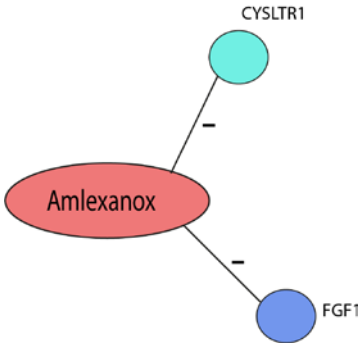
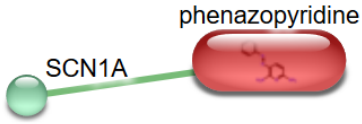
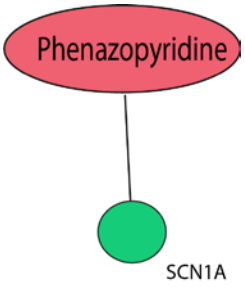
The compounds were thus filtered from those without positive target associations.

Taken together, a list of total 5 candidate drugs was generated for further investigations.

| Library | Compound | Predicted Target |
|---|---|---|
| Prestwick FDA | Tolnaftate | BCHE |
| Prestwick FDA | Amlexanox | CYSLTR1; FGF1 |
| Prestwick FDA | Nabumetone | PTGS1/2 |
| Prestwick FDA | Tiaprofenic Acid | PTGS1/2 |
| Prestwick FDA | Phenazopyridine | SCN1A |

**Table 8: 5 candidate drugs**

## 4.1. 2 Preliminary results HitPick: Target Prediction for 5 FDA candidate drugs

| Stitch Database Targets (www.stitch.embl.de) | HitPick Target Prediction (www.mips.helmholtz-muenchen.de/hitpick) | Target Description (www.genecards.org) |
|---|---|---|
|  |  | **CYSLTR1= Cysteinyl Leukotriene Receptor 1** Stimulated by leukotrienes and involved in bronchoconstriction, proliferation of smooth muscle and migration of eosinophils. Acitivates phosphatidylinositol-calcium second messenger system. Upregulated and associated with Asthma.<br><br>**FGF1= Fibroblast Growth Factor 1** involved in embryogenesis and organogenesis. Regulates cell growth, survival,differentiation and division. Ligand for FGFR1 at the presence of heparin. |
|  |  | **SCN1A = Sodium Voltage-Gated Channel Alpha Subunit 1** Regulates sodium transport into and out of a cell. Important for generating action potentials in muscle cells and neurons |

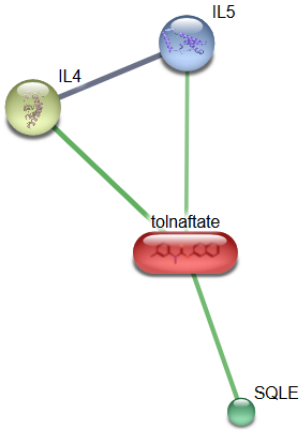
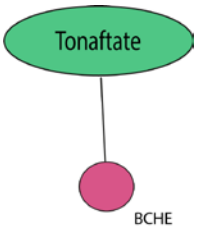
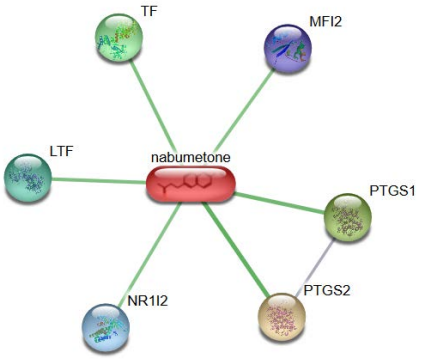
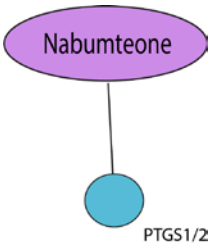
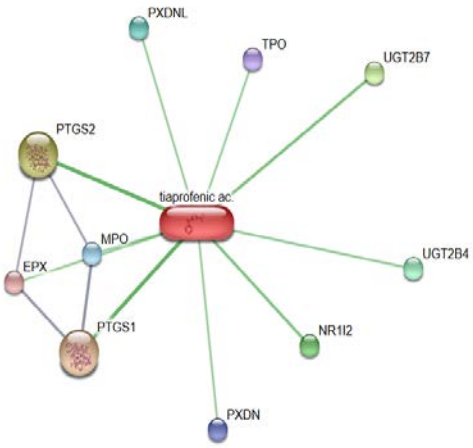
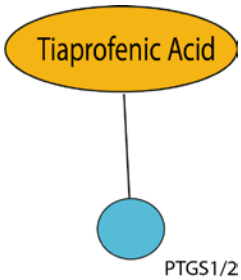
| Stitch Database Targets (www.stitch.embl.de) | HitPick Target Prediction (www.mips.helmholtz-muenchen.de/hitpick) | Target Description (www.genecards.org) |
|---|---|---|
|  |  | **BCHE = Butyrylcholinesterase.** Inactivates Acetylcholine. Is mostly expressed in the liver. |
|  |  | **Prostaglandin-Endoperoxide Synthase 1 and 2** Catalysizes the conversion from arachonidade to prostaglandin Contributes to cytoprotection and production of thromboxan A2, which promotes platelet activation and aggregation, vasoconstriction and proliferation of vascular smooth muscle cells. |
|  |  | **Prostaglandin-Endoperoxide Synthase 1 and 2** Catalysizes the conversion from arachonidade to prostaglandin Contributes to cytoprotection and production of thromboxan A2, which promotes platelet activation and aggregation, vasoconstriction and proliferation of vascular smooth muscle cells. |

**Table 9: Target Table.**

## 4.2 Literature Research

### 4.2.1 Initial Indications of FDA compounds

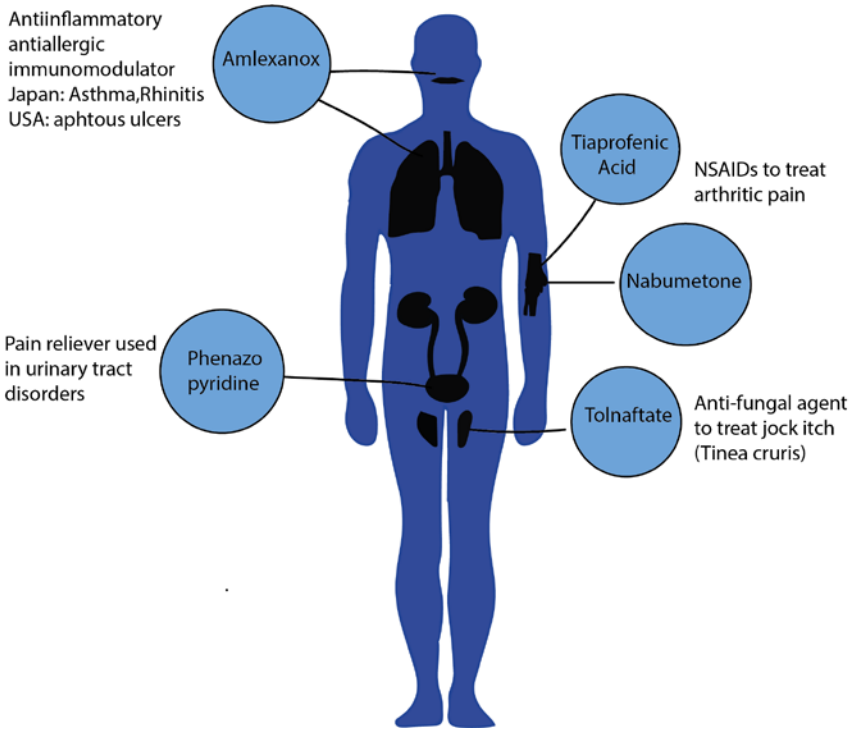



**Figure 10: Clinical indications of 5 FDA drugs.**
**Source:** https://pubchem.ncbi.nlm.nih.gov/ **accessed on Sept. 26, 2017.**

### 4.2.2 Pathways of compounds

#### 4.2.2.1 Tolnaftate

Although Tolnaftate is an FDA approved anti-fungal agent, little is known about its mechanism of action.

Is is assumed that Tolnaftate inhibits fungal growth by inhibiting squalen peroxidase which is needed for the synthesis of ergosterol, a main component of fungal membranes (Ryder, Frank, & Dupont, 1986).

According to recent literature, Tolnaftate has also been identified as a Hedgehog Inhibitor in a small molucule screening (Lipinski & Bushman, 2010) which used two independent assays using both mouse and human cell lines: immortilized PTC1 -/- mouse embryonic fibroblasts (MEFs) and Shh-responsive primary normal dermal human fibroblasts.

Like the Wnt pathway, hedgehog signalling plays a crucial role during embryionic development, regulating cell growth and differentiation and modulates the proper segmentation as a segment polarity protein.

A misregulation of hedgehog in humans is involved in many stem cell related diseases such as facial disformations and holopresencephalie, a failure of the forebrain to develop into two distinguished hemispheres (Belloni et al., 1996). Moreover, various forms of cancer, such as basal cell carcinoma, have high upregulation of hedgehog signaling

(Unden, Zaphiropoulos, Bruce, Toftgard, & Stahle-Backdahl, 1997; Xie et al., 1998). Vismodegib was the first FDA approved drug for treating basal cell carcinoma and it specificly inhibits hedgehog signalling; it has shown a tremendous reduction of cancer progression and thus shows the efficacy of modulating such stem cell signalling pathways. (Proctor, Thompson, & O'Bryant, 2014; Von Hoff et al., 2009)

It is interesting to mention that both Hedgehog and Wnt signalling share GSK3 and CK1 as pathway components and thus they influence and regulate each other in a form of cross signalling under certain contexts. Though the exact mechanism of the interplay remains elusive, recent literature suggests that there is an antagonistic effect between these pathways in a way that Wnt activation leads to an inhibition of Hedgehog signalling and vice versa. (Borday et al., 2012; M. Tang et al., 2010).

Considering that Hedgehog interacting protein (HHIP) has been shown to have decreased expression levels in COPD patients (X. Zhou et al., 2012), thus leading to an over-induction of hedgehog signalling, it implies that both Hedgehog and Wnt are both important signalling pathways contributing to the pathogenesis of COPD and may depend on each other. As Tolnaftate has been shown to be a Hh Inhibitor, its ability to be a  a Wnt pathwath activator in our screen may indicate potential direct or indirect effects on both pathways to induce Wnt signaling. Therefore, it may be an interesting candidate drug for modulating these deranged pathways in COPD patients. Investigating possible targets of Tolnaftate might also give new insights of underdstanding the interplay between Hedgehog and Wnt.

#### 4.2.2.2 Phenazopyridine

Phenazopyridine is currently used as a local anaesthetic in urinary tract disorders, yet its use is rather limited due to its toxic and its potential severe side effects like methemoglobinemia (Yu, Wang, & Chang, 2011).

Its mechanism of action is unknown and the amount of available literature illuminating this unresolved question is limited.

Interestingly, a previous link between tissue regeneration and the differentiation of embryonic stem cells has been made with Phenazopyridine (Suter, Preynat-Seauve, Tirefort, Feki, & Krause, 2009)

Here, the authors identified Phenazopyridine whithin a screening for molecules modulating neuronal differentiation of human embryonic stem cells.

To screen for enhancement of neuronal differentiation, lentivectors were used carrying a dual luciferase reporter system in murine CGR8 embryionic stem (ES) cell line. Phenazopyridine was identifed as a positive hit and then tested on H1 human ES, in which it enhanced neuronal differentiation and generated a monolayer of synchronized neural progenitors (Suter et al., 2009).

TWS119 another GSK3ß Inhibitor has been previously recognized to induce neurogenesis in murine P19 embryonic stem cells (Ding, Wu et al., 2003). Thus, similar processes might be involved both in  Phenazopyridine as with TWS119 either through GSK3ß Inhibition and thus Wnt activation or yet unknown mechanisms which determine cell fate and induce neuronal differentiation.

Indeed an overexpression of Wnt-1 has also been observed in a previous study (K. Tang et al., 2002) to induce neuronal differentiation in the same cell line P19, in the abscence

of retinoic acid, which is used for facilitating and fostering cell differentiation like in neurogenesis (Janesick, Wu, & Blumberg, 2015).

Collectively, this data supports the idea that Phenazopyridine may have induced its neurogenerative effect by activating Wnt/ß-catenin signaling.

Whether a similar effect can be observed in an emphysema model still needs to be determined but makes Phenazopyridine nevertheless an interesting candidate drug for Wnt modulation.

#### 4.2.2.3 Nabumetone and Tiaprofenic Acid

Both Nabumetone and Tiaprofenic acid are non-steroidal antiinflammatory drugs (NSAIDs) especially used for arthritic pain (Hedner et al., 2004; Sorkin & Brogden, 1985). Nabumetone is known to act through its metabolite 6-MNA. Both drugs inhibit cyclooxygenases (COX) and thus they diminish the production of prostaglandins causing inflammatory responses after injury.

Nabumetone was previously identified to be capable of enhancing reprogramming of murine embyrionic fibroblast cells (MEF) through COX2 inhibition and was also able to generate iPSCs without the presence of c-myc and Sox2, which are part of the 4 factors promoting iPSC induction (Yang, Lopez, & Rana, 2011). C-myc and Sox2 are both Wnt target genes and thus Nabumetone may have induced their expression through Wnt modulation.

The role of COX2 in developmental pathways, its inhibition by NSAIDs and its relation to Wnt though has not been previously identified.

The overlapping of Nabumetone from two independent screens for finding Wnt inducing molecules (see figure 9: Venn Diagram) however, supports its Wnt modulating ability.

#### 4.2.2.4 Amlexanox

Amlexanox is an anti-inflammatory, anti-allergic drug and has been used in Japan for treating asthma (Inagaki et al., 1992) and chronic rhinitis (Okubo et al., 2017) for many years. It is known to have antihistaminic effects by inhibiting IgE mediated histamine release from mast cells, possibly by elevating c AMP levels, an important regulator for mast cell histamine release (Makino, Saijo, Ashida, Kuriki, & Maki, 1987).

Moreover, it also inhibits lipoxygenase and thus reduces leukotriene release, which benefits asthma and COPD patients, as leukotrienes have bronchoconstrictive effects (Saijo et al., 1986).

In the USA, Amlexanox is used as a 5% oral paste against aphtous ulcers which may be associated with Behcets disease (a vasculitis, an inflammation of small blood vessels) or HIV but also appear in otherwise healthy individuals. It improves healing and relieves ulcer related pain (Bell, 2005; Khandwala, Van Inwegen, & Alfano, 1997).

In recent literature, Amlexanox was revealed to inhibit the interaction between S100A4 and EGF thus preventing the binding of EGF to the EGF receptor. It is therefore assumed to have antagonistic effects on EGFR related pathways (Cho, Chou, & Yu, 2016).Induction of EGFR activates signalling transduction pathways such as MEK/ERK, PI3K/AKT, m TOR and STAT (Jorissen et al., 2003) and is important for cell differentiation,proliferation, cell growth and survival.

In the context of lung diseases such as COPD, it has been shown that excessive mucus production, increase of MUC5AC and hyperplasia of goblet cells is associated with increased cytokine driven EGFR signalling (Takeyama et al., 1999; Vallath, Hynds, Succony, Janes, & Giangreco, 2014). The use of specific EGFR inhibitors were shown to diminish mucus overproduction (Kohri et al., 2002), hence suggesting that Amlexanox could benefit COPD patients in this respect as well and that this may be through EGF antagonism.

S100A4 has been preivously shown to be highly upregulated in intrapulmonary arteries of COPD patients and of mice with cigarette smoke induced emphysema and might thus contribute to the vascular remodelling in COPD (Reimann et al., 2015). Amlexanox may act competetively with S100A4 and might thus have a positive influence in the remodelling process. An increased availability of S100A4 in COPD might also explain the increased mucus production through EGFR signalling.

Supporting the results of HitPick target prediction, Amlexanox was found to be an inhibitor of fibroblast growth factor 1 (FGF1) release (Rajalingam et al., 2005) by directly binding to FGF1 and blocking its Cu2+ induced homodimerization, which is essential for extracellular release when cells are under stress.

In a study from Kranenburg et al., it was shown that FGF1,FGF2 and FGFR1 expression is increased in the bronchial epethelium of COPD lung tissue and possibly contributes to the airway remodeling (Kranenburg et al., 2005). Amlexanox as a potential FGF1 inhibitor might thus again fit the bill in counteracting the pathological conditions in COPD.

In addition to its usage as an anti-inflammatory drug for ulcer and asthma treatment, amlexanox has also shown promise as a new compound for obese patients or diabetic patients. Amlexanox administration improved insulin sensitivity, reduced weight and steatosis of obese mice and was shown to be a specific IKKe and Tank-binding kinase 1 (TBK1) inhibitor(Reilly et al., 2013). IKKe and TBK1 are highly elevated in fat and liver of obese mice upon NF-kB activation, a pro-inflammatory transcription factor.

The inhibition of this inflammation driving pathway was recently also under clinical trial with human diabetes type II patients (NCT number: NCT01975935), confirming the observation of increasing energy expenditure and improving glucose control under Amlexanox (Oral et al., 2017).

Chronic inflammation and Nf-kB not only play a role in this given example of obesity and diabetes but also in COPD and emphysema (Schuliga, 2015). Nf-kB activation was also shown to be necessary for MMP9 gene expression,a protease relevant in COPD and ECM remodelling, which was inhibited by BMS-345541, a specific IKK-2 Inhibitor in RAW 264.7 cells, a murine macrophage cell line (Rhee et al., 2007)

Targeting Nf-Kb and IKKs therefore seems reasonable. However, there are many discrepancies between different studies, in which it has been also proposed that IKK-2/Nf-kB signalling does not contribute to the inflammatory response of cigarette smoking and may not be relevant for COPD pathogenesis (Rastrick et al., 2013). However, this could be explained by using different in vivo models and thus involving different immunological responds of mice and rats.

The role of Nf-kB and IKKs in cigarette induced airway inflammation therefore remains controversial, and further studies have to be done.

In terms of Wnt/beta catenin signalling however, Amlexanox might provide an important link between IKKe Inhibition and ß-catenin activation (Chen et al., 2017). Here, the authors investigated whether the activation of ß-catenin is able to supress proliferation of colorectal cancer cells, which was indeed observed under IKKe inhibitor Amlexanox. They show that IKKe directly interacts with ß-catenin and induces phosphorylation of serine residues 675,680 and 681. A knockdown of IKKe moreover resulted in increase of c-myc and cyclinD1,which are both downstream of ß catenin.

All in all, one can summarize that all 5 FDA drugs show great potential to be used in Wnt modulation as described in previous literature. The challenge remains however to see comparable effects in different cell lines and different in vivo models and it is therefore hard to predict the efficacy within lung in vivo and in vitro models.

## 4.3 Results of Secondary Assays

### 4.3.1 Luciferase Assay: 5 FDA drugs are Wnt activators in 3T3 cells

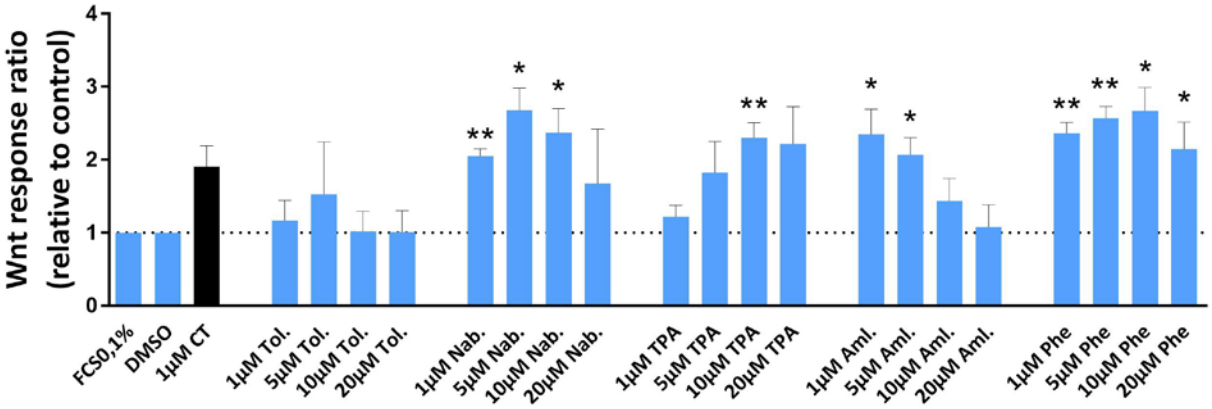



**Figure 11: Luciferase Assay: 5 FDA drugs are Wnt activators in 3T3 cells.** 3T3 cells stably expressing luciferase under the control of a Wnt responsive TCF/LEF promoter were treated for 24h with the 5 FDA compounds in 4 concentrations (1-20µM), lysed and analysed with a luminometer after adding luciferin. Data represents the mean of triplicates from 3 independant experiments +/- SDs. * P< 0,05, ** P< 0,01 ,using column statistics.

Repeating the luciferase assay with the 5 chosen FDA candidate drugs, the results validated the highthroughput screening and confirmed all 5 compounds as Wnt activators in 3T3 cells compared to DMSO and 1µM CHIR99021 (CT) as a positive control. While there is only a weak Wnt response for Tolnaftate visible, a dose dependent signal reduction can be observed in Tiaprofenic acid and Amlexanox. Statistical significance was verified for Nabumetone at 1µM,5µM and 10µM, for Tiaprofenic Acid at 10µM, for Amlexanox at 1µM and 5µM and for Phenazopyridine at all concentrations from 1 to 20µM.

### 4.3.2 WST1 Assay (3T3 cells)

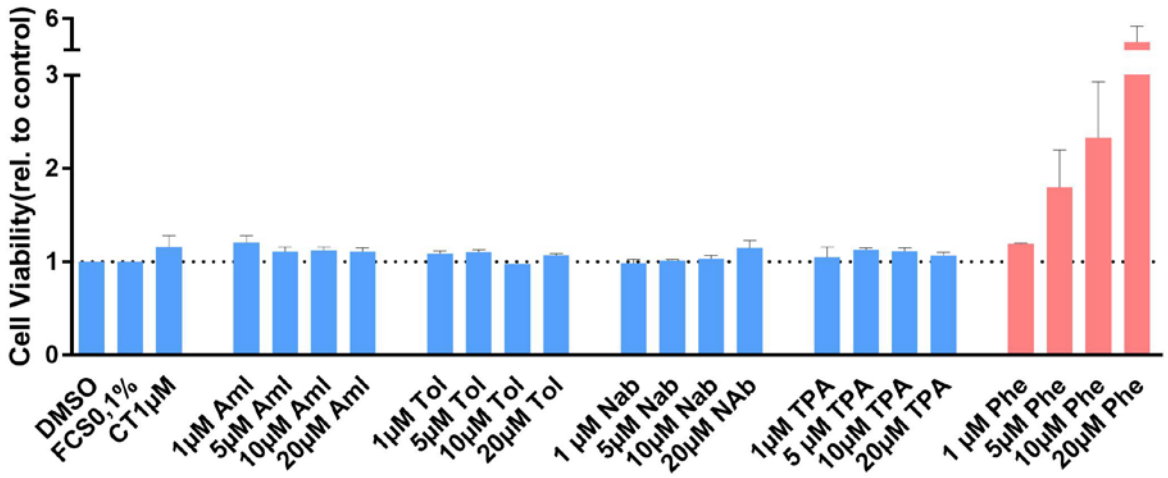



**Figure 12: WST1 Assay: Phenazopyridine as a colored compound shows a strong dose dependent induction.** 3T3 cells were exposed to compounds for 24h at indicated concentrations, supernatant was removed and then tested for cell viability using a WST1 assay. A noticeable induction was observed in the colored Phenazopyridine compound. Data represents the mean of triplicates from two independent experiments +/- SDs . Experiment was discontinued for further troubleshooting, optimization and testing whether the induction is color dependent and therefore false positive.

Testing the 5 FDA drugs in 3T3 cells for toxicity and to rule out that increases of cell number were just due to proliferation, we observed a striking dose dependent induction for Phenazopyridine in the WST1 assay. Knowing that Phenazopyridine is a reddish colored compound and reported to be rather toxic, we then assessed whether the increase in absorbance at 450 nm might be due to the increased absorbance attributed to the increased drug concentration and thus might have led to a false positive.

### 4.3.3 Wst1 Absorbance Measurement: Why to choose MTT and what to do when testing a colored compound reacting with reagent

An absorbance measurement of all FDA compounds at 20µM revealed that the colored Phenazopyridine absorbs clearly more light at 450nm than the other non colored compounds (see figure 13).

This also applied to Phenazopyridine containing no cells (NC, orange line) and Phenazopyridine as a pure compound, containing no cells and no WST1 substrate (NCNW, red line). Hence showing that the interference is due to the color and not due to any possible reducing components of the compound that might lead to a non-enzymatic reduction of the WST1 tetrazolium substrate to Formazan.

We therefore searched for a cell viability assay which would measure the absorbance in a range outside of which might interfere with our compounds (i.e. maximum absorbance far from 450nm).

The MTT assay seemed to be a reasonable alternative since the absorbance of the purple colored product Formazan is measured at 570nm and thus within the right range of measuring correct cell viability of cells treated with Phenazopyridine.

The comparison between the WST1 and MTT assay quantification of Phenazopyridine could confirm that the induction in WST1 was indeed color dependent (see figure 13).

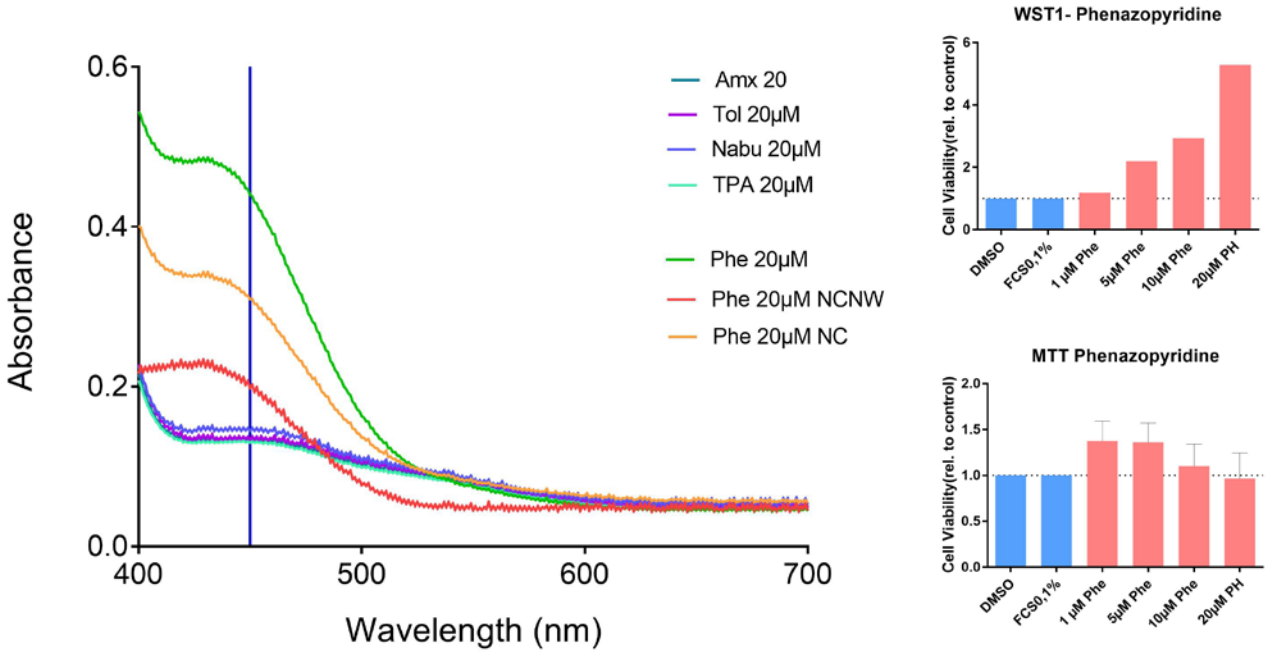



**Figure 13: WST1 Absorbance measurement.** a) Absorbance measurement revealed a color interference between Phenazopyridine and the WST1 product Formazan measured at 450nm. b) quantification of WST1 and MTT confirm that induction was color dependent as MTT Formazan absorbance is measured at 570nm. Both assays were done in 3T3 cells with a 24 hour treatment time of Phenazopyridine.

### 4.3.4 MTT Assay (3T3/MLE12 cells): Phenazopyridine and Tolnaftate show dose dependent toxicity in MLE12. No visible toxicity in 3T3 cells.

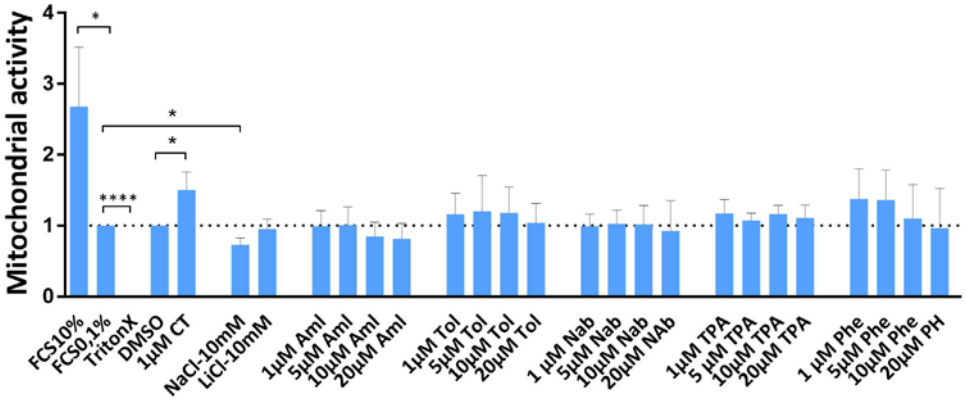



**Figure 14: MTT Assay in 3T3 cells shows no toxicity for 5 FDA compounds after 24h.** Cells were exposed to compounds for 24h at indicated concentrations. Data represents the mean of triplicates from 4 independant experiments +/- SDs. * , P<0,05 ;****,P< 0,0001 using Column statistics and hypothetical value = 1.

Optimizing the viability assay we not only switched to MTT due to the color interference but also covered the 96 well plates with Pol-L-Lysine to improve 3T3 cell attachment. Moreover NaCl, LiCl, Triton X (causing cell lysis) and FCS 10% (inducing cell growth) were added as further controls.

The results show that the 5 FDA compounds do notinduce proliferation, or result in increased metabolic activity nor are they toxic to the 3T3 cells after a treatment time of 24 hours within a concentration range of 1-20µM.

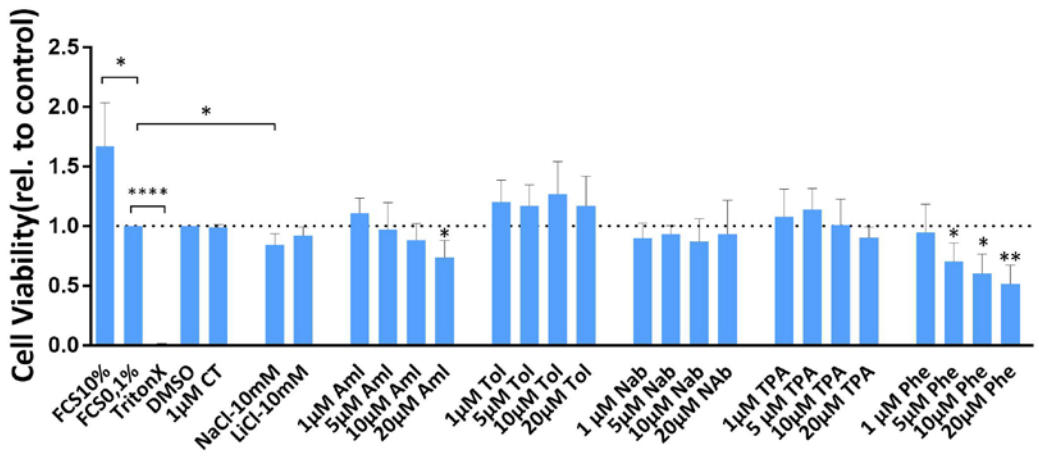



**Figure 15: MTT Assay in MLE12 cells: Phenazopyridine and Amlexanox show dose dependent toxicity.** Cells were exposed to compounds for 24h at indicated concentrations. Data represents the mean of triplicates from 4 independent experiments +/- SDs. *, P<0,05 ;**, P< 0,01; ****,P< 0,0001 using Column statistics and hypothetical value = 1.

Besides testing the 3T3 fibroblast cell line that was initially used in the highthroughput screening, the murine lung epithelium MLE12 cell line was further tested for toxicity as a next step as the lung epithelium cells are the cells of interest in which Wnt induction is proposed to be beneficial for inducing repair in lung alveoli. Repeating the MTT assay with murine lung epithelial cells MLE12 revealed a dose dependent toxicity for Phenazopyridine and Amlexanox.

### 4.3.5 Scratch Assay: Amlexanox, Nabumetone and TPA show wound closure after 72h at 1µM

#### 4.3.5.1 Wnt activation via Wnt3a and GSK Inhibition with CHIR99021 induces wound closure in MLE12 cells after 72h.

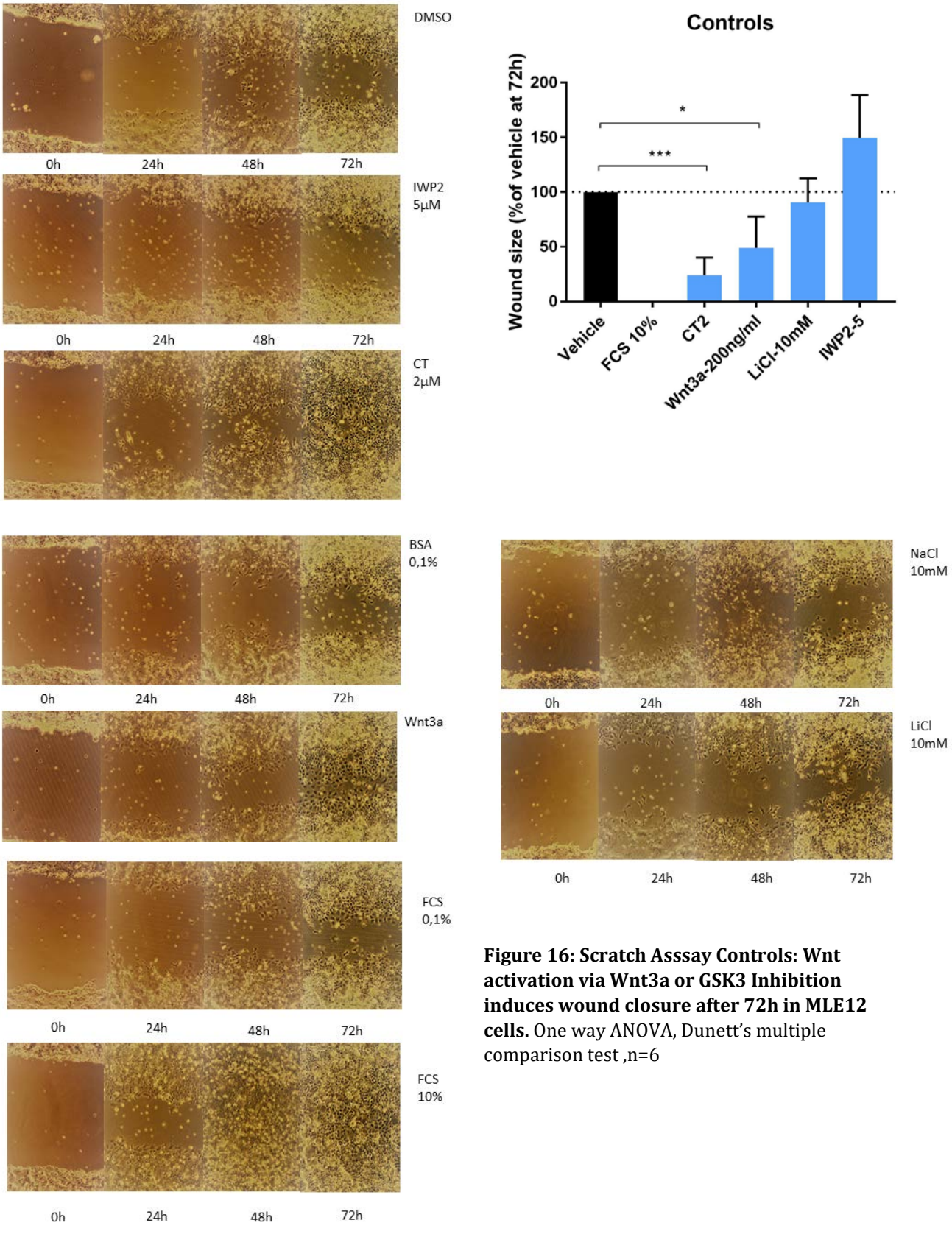



**Figure 16: Scratch Asssay Controls: Wnt activation via Wnt3a or GSK3 Inhibition induces wound closure after 72h in MLE12 cells.** One way ANOVA, Dunett's multiple comparison test ,n=6

To mimic cell migration during wound healing *in vivo* and verify that Wnt signaling is able to induce wound closure *in vitro*, we first tested the ability of Wnt3a, CHIR99021 and LiCl to close scratch wounds in murine lung epithelial cells. We used IWP2, a Porcn inhibitor and thus inhibitor of Wnt ligand secretion, as a negative control and FCS 0,1% vs FCS 10% for showing the functionality of the assay. Both Wnt3a and CHIR99021 demonstrated a visible wound closure after 72h. The GSK3 Inhibitor CHIR99021 showed the strongest wound closure capacity from all Wnt controls. Surprisingly, LiCl was not able to induce wound closure at a concentration of 10 mM.

### 4.3.5.2 Amlexanox, Nabutmetone and Tiaprofenic Acid show wound closure at 1µM treatment dose in MLE12 cells after 72h.

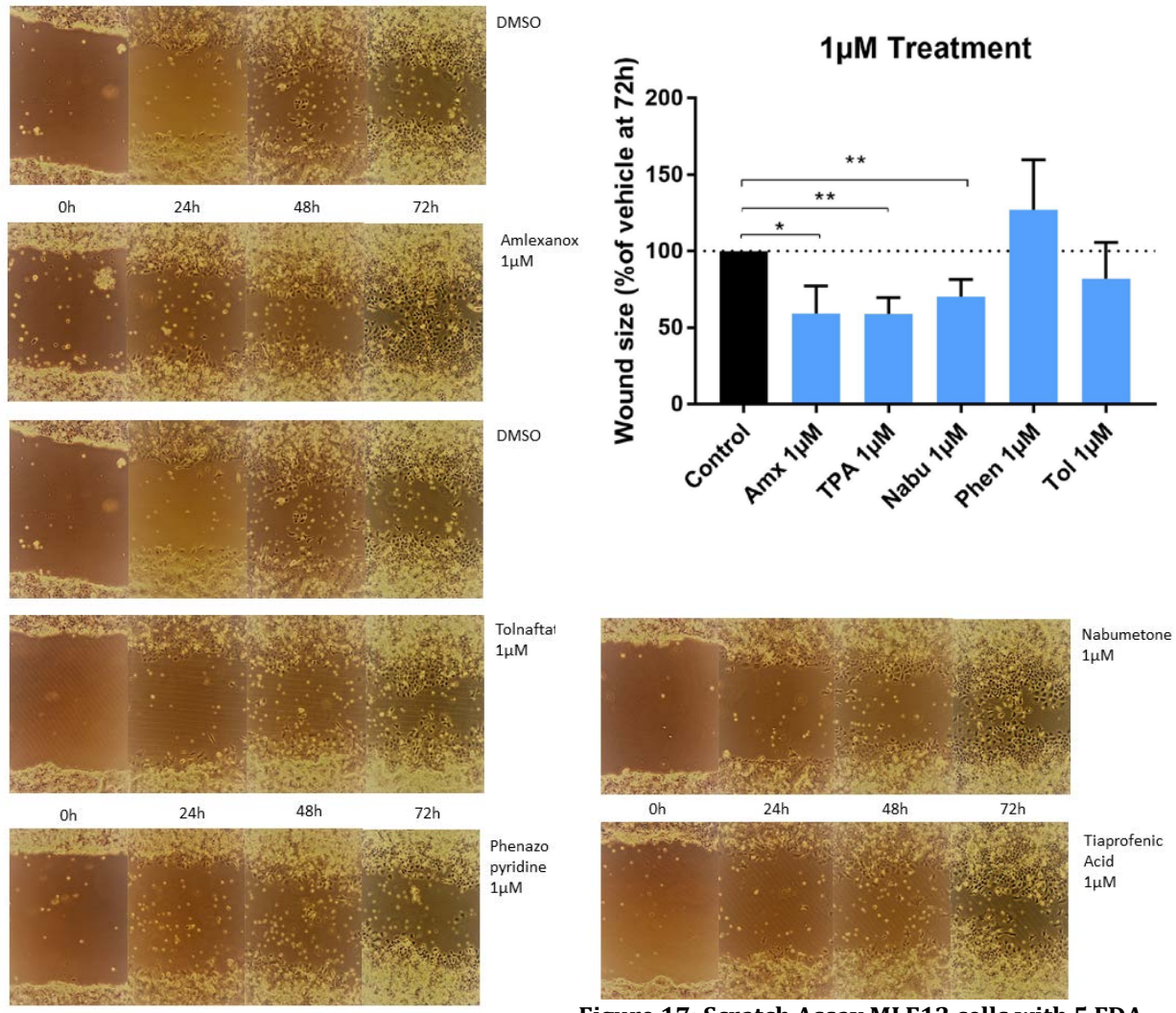


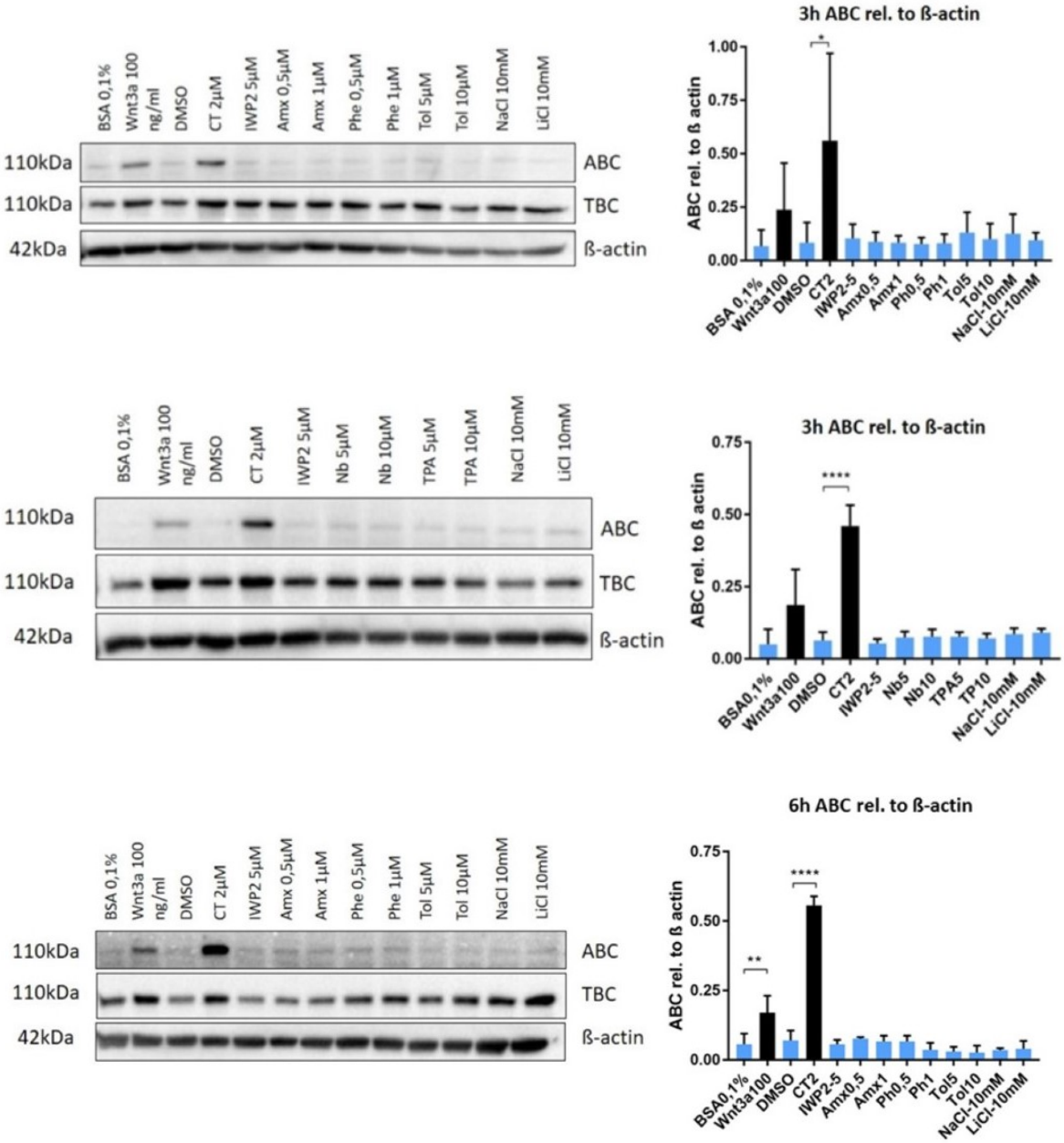

**Figure 17: Scratch Assay MLE12 cells with 5 FDA compounds: Amlexanox, Nabumetone and Tiaprofenic acid show wound closure at 1µM treatment after 72h.** ONE Way ANOVA Tukey's multiple comparison test (n=6). Representative images for 1µM treatment shown. *, P< 0,05; ** P< 0,01.

We next sought to test the wound closure ability of the 5 FDA compounds. We found that Amlexanox, Nabumetone and Tiaprofenic acid all induced wound closure at a low dose treatment of 1µM after 72h.

Both Tolnaftate and Phenazopyridine show toxicity signs for higher dose treatment than 1µM. This would go along with the results from the MTT assay where Phenazopyridine showed dose dependent toxicity.

### 4.3.6 Western Blot of Active beta-catenin:

#### 4.3.6.1 No ABC Induction in MLE12 cells after 3h, 6h and 24h treatment time.



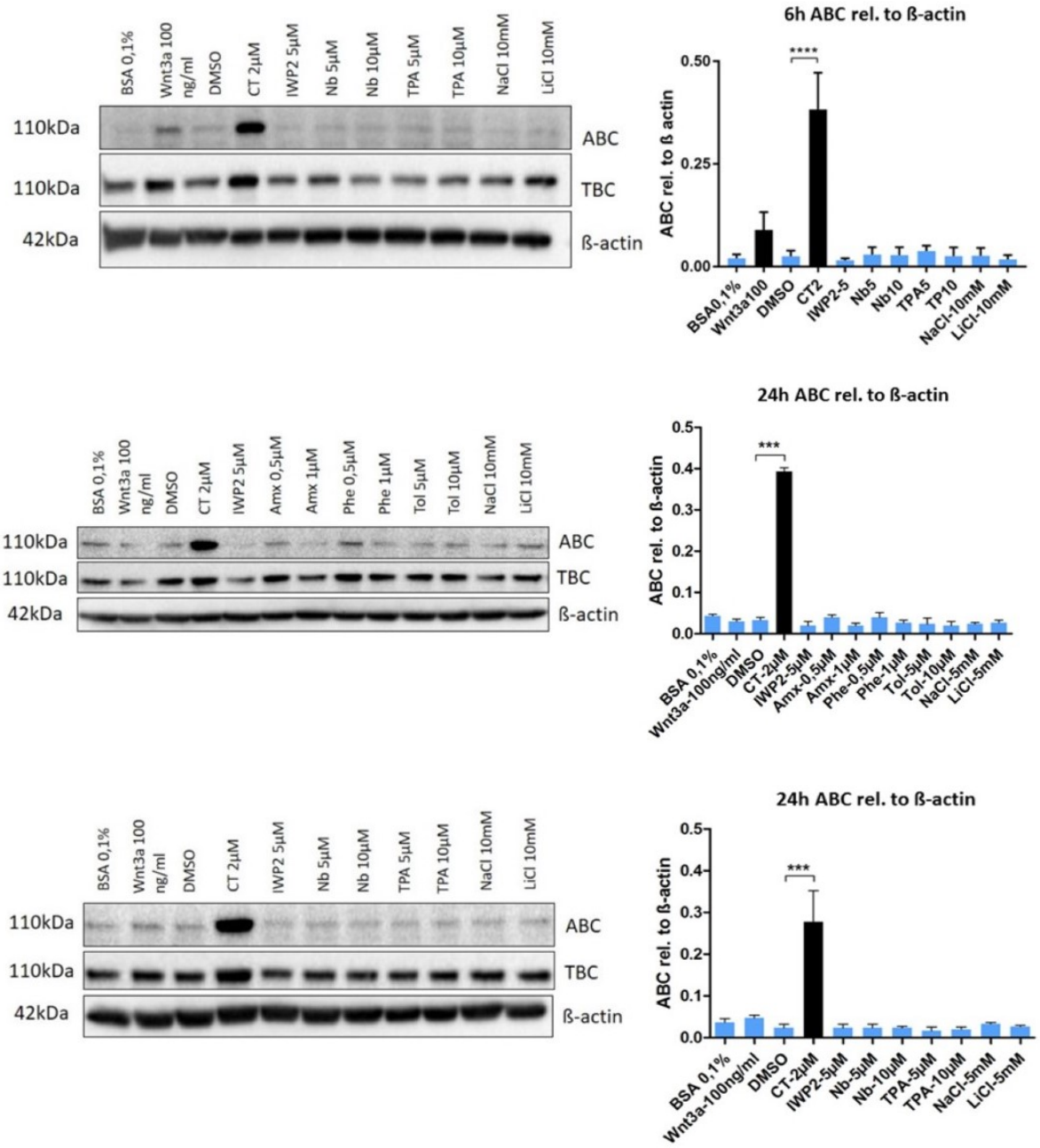



**Figure 18: Western Blot ABC. No active beta catenin induction in MLE12 cells.** Immunoblot and quantification of ABC in MLE12 cells (n=3), determined by one-way Anova. Followed by Tukey's multiple comparison test. ****, P < 0,0001,***,P<0,001, **, P < 0,01.

Active beta catenin (ABC) is an indicator for active Wnt signalling. When translocated into the nucleus it forms a complex with the TCF/LEF binding site and thus induces the transcription of Wnt target genes.

As canonical Wnt signalling is downregulated in human airway epithelium of COPD patients (Wang et al., 2011), murine lung epithelium (MLE12) cells were used as an in vitro model to mimic the state of injured lung alveoli with Wnt activity as an indicator for inducible regeneration.

The Western blot shows that none of the 5 FDA compounds considerably induced active beta catenin in MLE12 cells. As this translocation is known to be time dependant, we examined different time frames ranging from 3h to 6h and to 24h. However, we could

not observed any significant time dependant effect for any of the FDA compounds in MLE12 cells.

The positive control CHIR99021 as a highly potent GSK3 Inhibitor shows a consistent induction of active beta catenin throughout all timepoints, thus indicating that GSK3 inhibition results in ABC induction in MLE12 cells. Further, Wnt3a induced ABC visibly after 3 and 6 hours but not after 24 hours While LiCl failed to induce ABC throughout all the experiments.

# 5.Discussion

## 5.1 Summary of results

While the highthroughput screening could be validated by the luciferase assay and thus confirm that all 5 candidate drugs induce Wnt/beta catenin signaling after 24h in murine 3T3 mesenchymal fibroblasts and are not toxic for this cell line, major discrepancies were observed for murine MLE12 lung epithelium cells. Here, no induction of active beta catenin was visible for any of the compounds in the Western Blot experiment, also not for different time frames ranging from 3h to 6h and to 24h.

In the MLE12 cell line MTT revealed dose dependent toxicity for Tolnaftate and Phenazopyridine, which was also confirmed by the MLE12 scratch assay.

The scratch assay itself showed that Amlexanox, Nabumetone and Tiaprofenic acid all induced wound closure at a low dose treatment of 1µM after 72h, though it is not clear whether this is Wnt dependent or not.

## 5.2 Limitation of methods

One major limitation of the project is, that the screen started off with an immortilized mesenchymal 3T3 fibroblast cell line, though the secondary assays were implemented in MLE12 epethelial cells. Using an immortilized cell line was necessary to conduct a screen in a highthroughput manner. However, as Wnt/beta catenin signalling is downregulated in airway epithelial cells of COPD patients and to mimick these conditions of a lung, murine lung epethelial cells seemed the best choice. Gene expression, active beta catenin protein induction and the Wnt luciferase assays are all highly dependant on the cell type and thus Wnt activating compounds identified in a mesenchymal cell line may not activate the Wnt pathway in an epithelial cell. Moreover, the 3T3 cells were stably transfected with a Wnt reporter while testing MLE12 cells for Wnt induction would be TOP/FOP plasmid based, making it thus difficult to compare.

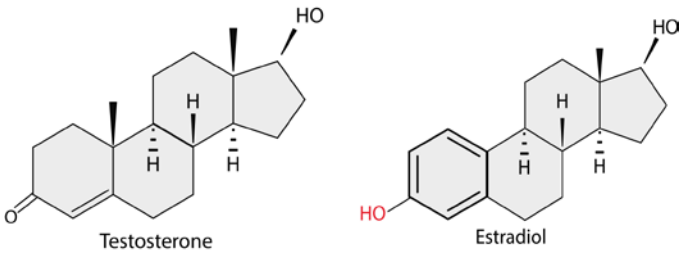
The fact that the Western Blot did not show induction of active- beta catenin in MLE12 cells under compound treatment supports this potential drawback.

Either the Wnt induction by the compounds was cell line specific and limited to 3T3 cells, the epitope was masked by the compounds preventing the antibody from binding or the compounds acted directly on luciferase activity without inducing TCF/LEF via beta-catenin translocation into the nucleus. It has been mentioned previously in literature though that the anti-beta catenin antibody 8E7 that recognizes ABC when unphosphorylated at S37 and T41 also shows cross-reaction with an unspecific nuclear antigen and might therefore be used with caution (Maher et al., 2009). So whether active beta catenin induction is really absent in MLE12 cells under compound treatment or whether it remains invisible due to the limitations of the antibody is not clear.

Further complementary assays would have been needed to answer this questions.

When selecting compounds based on target predictions, there is also a risk of choosing candidate drugs which were false positives in the initial HTS. The HitPick software is based on similarity search comparing the similarities of fragments from the chemical structure. However, it is not always the case that similar compounds share the same bioactivity. Hence, candidate drugs should always be validated using several assays to further characterize the compounds activity.

A classical and vivid example of similar structure but different biological activity are the sex hormones testosterone and estradiol. Both are steroids and share a similar structure yet the slight difference in the substitutents alter their biological function dramatically.




In silico methods are therefore a powerful tool in recognizing patterns and screening virtual databases but to determine a target of a new compound the precision for making a right prediction is currently rather low.

**Figure 19: Sexual Hormones.** Similar structure but different properties.

## 5.3 Is Wnt/beta catenin the right target for emphysema treatment?

The ubiquity of Wnt signalling from development to various diseases makes Wnt a promising and powerful target. At the same time, the ubiquity turns Wnt into a double edged sword as pharmaceutical modulation might induce negative side effects.

Given the complexity of COPD pathophysiology as previously described, it is likely not possible that the activation of one single pathway is able to cure a disease.

The interconnectivness of signalling pathways in general and the fact that the mechanism of Lithium and the consequence of GSK3 inhibition is not entirely known, it is more likely that several pathways are simultanously modulated by lithium leading to the observed attenuation of emphysema in the animal model.

GSK3beta, the main target of Lithium and CHIR99021 for instances, is next to Wnt signalling also known to be involved in hedgehog signalling, glycogen synthase, notch signalling and Reelin signalling. Even Lithium itself is rather promiscous and interacts with inositol monophosphatase, which is involved in IP-PKC pathway (Phiel & Klein, 2001), all highlighting the interconnectivness of various pathways.

The 5 compounds might be indeed Wnt activators, but the real targets within the pathway beyond the HitPick prediction have not been revealed and will be important to elucidate. Crosstalk reaction must be therefore considered leading to an indirect increase of Wnt signalling but may not cover those pathways that are also modulated by Lithium mediated GSK3 inhibition which might be necessary for the emphysema attenuating effect.

Moreover, it should be considered that constitutive upregulation of Wnt is known to cause cancer (Bienz & Clevers, 2000), which would already imply that Wnt therapy would have to be time restricted and maybe only used for those patients who have a detactable downregulation of this pathway that could be somehow monitored during the time of therapy.

Further in vitro experiments which help to elucidate the potential targets of the 5 FDA drugs and further in vivo experiments are needed to answer the question, whether any of the 5 FDA compounds can be used clinically for COPD.

## 5.4 Conclusion

In conclusion one can say, that the 5 compounds activate Wnt in mesenchymal 3T3 cells, though it remains unclear whether Wnt induction is also possible in MLE12 cells.

The results from the scratch assay show that there is indeed some regenerative potential for the compounds Amlexanox,Nabumetone and Tiaprofenic acid that all induced wound closure. Whether this is Wnt dependant or caused by a different mechanism can not be told.

From all 5 candidate drugs Amlexanox seems to be the most promising one as showing various targets that all contribute to the pathogenesis of COPD and emphysema.

In that way, COPD patients might benefit from Amlexanox not only due to Wnt induction which induces repair but also due to the anti-inflammatory features, its potential to reduce mucus production via EGF inhibition and by inhibiting bronchoconstriction as a lipoxigenase inhibitor, counteracting air way remodelling as a FGF1 and S100A4 inhibitor and inhibiting histamine release as a cAMP inducer.

Amlexanox shows many positive features that might benefit COPD patients. It has been used in Japan for Asthma treatment for a long time but is not used in other countries for this indication.The previously mentioned clinical trial (Oral et al., 2017) focusing on diabetes patients showed no severe side effects and we would therefore propose further in vitro and in vivo studies to investigate COPD modifying effects in a murine emphysema model that may be modulated by Amlexanox .

## 5.5 Future Perspectives: General thoughts on regenerative medicine

One major question that remains unresolved within the scientific community is why animals hugely differ in their regenerative capacity. While human and mammals in general have a rather low regenerative ability, creatures as flatworms, fish and salamander are able to regenerate entire limps when appendages have been removed.

Also, it is unclear why amphibians as tadpoles and axolotls which are in their aquatic larval stage loose their ability to regenerate once they go through metamorphosis becoming terrestrial adults.

Axolotls have a special role concerning this development as they will remain in neoteny due to a lack of thyroid stimulating hormone (TSH) unless metamorphosis is induced by the induction of iodine or thyroid hormones. This raises the question what kind of role iodine and the thyroid have in terms of regeneration and whether water might be a crucial element to enable scar free wound healing regaining full restoration of form and function.

It has been also proposed that the devolpment of a more mature and strong adaptive immune system is responsible for this loss of regenerative capacity as terrestrial life would also mean a greater exposure to pathogens.

Considering that frogs develop a more mature immune system after metamorphosis while likewise loosing regenerative capacity, this would also support this idea (Godwin & Brockes, 2006; Godwin & Rosenthal, 2014). In addition to that, it has been observed that mice lacking macrophages and neutrophils and thus genetically not able to full

immune response are able to induce scar-free healing comparable to the embryionic state where wound healing also occurs without immune response. (Martin et al., 2003) This would underline the neccesity to find new drugs for COPD patients that are not only able to induce repair through induction of developmental pathways as Wnt but also to diminish immune response to enable scar free wound healing.

Understanding the concept of tissue regeneration of amphibians as axolotls might thus give crucial hints on how regeneration in human occurs, what the major differences are and how it may be induced through Wnt/beta catenin signaling modulation.

Refering to amphibians, it has been shown that Wnt is important especially during the early stages of limb regeneration and that limb recovery is strongly impaired once Wnt is downregulated (Whyte, Smith, & Helms, 2012).

There is strong evidence supporting the idea that elevating Wnt/beta catenin signaling in animals with limited regenerative capacity will induce regeneration. Postmetamorphic frogs for instance will grow limbs after amputation when Wnt is upregulated which would normally end up with low functioning spikes without any appendages. Though this observation was highly stage dependant during an early phase after amputation (Kawakami et al., 2006; Whyte et al., 2012).

Moreover, the process of Wnt driven embryogenesis and regenerationn is also concentration dependant, meaning when spread through the tissue a Wnt gradient is formed(Kestler & Kuhl, 2011; Solis, Luchtenborg, & Katanaev, 2013).

Altogether one can say, that the idea of upregulation of Wnt/beta catenin through pharmacological modulation to induce regeneration is very promising, hoewever many questions remain unresolved and limitations have to be considered concerning the dependency on time, concentration and what factors are necessary to recruit stem cells to the site of injury.

# 6. Appendix

## 6.1 Abbreviations

| | |
|---|---|
| HTS | High Throughput Screening |
| NIH | National Institute of Health |
| COPD | Chronic Obstructive Pulmonary Disease |
| FDA | Food and Drug Administration |
| Fzd4 | Frizzled 4 protein |
| Lrp5/6 | Lipoprotein related receptor 5/6 |
| APS | Adenomatosis polyposis coli |
| TCF/LEF | T-cell factor/lymphoid enhancer factor |
| GSK3 | Glycogen Synthase Kinase 3 |
| CK1 | Casein Kinase 1 |
| DMSO | Dimethyl sulfoxide |
| Dkk | Dickkopf protein |
| Dsh | Dishevelled protein |
| FCS | Fetal Calf Serum |
| WST1 | Water Soluble Tetrazolium 1 |
| MTT | 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide |
| Amx | Amlexanox |
| TPA | Tiaprofenic Acid |
| Tol | Tolnaftate |
| Phe | Phenazopyridine |
| Nb | Nabumetone |

## 6.2 List of figures

## 6.3 List of tables

## 6.5 Eidesstattliche Versicherung

Hiermit erkläre ich, Astrid Laura Staschewski, an Eides statt, dass ich die vorliegende Dissertation mit dem Thema

*“ Identification of novel compounds for Wnt/beta-catenin induced lung repair in COPD ”*

selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

Ich erkläre des Weiteren, dass die hier vorgelegte Dissertation nicht in gleicher oder in ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht wurde.

München, 04.06.2021 Astrid Staschewski

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