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RESEARCH ARTICLE

A drug screen with approved compounds identifies amlexanox as a novel Wnt/β-catenin activator inducing lung epithelial organoid formation

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Background and Purpose: Emphysema is an incurable disease characterized by loss of lung tissue leading to impaired gas exchange. Wnt/β-catenin signalling is reduced in emphysema, and exogenous activation of the pathway in experimental models in vivo and in human ex vivo lung tissue improves lung function and structure. We sought to identify a pharmaceutical able to activate Wnt/β-catenin signalling and assess its potential to activate lung epithelial cells and repair.

Experimental Approach: We screened 1216 human-approved compounds for Wnt/β-catenin signalling activation using luciferase reporter cells and selected candidates based on their computationally predicted protein targets. We further performed confirmatory luciferase reporter and metabolic activity assays. Finally, we

Abbreviations: ADAMTS4, A disintegrin and metalloproteinase with thrombospondin motifs 4; COPD, chronic obstructive pulmonary disease; EMA, European Medicine Agency; FDA, Food and Drug Administration; GSK-3β, glycogen synthase kinase-3β; HGF, hepatocyte growth factor; IKK, IκB kinase; IRF3, IFN regulatory factor 3; LEF, lymphoid enhancer-binding factor; TBK1, TANK-binding kinase 1; TCF, T-cell factor.

studied the regenerative potential in murine adult epithelial cell-derived lung organoids and in vivo using a murine elastase-induced emphysema model.

Key Results: The primary screen identified 16 compounds that significantly induced Wnt/β-catenin-dependent luciferase activity. Selected compounds activated Wnt/β-catenin signalling without inducing cell toxicity or proliferation. Two compounds were able to promote organoid formation, which was reversed by pharmacological Wnt/β-catenin inhibition, confirming the Wnt/β-catenin-dependent mechanism of action. Amlexanox was used for in vivo evaluation, and preventive treatment resulted in improved lung function and structure in emphysematous mouse lungs. Moreover, gene expression of *Hgf*, an important alveolar repair marker, was increased, whereas disease marker *Eln* was decreased, indicating that amlexanox induces pro-regenerative signalling in emphysema.

Conclusion and Implications: Using a drug screen based on Wnt/β-catenin activity, organoid assays and a murine emphysema model, amlexanox was identified as a novel potential therapeutic agent for emphysema.

KEYWORDS

amlexanox, chronic obstructive pulmonary disease, emphysema, organoids, regenerative medicine, Wnt/β-catenin signalling pathway

1 | INTRODUCTION

Respiratory diseases are increasing in prevalence and are associated with significant mortality, representing a medical challenge with unmet needs (Li et al., 2020). Chronic obstructive pulmonary disease (COPD) is the third leading cause of death in the world (Quaderi & Hurst, 2018) with two main characteristics: small airway disease and emphysema. The main risk factors for COPD include cigarette smoke, advanced age and long-term exposure to air pollutants and chemicals. Defined as a clinical syndrome that manifests as a heterogeneous and slowly progressing lung disease, COPD results in airflow limitation, as well as excessive inflammation and parenchymal tissue destruction. The tissue damage in the alveolar region is called emphysema, which results in a reduction of the gas exchange area of the lung (Agusti & Hogg, 2019; Celli & Wedzicha, 2019). Lung transplantation is the only curative treatment option for COPD. However, organ availability, candidate criteria and eligibility, and tissue rejection and other associated complications make lung transplantation an unreliable treatment for most cases (Siddiqui & Diamond, 2018). Other treatment options, such as bronchodilators, are effective in relieving symptoms but are unable to reverse disease progression (Singh et al., 2019).

As with other respiratory diseases, there are great challenges in the development of effective therapies for emphysema/COPD. Over the past 40 years, many of the drugs introduced for the treatment of COPD have been improvements in existing drug classes, such as inhaled corticosteroids and long-acting β_2-adrenoceptor agonists (Barnes et al., 2015). Repurposing old drugs is an attractive approach to accelerate drug development (Pushpakom et al., 2019).

What is already known

- Wnt/β-catenin signalling is down-regulated in COPD lungs and activation induces tissue repair.

What does this study adds

- A proof-of-concept study identified a drug activating Wnt/β-catenin signalling and initiating lung repair.
- Lung organoids are good test systems for pro-regenerative drug discovery in emphysema and COPD.

What is the clinical significance

- Amlexanox, an approved drug with potential for emphysema treatment, can rapidly translate into clinical use.

Regenerative pharmacology for COPD has been discussed in the lung field (Hind & Maden, 2011; Ng-Blichfeldt, Gosens, et al., 2019). Different studies suggest the lung remains largely quiescent after development but, following injury, multiple cell types can proliferate and restore the lung structure (Liu et al., 2019; Nabhan et al., 2018; Zacharias et al., 2018). The signalling pathways involved in these processes and their alteration in COPD represent an area of active research.

Wnt/β-catenin signalling is an important pathway during lung development, homeostasis and repair (Baarsma & Konigshoff, 2017). We and others have shown it to be decreased in COPD (Baarsma et al., 2017; Jiang et al., 2016; Kneidinger et al., 2011; Skronska-Wasek et al., 2017; Uhl et al., 2015; Wang et al., 2011). We have also previously demonstrated that Wnt/β-catenin signalling is a promising target for lung repair in COPD/emphysema both in in vivo and ex vivo studies, using precision-cut lung slices derived from COPD patients (Conlon et al., 2020; Kneidinger et al., 2011; Uhl et al., 2015). Although lithium chloride (LiCl) and CHIR99021 (laduviglusib) are two well-known glycogen synthase kinase-3β (GSK-3β) inhibitors used to experimentally activate Wnt/β-catenin signalling, there are toxicity concerns regarding their systemic use in humans (De Meyer et al., 2011; Uhl et al., 2015).

Here, we applied an Food and Drug Administration (FDA), European Medicine Agency (EMA) and other agency-approved drug screen to identify novel Wnt/β-catenin signalling activators with the potential to initiate lung repair and thus could be used for the treatment of COPD.

2 | METHODS

2.1 | Cell culture

LEADING LIGHT Wnt Reporter cells (Enzo Life Sciences) and NIH/3T3 (ATCC CRL-1658, ATCC, RRID:CVCL_0594) cells, both fibroblast cell lines, were cultured in high-glucose DMEM (Gibco), supplemented with 4-mM L-alanyl–L-glutamine dipeptide (Gibco), 10% (w/v) FBS (PAN-Biotech) and 100-U·ml^{-1} penicillin and streptomycin (Gibco). For all experiments using NIH/3T3 and LEADING LIGHT Wnt Reporter cells, well plates were coated with poly-L-lysine (Sigma-Aldrich). MLg (ATCC CCL206, ATCC, RRID:CVCL_0437) cells were cultured in DMEM/Ham's F12 media (Gibco) supplemented with 10% (w/v) FBS and 100-U·ml^{-1} penicillin and streptomycin. Cells were grown at 37°C with 5% CO_2 in humidified conditions.

2.2 | High-throughput small compound screen

For the compound screen, LEADING LIGHT Wnt Reporter cells stably expressing firefly luciferase under the control of T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) were used (Figure S1); 1×10^4 cells in 50 µl of 10% (w/v) FBS-supplemented media were dispensed in white opaque 384-well plates (PerkinElmer) and allowed to adhere for 24 h at 37°C in 5% CO_2 in humidified conditions. Cells were serum starved with 0.1% (w/v) FBS media for 24 h to allow for cell synchronization prior to treatment. Cells were then treated for 24 h with 10 µM of 1216 compounds belonging to the Prestwick Chemical Library (Prestwick Chemical) in 0.1% (w/v) FBS-supplemented medium; 1% (w/v) DMSO (Carl Roth) and 10-µM CHIR99021 (Tocris), a GSK-3β inhibitor, were used as negative and positive controls, respectively. Media were removed, and 25 µl of Bright-Glo Luciferase Assay System (Promega) was added to each well. Luminescence was measured using an EnVision 2102 Multilabel Reader (PerkinElmer). Luminescence results were normalized to control. The quality and robustness of the assay were calculated using the Z' factor and signal window (SW) (Figure S2). Hit threshold was set above plate average signal of the compounds plus three times the SD.

2.3 | Drug selection

Computational analysis of potential false positives was performed in the compounds active in both the high-throughput small compound screen and in multiple assays, by comparison of the number of screens publicly available in PubChem (RRID:SCR_004284) and ChemBank (RRID:SCR_007592) databases. The activity of hits in the ChemBank screens was determined using an optimized B-score method that showed better performance than other hit identification methods. The activity of compounds in the PubChem bioassays was taken as provided by the database. After drug promiscuity assessment, drug targets were predicted using the software HitPick (Liu et al., 2013) (Figure S3). Final candidate drugs were selected on the basis of the number (i) of hits of a PubMed literature search composed by 'predicted target' + 'COPD' or 'predicted target' + 'Wnt' and (ii) of predicted targets belonging to the gene ontology terms 'epithelial cell proliferation' (GO:0050673) and 'regulation of inflammatory response' (GO:0050727), relevant in COPD.

2.4 | Luciferase reporter assay

LEADING LIGHT Wnt Reporter cells were lysed with Glo Lysis Buffer (Promega) 24 h after compound treatment. Luciferase activity was determined using a luminescence plate reader (Berthold Technologies) and the Bright-Glo Luciferase Assay System according to the manufacturer's instructions. The measured values were analysed with WinGlow Software, MikroWin 2000, Version 4.41 (Berthold Technologies) and normalized to control. Quantifications were based on at least three independent experiments, each condition performed in triplicates.

2.5 | Cell viability assay

Cell viability analysis was performed using CellTiter-Glo (Promega) according to the manufacturer's instructions, in NIH/3T3 cells. The assay was performed after 24 h of compound treatment, and measurements were done in triplicates. The measured values were analysed with WinGlow Software, MikroWin 2000, Version 4.41 (Berthold Technologies) and normalized to control. Quantifications are based on at least three independent experiments.

2.6 | Animals

All animal care and experimental procedures were conducted under strict governmental and international guidelines, and approved under the regulatory guidelines of University of Colorado Institutional Animal Care and Use Committee. Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020). Eight- to 12-week-old pathogen-free wild-type mice weighing 20 to 22 g were used in this study. C57BL/6N mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and C57BL/6J mice from The Jackson Laboratory (Maine, USA, RRID: IMSR_JAX:000664). Animals were housed in rooms maintained at constant temperature and humidity, with a 12-h light cycle, and were allowed food and water ad libitum.

2.7 | Lung epithelial cell isolation

Lung epithelial cells were isolated as previously described (Messier et al., 2012; Mutze et al., 2015; Ng-Blichfeldt et al., 2018) with slight modifications. C57BL/6N female mice were anaesthetized with a mixture of 30-mg·kg^{-1} **ketamine** (bela-pharm) and 100-mg·kg^{-1} **xylazine** (CP-pharma) and killed, through exsanguination by cutting the vena cava, and lungs were flushed with PBS. Mouse lungs were intratracheally inflated with dispase (BD Bioscience) followed by 300-µl instillation of 1% low gelling temperature agarose (A9414, Sigma-Aldrich). Lungs were excised, minced and filtered through 100-, 20- and 10-µm nylon meshes (Sefar). White blood cells were depleted with CD45, and epithelial cells were selected using CD326 (EpCAM) magnetic beads, respectively (130-052-301 and 130-105-958, Miltenyi Biotec), according to the manufacturer's instructions. EpCAM^{+} cells were resuspended in DMEM containing 2-mM L-alanyl–L-glutamine dipeptide (Gibco), 100-U·ml^{-1} penicillin and streptomycin (Sigma-Aldrich), 3.6-mg·ml^{-1} glucose (AppliChem) and 10-mM HEPES (Life Technologies) until further use.

2.8 | Organoid culture

Organoids were cultured as previously described (Barkauskas et al., 2013; Hu et al., 2020; Ng-Blichfeldt et al., 2018, Lehmann et al., 2020) with some modifications. Briefly, MLg mouse lung fibroblasts were proliferation-inactivated by incubation in culture medium containing 10-µg·ml^{-1} **mitomycin C** (Merck) for 2 h, followed by three washes in warm PBS (Life Technologies), and let to recover in culture media for at least 1 h; 20,000 EpCAM^{+} cells suspended in 50-µl growth factor-reduced Matrigel (Corning) were diluted 1:1 with 20,000 MLg cells in 50-µl DMEM/F12 containing 10% (w/v) FBS and seeded into 24-well plate 0.4-µm pore transwell inserts (Corning). Cultures were maintained in DMEM/F12 containing 5% FBS, 100-U·ml^{-1} penicillin/streptomycin, 2-mM L-alanyl–L-glutamine dipeptide (Gibco), 2.5-µg·ml^{-1} amphotericin B (Gibco), 1× insulin–transferrin–selenium (Gibco), 0.025-µg·ml^{-1} recombinant human **EGF** (Sigma), 0.1-µg·ml^{-1} cholera toxin (Sigma-Aldrich), 30-µg·ml^{-1} bovine pituitary extract (Sigma-Aldrich) and 0.01 µM of freshly added all-*trans* retinoic acid (Sigma-Aldrich) beneath the inserts; 10-µM **Y-27632** (Tocris), a Rho-associated kinase (ROCK) inhibitor, was added to the media for the first 48 h of culture. Treatments were added to the organoid media from Day 0 and in every culture media exchange. Media were refreshed every 2–3 days.

2.9 | Organoid quantification

Microscopy for organoid quantification at Day 14 was performed using a microscope Axiovert 40 (Zeiss). Individual organoids from multilayer Z-stacks, with a minimum size of 50 µm, were manually selected using the Fiji image processing package (Schindelin et al., 2012, RRID:SCR_002285). The total number of organoids was then normalized to treatment control.

2.10 | Immunofluorescence

Immunofluorescence staining of organoids was performed as previously described (Ng-Blichfeldt et al., 2018). Organoids were kept in well inserts and fixed at Day 14 with ice-cold acetone–methanol mixed 1:1 (AppliChem) for 15 min at −20°C. Next, organoids were blocked with 5% (w/v) BSA (Sigma-Aldrich) dissolved in PBS and further stored at 4°C. The following day, organoids were incubated with primary antibodies diluted in PBS with 0.1% (w/v) BSA and 0.1% (v/v) Triton X-100 solution (AppliChem) at 4°C. The next day, organoids were washed with PBS and incubated with secondary antibodies, diluted 1:200 at 4°C. Finally, on the third day, cell nuclei were stained with DAPI (Roche) diluted 1:1000 for 10 min. Antibodies and dilutions used were E-cadherin 1:200 (610181, BD Bioscience, RRID:AB_397580), prosurfactant protein C 1:100 (AB3786, Millipore, RRID:AB_91588), acetylated α-tubulin (ACT) 1:2000 (ab24610, Abcam, RRID: AB_448182), donkey anti-mouse IgG conjugated with Alexa Fluor 647 1:200 (ab150107, Abcam, RRID:AB_2890037), goat anti-mouse IgG conjugated with Alexa Fluor 568 1:200 (A11031, Invitrogen, RRID:AB_144696) and goat anti-rabbit IgG conjugated with Alexa Fluor 488 1:200 (ab150077, Abcam, RRID:AB_2630356). Immunofluorescence was visualized using either a confocal microscope LSM710 or a microscope Axio Imager M2 (Zeiss). Images were processed using ImageJ (Schindelin et al., 2015, RRID: SCR_003070). The immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology* (Alexander et al., 2018).

2.11 | Amlexanox preparation and in vivo administration

Amlexanox was administered to C57BL/6J mice by oral gavage as previously described (Reilly et al., 2013). Amlexanox was first dissolved in 150-mM NaOH, to a final concentration of 20 mg·ml^{-1}. The solution was further diluted using a 1:1 mixture of water and 1.5-M Tris buffer pH 8.8, to achieve a final concentration of 10 mg·ml^{-1}, which was further buffered to a pH of >7.5, to avoid amlexanox precipitation. The same solution without amlexanox was used as vehicle control. Mice were treated on a daily basis, from Day −1 (the day before elastase instillation) to Day 6, in a preventive regimen, with either 50-mg·kg^{-1} amlexanox or vehicle. Mice were randomized and divided into the following groups: diseased controls (elastase-challenged receiving vehicle) and diseased treated mice (elastase-challenged receiving amlexanox treatment). Experiments were performed with 10 animals per group (five male and five female). One mouse died before the end of the experiment, resulting in a final *n* of 9 for the amlexanox treatment group.

2.12 | Elastase mouse model of emphysema

Emphysema was induced as described previously (Kneidinger et al., 2011), using elastase, an enzyme known to induce destruction of lung tissue. The elastase-induced injury model is widely used to study emphysema and well documented (Wright et al., 2008). Briefly, porcine pancreatic elastase (Sigma-Aldrich) was dissolved in sterile PBS (Gibco) and applied orotracheally, 40-U·kg^{-1} in 80-µl PBS, in C57BL/6J mice anaesthetized with 3% isoflurane. Animal health status and weights were closely monitored daily.

2.13 | Lung function measurement

C57BL/6J mice were anaesthetized with ketamine–xylazine, tracheostomized, intubated and connected to a flexiVent FX system (SCIREQ) (John-Schuster et al., 2014). Mice were ventilated with a tidal volume of 10 ml·kg^{-1} at a frequency of 150 breaths min^{-1} to reach a mean lung volume similar to that of spontaneous breathing. Testing of lung mechanical properties including dynamic lung compliance and resistance was carried out by a software-generated script. Measurements were repeated four times per animal. The coefficient of determination (COD), a quality control parameter comparing the experimental signals and the lung function software models, was determined by the system. Only datasets with a coefficient higher than 0.95 were included in the analysis. Following lung function measurements, anaesthetized animals were killed, and lungs were collected.

2.14 | RNA extraction, cDNA synthesis and quantitative real-time PCR

Fresh lung tissue was snap frozen in liquid nitrogen and kept at −80°C until further processing. Frozen tissue was pulverized using a TissueLyser II (Qiagen). Total RNA from lung tissue homogenate was isolated using QIAzol (Qiagen) and purified with the peqGOLD Total RNA Kit (Peqlab), according to the manufacturer's instructions. cDNA was synthesized using reverse transcription and M-MLV Reverse Transcriptase (Invitrogen). mRNA expression of target genes was compared with reference control hypoxanthine-guanine phosphoribosyltransferase (Hprt)-1, using SYBR Green (Roche) and a LC480 Light Cycler (Roche). Primers were used at a final concentration of 500 nM with the following sequences: *Hprt* forward 5′-CCTAAGATGAGCGCAAGTTGAA-3′, reverse 5′-CCACAGGACTAGAACACCTGCTAA-3′; *Axin2* forward 5′-AGCAGAGGGACAGGAACCA-3′, reverse 5′-CTGAACCGATTCATGACCAC-3′; Fzd4 forward 5′-TCCAGCCAGCTGCAGTTCTTCC-3′, reverse 5′-CTGAAAGGCACATGCCACCGC-3′; Fgf7 forward 5′-AGGCTCAAGTTGCACGAGGC-3′, reverse 5′-GCGGTTGCTCCTTGACTTTTGTT-3′; Hgf forward 5′-TGCCTGTGCCTTGACTTAGCG-3′, reverse 5′-CCGGGCTGAAAGAATCAAAGCA-3′; Adamts4 forward 5′-AACCAAGCGCTTCGCTTCTCT-3′, reverse 5′-AGCTGCCATAACCGTCAGCA-3′; and *Eln* forward 5′-GGCGTCTTGCTGATCCTCT-3′, reverse 5′-ATAATAGACTCCACCGGGAACT-3′. Relative transcript expression of a gene was calculated as ΔCt (ΔCt = Ct reference gene − Ct target gene).

2.15 | Mouse lung histology

Lungs were fixed at a constant pressure (20 cmH_2O) by intratracheal instillation of PBS-buffered 6% paraformaldehyde (PFA). The trachea was sealed with a ligature, and lungs and heart were removed en bloc, stored for 24 h in 4% PFA and then kept in PBS at 4°C. Lobes were embedded in paraffin for histological analysis of haematoxylin–eosin (H&E)-stained slides (3-µm sections). Images were acquired at 10× and 20× magnifications using a microscope Axio Imager M2 (Zeiss). Images were processed using ImageJ (Schindelin et al., 2015).

2.16 | Data and statistical analysis

To decrease unintentional bias, personnel were blinded to experimental groups during sample processing and data analysis. During the blinding process, samples were randomly excluded so that sample sizes were the same between treatment groups during processing and analysis. Data were analysed using GraphPad Prism 9.1.0 (RRID: SCR_002798) and presented as mean ± SD. *n* is described in the figure legends and refers to number of independent experiments. Data from the luciferase reporter assay, cell viability assay and organoid number quantification were normalized to respective control treatment and analysed using a one-sample Wilcoxon signed-rank test

comparing with a hypothetical value of 1. For calculation of significances between different treatments a Kruskal–Wallis test, followed by a Dunn's multiple comparisons test was used. Mouse lung function and mouse lung gene expression data were analysed using a Mann–Whitney test. Differences at $P < 0.05$ were considered significant. The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018).

2.17 | Materials

The 1216 compounds from the Prestwick Chemical Library were supplied by Prestwick Chemical (Illkirch-Graffenstaden, France). Ketamine was supplied by bela-pharm (Vechta, Germany) and xylazine by CP-pharma (Burgdorf, Germany). CHIR99021 (Tocris, Minneapolis, MN, USA), a GSK-3β inhibitor; **amlexanox** (Cayman Chemical, Ann Arbor, MI, USA), a IκB kinase ε (**IKKε**) and TANK-binding kinase 1 (**TBK1**) inhibitor; **nabumetone**, a **COX** inhibitor; phenazopyridine hydrochloride; tolnaftate (Santa Cruz Biotechnology, Texas, USA); and tiaprofenic acid (Sigma-Aldrich, St Louis, MO, USA) were dissolved in DMSO to a stock concentration of 20 mM. PKF115-584 (calphostin C) and iCRT14 (Tocris), antagonists of the TCF/β-catenin complex, were dissolved in DMSO to a stock concentration of 1 and 75 mM, respectively. LiCl (Sigma-Aldrich), a GSK-3β inhibitor, was dissolved in culture media to a stock solution of 2 M. Reagents were stored as stock solutions at −20°C.

2.18 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander, Christopoulos et al., 2019; Alexander, Fabbro et al., 2019).

3 | RESULTS

3.1 | Wnt/β-catenin luciferase reporter cell-based screening identifies human-approved drugs as Wnt activators

To identify novel compounds, which could activate Wnt/β-catenin signalling, we screened 1216 compounds that were also clinically approved by the EMA, FDA and other agencies, using a Wnt luciferase reporter cell line (Figure 1a,b). The hit threshold for our assay was set above the mean signal of all compounds together, shifted by three times the SD, specific for each assay plate. We identified 16 drugs that increased β-catenin-dependent luciferase activity (Figure 1c); 12 drugs decreased luciferase activity and were in the Wnt/β-catenin inhibitory range of the assay. Next, we selected five drugs out of the 16 hits based on the following algorithm: (i) promiscuity analysis in ChemBank and PubChem databases, regarding the ratio of the number of assays the hits were tested and active, to exclude potential false positives (Table S1); (ii) predicted protein targets, using the bioinformatic tool HitPick (Figure S4); and (iii) literature search, performed on the drug's predicted protein targets, associated with either the keywords 'Wnt' or 'COPD' or the GO terms 'epithelial cell proliferation' or 'regulation of inflammatory response', to study their potential involvement in the Wnt/β-catenin pathway and disease relevance (Figure 1a). The five selected candidate compounds were **amlexanox**, **phenazopyridine** hydrochloride, tolnaftate, **nabumetone** and tiaprofenic acid (Figure 1c and Table S2).

3.2 | Inhibition of β-catenin reduces Wnt-dependent luciferase activity in amlexanox- and phenazopyridine hydrochloride-treated cells

Next, we aimed to validate that the candidate drugs amlexanox, phenazopyridine hydrochloride, tolnaftate, nabumetone and tiaprofenic acid, selected in our primary screen, regulate β-catenin signalling. To do so, we treated the Wnt/β-catenin reporter cell line with the candidate drugs alone or together with PKF115-584, a well-known compound inhibiting the TCF–β-catenin complex (Lepourcelet et al., 2004). All five compounds significantly increased luciferase activity, confirming the results of our drug screen (Figure 2a). Wnt/β-catenin signalling induction was achieved at all concentrations, except for the lower dose of tiaprofenic acid. Inhibition of β-catenin led to a significant decrease in luciferase activity, stimulated by either amlexanox or phenazopyridine hydrochloride. For tolnaftate, nabumetone and tiaprofenic acid, there was also a decrease in luciferase activity, but not significant. At the same time, we observed that LiCl, a classic inhibitor of GSK-3β and the only drug known to activate Wnt/β-catenin signalling in patients, induced luciferase activity in our reporter system. However LiCl induced less luciferase activity than the candidate drugs. Second, the five selected candidate hits were analysed in a concentration dependent manner with respect to their proliferative capacity, to rule out the possibility that the increase in luciferase activity was due to an increase in cell number (Figure 2a). We observed that there were no significant changes in the metabolic activity of the cells treated with the candidate drugs, measured by ATP levels, accounting for both proliferation and toxicity (Figure 2b). Based on these results, we focused our further studies on the compounds amlexanox and phenazopyridine hydrochloride.

3.3 | Amlexanox and phenazopyridine hydrochloride induce primary adult lung epithelial cell-driven organoid formation

We next investigated the potential effects of the candidate drugs on lung regeneration. Amlexanox and phenazopyridine hydrochloride,

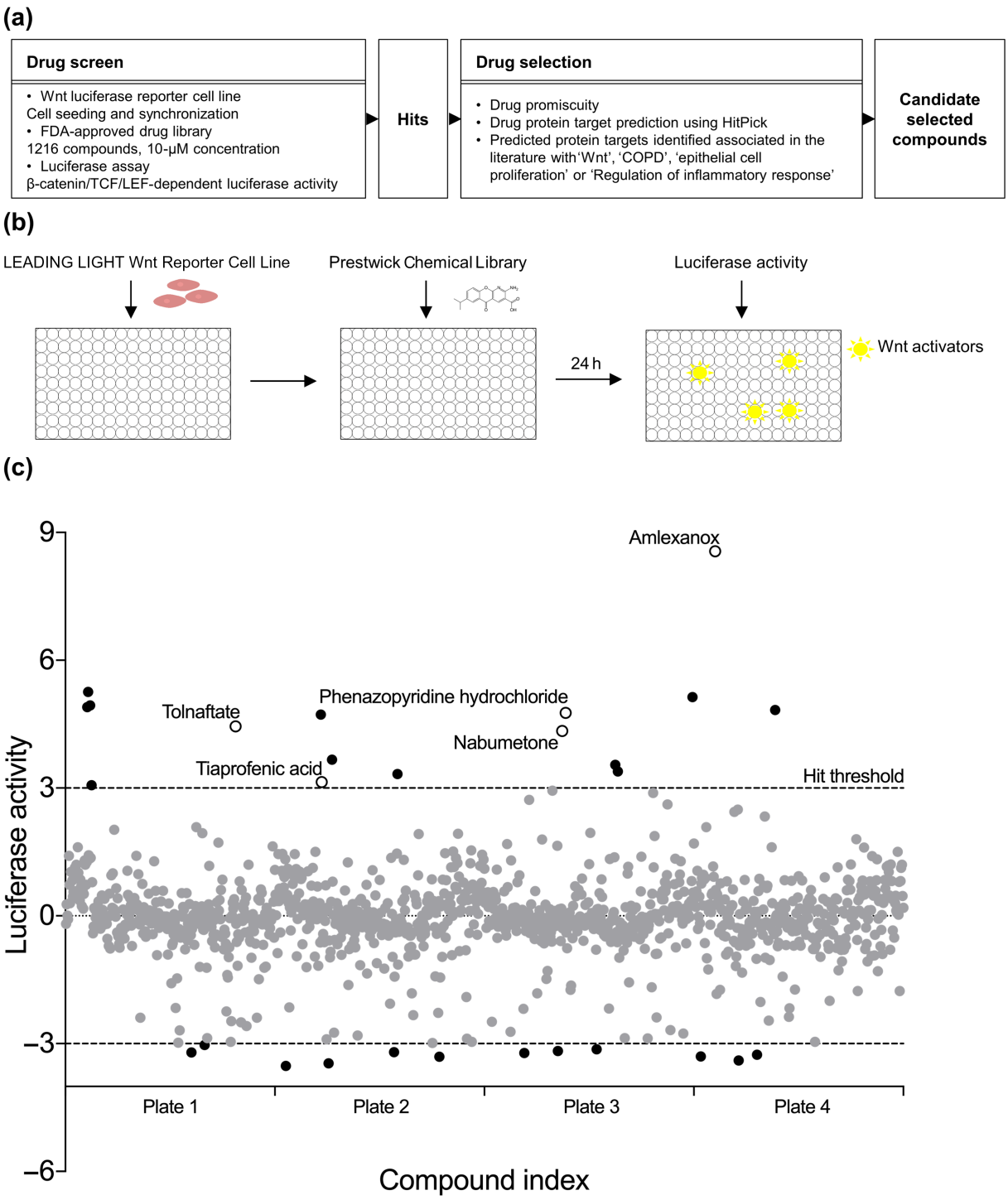

FIGURE 1 High-throughput drug screen and candidate compound selection. (a) Diagram with outline of candidate compound identification and selection process. Hits were identified following a cell-based Wnt reporter screen of drugs already approved by the FDA, EMA and other comparable agencies. Candidates were selected on the basis of their predicted protein targets (HitPick), associated with 'Wnt', 'COPD', 'epithelial cell proliferation' or 'regulation of inflammation'. (b) Schematic overview of the drug screening experimental procedure. LEADING LIGHT Wnt Reporter Cell Line, expressing firefly luciferase reporter gene under the control of Wnt-responsive promoters (TCF/LEF), was seeded in a 384-well plate, serum starved (0.1% FBS) for 24 h and treated for another 24 h with 10 μM of each compound. Luciferase assay was performed, luminescence values were analysed and luciferase activity was calculated relative to negative control (DMSO); 10-μM CHIR99021 was used as positive control. (c) Results of the cell-based Wnt reporter drug screen. $N = 1$. Luciferase activity represents the calculated Z score for the luminescence signal of each compound treatment relative to control. Hits Z score was set as greater than the average signal of all compounds plus three times the SD. This compound screen identified 16 Wnt/β-catenin/TCF/LEF activators. White dots highlight the five selected candidate compounds: amlexanox, phenazopyridine hydrochloride, tolnaftate, nabumetone and tiaprofenic acid

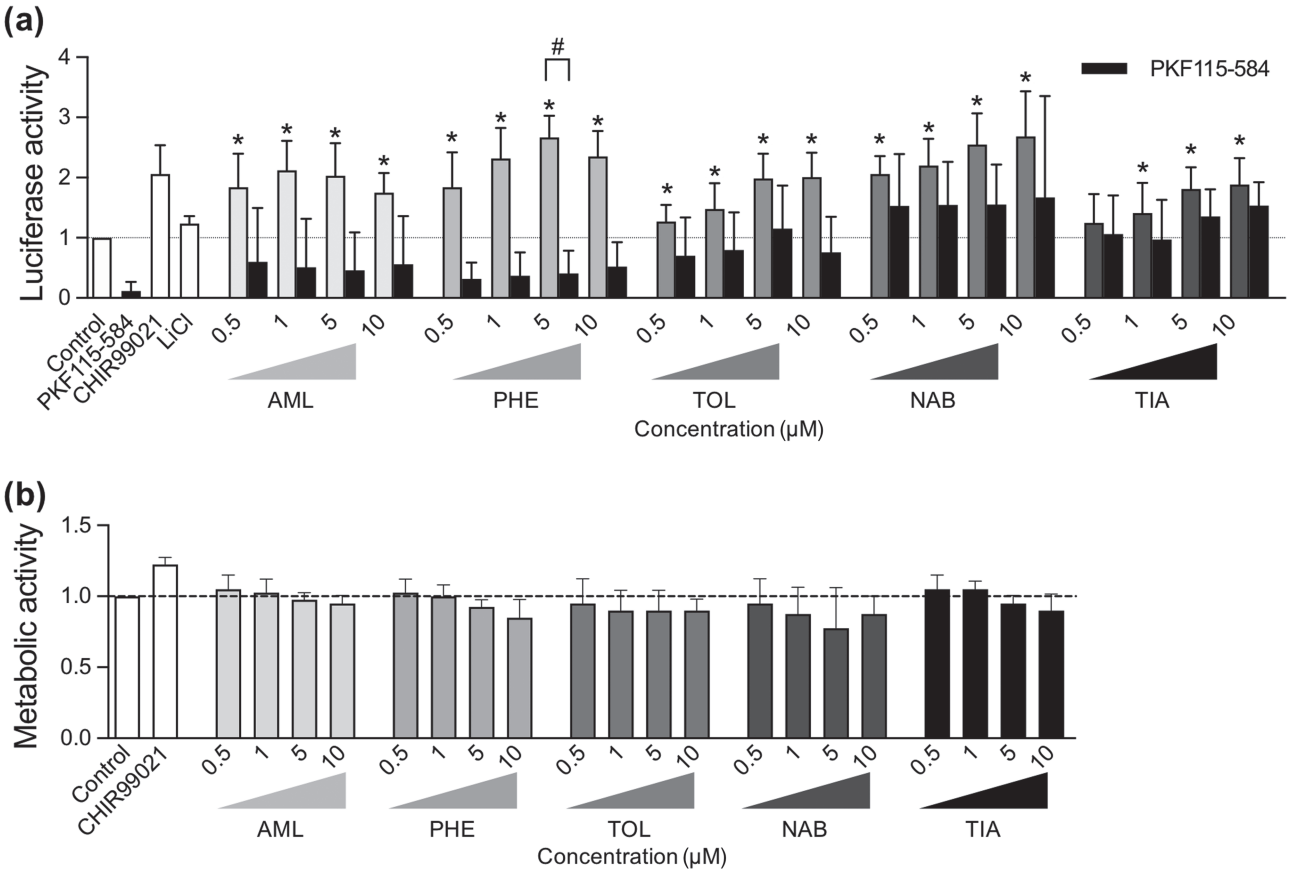

FIGURE 2 Confirmation of selected candidate compounds: Wnt modulation and metabolic activity. (a) Luciferase assay in LEADING LIGHT Wnt Reporter Cell Line treated with 0.5, 1, 5 or 10 μM of candidate compounds for 24 h with or without 1-μM PKF115-584, an antagonist of TCF/β-catenin complex. GSK-3β inhibitors, 1-μM CHIR99021 and 10-mM lithium chloride (LiCl) were used as positive controls. Luciferase activity was calculated relative to negative control (DMSO). $N = 3–10$. (b) Metabolic activity of NIH/3T3 cells treated with 0.5, 1, 5 or 10 μM of candidate compounds for 24 h was evaluated by ATP levels, measured with CellTiter-Glo luminescent cell viability assay; 1-μM CHIR99021 was used as a positive control. Metabolic activity was calculated relative to negative control (DMSO). Data are presented as mean ± SD of independent experiments; $N = 4$., $^{*}P < 0.05$, significantly different from a hypothetical value of 1; one-sample Wilcoxon signed-rank test. $^{\#}P < 0.05$. significantly different as indicated; Kruskal–Wallis test with Dunn's post test. AML, amlexanox; NAB, nabumetone; PHE, phenazopyridine hydrochloride; TIA, tiaprofenic acid; TOL, tolnaftate

both of which showed previous β-catenin activation, were applied in a well-established 3D organoid assay using adult mouse epithelial cells (Figure 3a) (Lehmann et al., 2020). We tested whether these compounds increase the number of distal lung organoids using a standard protocol including epithelial progenitor cells co-cultured with MLg lung fibroblasts cell line in Matrigel (Hu et al., 2020; Ng-Blichfeldt et al., 2018). Importantly, we and others have shown that organoid formation in this assay is dependent on Wnt/β-catenin signalling (Frank et al., 2016; Hu et al., 2020). We tested both compounds using two different concentrations, 0.5 and 1 μM, which were sufficient to induce Wnt/β-catenin signalling in the Wnt reporter luciferase assay (Figure 2a). Organoid formation quantified by number of organoids was induced by LiCl (used as a positive control), as well as by amlexanox and lower doses of phenazopyridine hydrochloride (Figure 3b,c). In parallel, we observed that compound treatment for 14 days did not alter E-cadherin expression (Figure 3d). Organoids expressed lung epithelial cell markers for both ACT and pro-surfactant C protein (SFTPC), markers for airway and alveolar epithelial cells, respectively, as highlighted by the red and green arrows (Figure 3e). These results show that amlexanox and phenazopyridine hydrochloride can contribute to Wnt/β-catenin signalling-driven lung progenitor cell function.

3.4 | Inhibition of β-catenin reduces amlexanox and phenazopyridine hydrochloride organoid formation capacity

As described above, lung epithelial cell progenitor capability to form organoids in vitro requires Wnt/β-catenin signalling (Hu et al., 2020). We next determined whether induction of organoid formation by amlexanox and phenazopyridine hydrochloride was mediated by the Wnt pathway. To this end, we used a known potent Wnt/β-catenin signalling inhibitor iCRT14, which interferes with TCF binding to DNA in addition to altering TCF/β-catenin interactions (Gonsalves et al., 2011). iCRT14

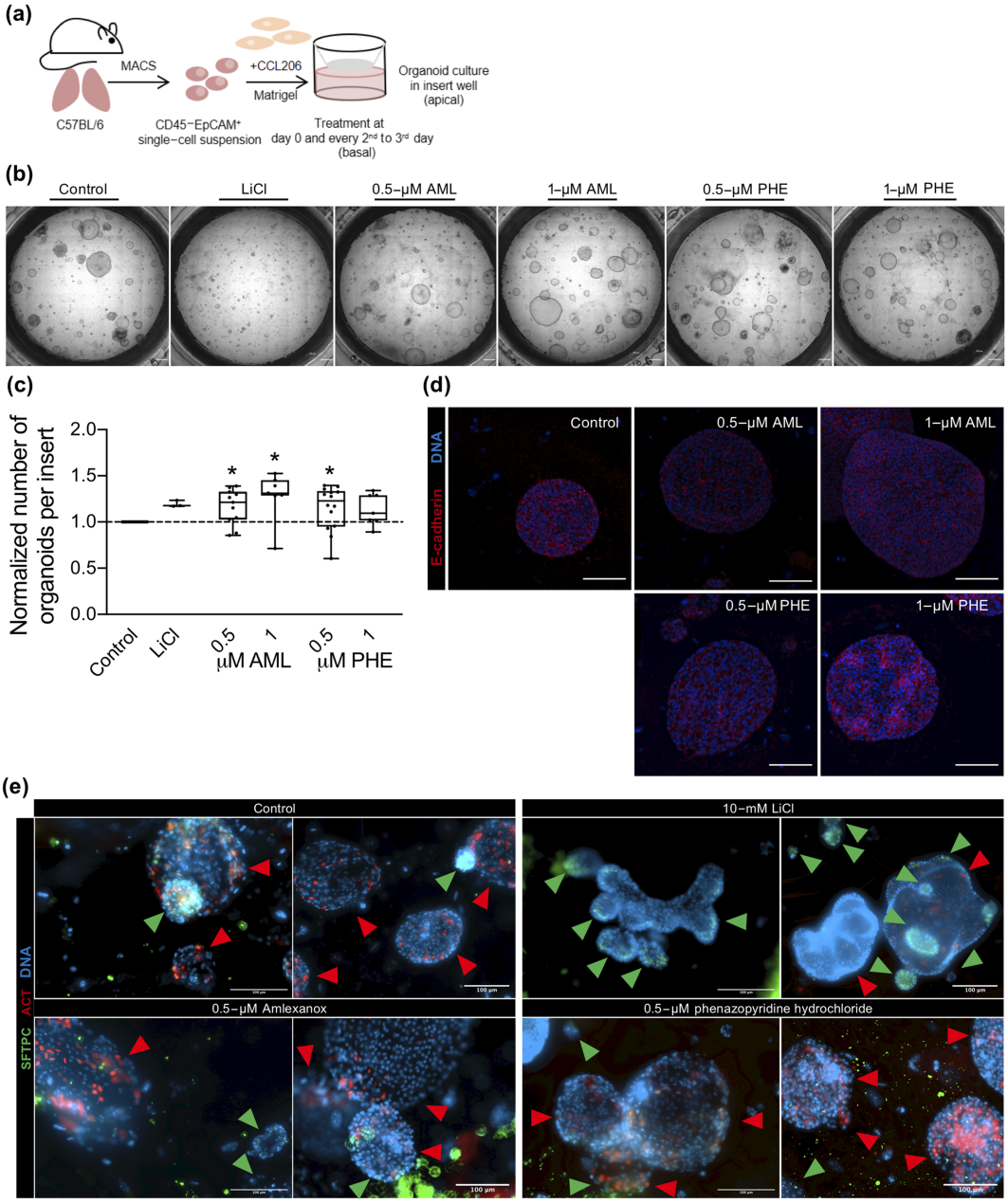

FIGURE 3 Assay of the alveolar regeneration potential of the selected compounds, using primary mouse alveolar type II cell-derived 3D lung organoids. (a) Scheme of mouse organoid experimental set-up. Primary mouse ATII cells were isolated from 10- to 12-week-old wild-type C57BL/6 mice lungs after enzymic dissociation with dispase and MACS sorting of CD45-negative and EpCAM-positive cells. Cells were seeded in Matrigel together with non-proliferating CCL206 lung fibroblasts in well inserts. Media with treatments were changed every second to third day. (b) Representative phase-contrast images of whole insert organoids (top) and inset images (bottom). Treatments with DMSO, 10-mM lithium chloride (LiCl), 0.5- and 1-μM amlexanox (AML), and 0.5- and 1-μM phenazopyridine hydrochloride (PHE) started at Day 0 and continued every second to third day for 14 days. Scale bar = 500 μm, $n = 3$–14. (c) Normalized total number of organoids per insert to control (DMSO). $N = 3$–14 from individual cell isolations. $^*P < 0.05$. significantly different from a hypothetical value of 1; one-sample Wilcoxon signed-rank test. (d) Maximum intensity projection images of whole mount immunofluorescence staining of Day 14 organoids. E-cadherin (red) and DAPI (blue). Scale bar = 100 μm. $N = 3$. (e) Images from whole mount immunofluorescent-stained Day 14 organoids. Prosurfactant protein C (SFTPC, green), acetylated α-tubulin (ACT, red) and DAPI (DNA, blue). Scale bar = 100 μm, $n = 3$

treatment of organoids significantly reduced organoid numbers when used alone and, importantly, also in combination with amlexanox or phenazopyridine hydrochloride (Figure 4a,b). These data further support the notion that the effect of both compounds on organoid formation is mediated by Wnt/β-catenin signalling.

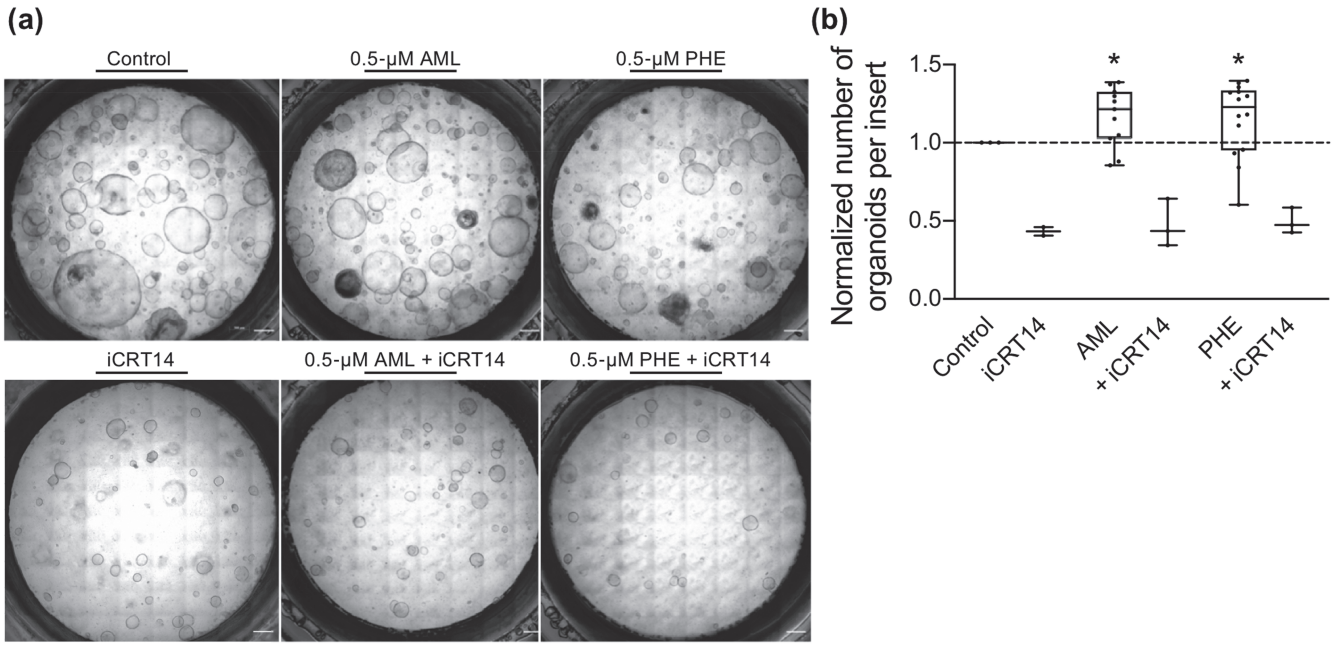

FIGURE 4 Wnt modulation and organoid formation. (a) Representative phase-contrast images of whole insert Day 14 organoids after treatment with the candidate compounds (0.5 μM) amlexanox (AML) and phenazopyridine hydrochloride (PHE), with or without 10-μM iCRT14, a potent Wnt inhibitor that blocks β-catenin/TCF4 binding. Scale bar = 500 μm. (b) Normalized total number of organoids per insert to control (DMSO). Data are presented as box plots with median of independent experiments; $n = 2–14$. $^*P < 0.05$. significantly different from a hypothetical value of 1; one-sample Wilcoxon signed-rank test

3.5 | Amlexanox treatment improves lung function and induces lung repair-related genes while reducing disease markers in a mouse model of lung emphysema

Finally, we asked the question whether the regenerative effect observed with amlexanox in primary adult lung epithelial cell-derived murine organoid cultures could also be translated to an in vivo model. To address this, we used the well-accepted model of tissue destruction seen in emphysema by subjecting mouse lungs to orotracheal administration of elastase (Kneidinger et al., 2011; Wright et al., 2008) (Figure 5a). Mice were treated preventively with 50 mg·kg^{-1} of amlexanox twice before being challenged with elastase and afterwards daily for 7 days. Notably, elastase-challenged mice treated with amlexanox presented with improved lung function, as measured by a lower lung compliance, compared with that in mice treated only with vehicle (Figure 5b). Moreover, treatment with amlexanox also led to attenuated tissue destruction compared with the elastase vehicle group, as observed by histology (Figure 5c). No changes were observed in healthy mice subjected to amlexanox, as shown in Figure S5 (lung function) and Figure S6 (lung structure). Although we did not observe significant changes in Wnt target genes, such as *Axin2*, frizzled class receptor 4, *Fzd4* or *Fgf7*, after 7 days treatment, we observed that amlexanox treatment led to a significant increase in the transcript expression of hepatocyte growth factor, *Hgf*, a protein that has been associated with lung repair (Hegab et al., 2008; McQualter et al., 2010; Yanagita et al., 1993) (Figure 5d). Lung expression of *Eln*, the gene for elastin, a known marker of aberrant repair attempts in COPD (Houghton et al., 2006; Uhl et al., 2015) was significantly decreased with amlexanox treatment. Similar changes were observed with a lower dose of amlexanox (25 mg·kg^{-1}, Figure S7).

4 | DISCUSSION AND CONCLUSIONS

The field of regenerative medicine is evolving rapidly with the growing understanding of molecular and cell-specific features during organ development, injury and regeneration, provided mainly by stem cell, single-cell sequencing and cell tracing studies and also by the improvement of experimental models. On the other hand, drug development is still a long process, in particular for respiratory disease. Currently, the importance of rapid drug discovery for finding new treatments for emerging respiratory diseases is being highlighted every day. Drug repurposing can shorten the drug development cycle from 10–17 to 3–12 years and thus represents an advantageous approach (Dudley et al., 2011).

Our study provides an early proof of concept for the potential for rapid drug translation. Here, we identify amlexanox as a potential candidate for the treatment of lung emphysema, which is characterized by loss of lung tissue, often found in COPD patients. We identified amlexanox as a potential drug for regenerative pharmacology using a drug screen based on Wnt/β-catenin signal activation.

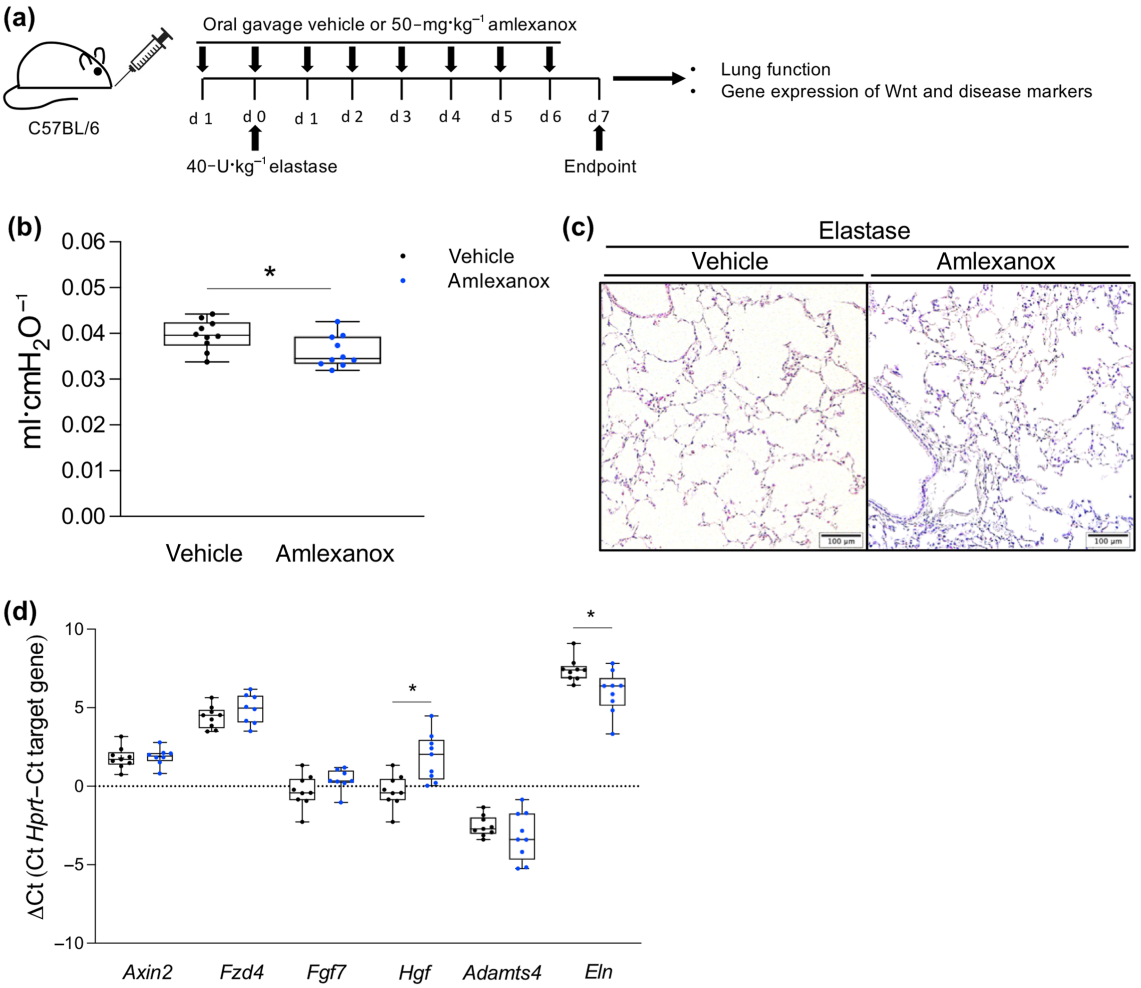

FIGURE 5 Functional assessment, in vivo, of amlexanox in a mouse model of emphysema. (a) Treatment scheme of wild-type C57BL/6 mice, with amlexanox. Mice were treated preventively with either 50 mg·kg^{-1} of amlexanox or vehicle a day before being challenged with 40-U·kg^{-1} porcine pancreatic elastase and every day for 7 days via oral gavage. Lung function and gene expression were analysed. (b) Dynamic compliance from lung function analysis. $N = 10$. (c) Representative histological images of mice lung sections stained with haematoxylin and eosin. $N = 4–5$. Scale bar = 100 µm. (d) Lung gene expression analysis of Wnt, tissue repair and COPD-related genes *Axin2*, *Fzd4*, *Fgf7*, *Hgf*, *Adamts4* and *Eln*. $N = 8–9$. Data are presented as mean ± SD. *$P < 0.05$, significantly different as indicated; Mann–Whitney test

Our unbiased drug screen approach identified 16 approved compounds that were able to significantly induce Wnt-dependent luciferase activity. Our results overlap with other studies performing similar Wnt/β-catenin-based independent screening in cells (Figure S8), which reinforces the reproducibility of our findings (Biechele et al., 2010; Zhan et al., 2019; Zhao et al., 2012). Unique compounds identified in our screen are likely to be due to differences in the drug screen set-up with distinct results based on the cell line used or chemical library applied. Furthermore, only compounds that showed no promiscuity in publicly available bioassays were considered in our study. Thus, we focused on drugs with specific effect on Wnt/β-catenin signalling, rather than an overall interference with gene transcription or protein translation. We selected five candidate drugs based on a platform for target prediction called HitPick (Liu et al., 2013). Using HitPick, targets are predicted by molecular comparison of the hits with other compounds for which targets are known. After a literature search, hits in which predicted targets were found to be associated with either 'Wnt' or 'COPD' or belonging to the GO terms 'epithelial cell proliferation' or 'regulation of inflammatory response' were selected. Our approach is in line with recent strategies in place for computational drug repurposing (Dudley et al., 2011). We have incorporated both drug-based strategies, through chemical similarities with other compounds for target prediction, as well as disease-based strategies, through the association of the hits' predicted targets to current knowledge of disease and pathology, to select final drug candidates with strong potential for COPD/emphysema treatment.

We focused our further analysis on amlexanox, phenazopyridine hydrochloride, tolnaftate, nabumetone and tiaprofenic acid. All five drugs induced Wnt/β-catenin reporter luciferase activity in a confirmatory screening, using the same cell line we used in the initial screen. To exclude the possibility that luciferase increases were due to cell proliferation, we measured ATP changes

after drug treatment and we did not observe significant increase in number of cells, as demonstrated by cell metabolic activity (Rulifson et al., 2007).

Upon Wnt/β-catenin signalling inhibition, two of the five compounds showed a significant decrease of their capacity to induce Wnt/β-catenin capacity in the monocultures. On the one hand, this indicates Wnt/β-catenin specificity, but on the other hand, it could represent a challenge in the disease context, where the tissue environment contains factors, which dampen Wnt/β-catenin signalling (Baarsma et al., 2017). This may indicate that a multi-targeted approach is necessary using a combination of pharmacological approaches to initiate functional repair.

Our initial screen was based on results observed in embryonic fibroblasts, so we applied a lung epithelial cell-based organoid assay, to validate our findings in a primary cell set-up and further to predict pharmacological effects, which could not be detected in 2D. Importantly, we and others have demonstrated that organoid formation in this system requires Wnt/β-catenin signalling (Frank et al., 2016; Hu et al., 2020). Both amlexanox and phenazopyridine hydrochloride exhibited a significant increase in lung organoid formation and led to differentiation of adult epithelial cells, indicating a potential to induce lung regeneration. E-cadherin protein expression is essential in maintaining epithelial tissue integrity and known to be reduced in many epithelial cell malignancies, such as cancer (Yu et al., 2019). In our hands, Wnt/β-catenin signalling activation using amlexanox and phenazopyridine hydrochloride did not seem to have an effect on E-cadherin expression in adult lung epithelial cells. Because amlexanox performed better in the organoid assay than phenazopyridine hydrochloride, we selected amlexanox for further investigation in vivo.

Our in vivo results demonstrate that amlexanox treatment attenuates emphysema development potentially by initiating a pro-regenerative environment in the lung. Importantly, mice, treated with amlexanox, exhibited improved lung function and structure. In line with this, we observed transcriptional changes in several genes previously implicated in emphysema and COPD (Barnes, 2014; Ezzie et al., 2012; Houghton et al., 2006). Among these, HGF is well described as a primarily mesenchyme-produced factor that has been implicated in adult lung repair (Hegab et al., 2008; McQualter et al., 2010; Yanagita et al., 1993). The increase in HGF, observed in vivo, points to potential additional effects of amlexanox—either directly on mesenchymal (or other) cells or indirectly due to changes in (Wnt/β-catenin-driven) lung epithelial signalling induced by amlexanox. Along these lines, it is important to note that our organoid system includes a fibroblast cell line as support cells, and we have previously found that HGF expression from fibroblasts contributes to organoid formation (Ng-Blichfeldt, de Jong, et al., 2019). A stromal-free lung epithelial progenitor cell culture (McQualter et al., 2010) could be helpful to further dissect the effects of amlexanox on epithelial, as distinct from stromal, cells to induce organoid formation and lung repair.

We did not observe changes in Wnt target genes in our study when analysing lung tissue 7 days after amlexanox treatment. This might be due to the temporal expression of Wnt/β-catenin targets gene, which are described to be direct feedback genes (Katoh, 2008; Pedone & Marucci, 2019). However, further studies are needed that expand on in vivo monitoring of Wnt/β-catenin activity upon amlexanox treatment.

Amlexanox is clinically used for the treatment of aphthous ulcers, allergic rhinitis and asthma. The drug is described to be anti-inflammatory and anti-allergic with immunomodulatory properties. The mechanism of action has been described to be through reduction of the release of **histamine** and leukotrienes (Inagaki et al., 1992). Different studies indicate that amlexanox administration is associated with weight loss and inflammation reduction (Ozasa et al., 2019; Reilly et al., 2013). Currently, amlexanox is under clinical trial for the treatment of Type 2 diabetes and obesity (NCT01842282). Amlexanox was shown to have a bronchodilator effect in aspirin-induced asthma patients (Imokawa et al., 1993). Therapeutic-targeting of leukotrienes in COPD is an option, as amlexanox has anti-inflammatory as well as bronchodilator effects on the airway in a few subsets of patients (Lee et al., 2015). Only recently, a study demonstrated that amlexanox activated Wnt/β-catenin signalling (Bordonaro & Lazarova, 2019). Wnt/β-catenin signalling induction by amlexanox has been ascribed to inhibition of IKKε/TBK1 (Reilly et al., 2013). IKKε is known to interact with β-catenin (Chen et al., 2017). Additionally, IKKε and TBK1 can activate IFN regulatory factor 3 (IRF3) signalling (Fitzgerald et al., 2003). Inhibition of both these processes has been shown to lead to β-catenin signalling activation (Chen et al., 2017; Tian et al., 2020). It is important to note that these mechanisms need to be further confirmed in the lung.

In the present study, we focused mainly on the phenotypic effect of amlexanox. Our data add to the recent observations that epithelial (progenitor) cells exhibit active Wnt/β-catenin signalling capacity. A recent study characterized stem cell clone libraries of COPD patient's lungs in advanced stages and identified normal lung stem cells present and three more predominant pathogenic stem cell variants (Rao et al., 2020). These stem cell variants were found to contribute to COPD, and targeting them represents an opportunity to treat the disease. This study indicates however that COPD patients retain normal lung stem cell niches that could potentially regenerate the lung. Discovery of Wnt/β-catenin signalling modulators that selectively affect one or more of these cell populations could potentially induce regeneration in an adult and diseased lung.

Our study adds to a growing body of evidence that modulation of the Wnt/β-catenin pathway can be harnessed for the induction of lung regeneration in lung emphysema. However, certain limitations of our study need to be addressed to further our understanding of amlexanox as a potential therapeutic agent. In particular, further in vivo analysis is required using both therapeutic and preventive approaches in COPD/emphysema models, including the cigarette smoke exposure model. Moreover, ex vivo studies regarding regenerative potential in COPD patient's lungs are needed to understand the regenerative potential of amlexanox in the lung. This could be done by using organoids derived from COPD/emphysema lungs and/or tissue-based models derived from

COPD. For example, we have shown that Wnt/β-catenin signal activation and lung repair can be achieved by the GSK-3β inhibitor LiCl in COPD patient-derived precision-cut lung slices (Uhl et al., 2015).

In summary, amlexanox was identified as a potential new candidate for the treatment of lung emphysema, based on a combination of different phenotypic and computational readouts. Many other undiscovered applications of already approved drugs exist, and the drug discovery approaches described in this study are an important and necessary step towards therapeutic advances for patients with emphysema and COPD.

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AUTHOR CONTRIBUTIONS

R.C., D.E.W., M.L., H.A.B. and M.K. conceived and designed the research. R.C., A.D., M.M.D.S., K.S., I.R., X.L. and M.C. performed the experiments, analysed the data and prepared the figures. R.C., D.E.W., M.L., O.S., A.Ö.Y., K.H. and M.K. interpreted the results. R.C., D.E.W. and M.K. drafted the manuscript. All authors critically edited, revised and approved the manuscript.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

DATA AVAILABILITY STATEMENT

Data underlying the findings described in this manuscript may be obtained upon request to the authors.

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