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Manuscript title:

Small molecule screen employing patient-derived iPSC hepatocytes identifies LRRK2 as a novel therapeutic target for Alpha1 Antitrypsin Deficiency

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Conflict-of-interest statement:

The authors have declared that no conflict of interest exists.

Abstract:

Alpha-1 antitrypsin deficiency (A1ATD) is a life-threatening condition caused by inheritance of the SERPINA1-Z genetic variant (PiZ) leading to protein misfolding and liver toxicity. There remain no approved medicines for this disease. Here we report the results of a small molecule screen performed in patient-derived iPSC hepatocytes that identified Leucine-rich repeat kinase-2 (LRRK2), as a potentially new therapeutic target. Genetic deletion of LRRK2 in PiZ mice reduced both intrahepatic misfolded polymers and fibrosis leading us to test a library of commercially available LRRK2 inhibitors across several cell models. One of these molecules, CZC-25146, a candidate with favourable pharmacokinetic properties, reduced polymer load, increased normal AAT secretion and reduced inflammatory cytokines in both cells and the PiZ mouse. Mechanistically, this effect was achieved via inhibition of LRRK2 kinase activity and autophagy induction. These findings support the use of CZC-25146 (and potentially LRRK2 inhibitors more broadly) in hepatic proteinopathy disease research and as potential new therapies.

Main Text

Introduction:

The ability of a cell to tightly regulate protein quality is fundamental to life¹. Failure of these processes can result in the accumulation of protein aggregates (proteinopathy) with devastating repercussions². Accumulation of Alpha-1 antitrypsin (AAT) protein aggregates is an archetypal example of how dysregulation in these mechanisms can lead to life-threatening human disease^{3–5}. In healthy individuals, AAT is a 52 kDa glycoprotein product of the *SERPINA1* gene secreted from the liver's hepatocytes to the lungs, where it acts as a neutrophil elastase inhibitor. The 'Z' variant describes a single base pair mutation in Exon 5 of the *SERPINA1* gene that results in an amino acid substitution (Lysine for Glutamate) at position 342⁵. This substitution leads to structural changes in the hinge region of the AAT protein, altered folding dynamics, and predisposition to aggregation of retained polymeric protein (termed ATZ) within the endoplasmic reticulum. Toxic accumulation of intra-hepatocytic ATZ, in turn, triggers inflammation, fibrosis, and downstream liver cancer and/or liver failure. Lack of AAT reaching the lung leads to uninhibited neutrophil elastase mediated parenchymal destruction and emphysema. Epidemiological studies suggest that there may be over 100,000 homozygous (ZZ) and over 100 million heterozygous (MZ, SZ) patients suffering from associated liver disease⁶. Despite significant advances in our understanding of pathology driven by ATZ accumulation, there remains no approved medicine to address this problem, with liver transplantation still the only curative treatment option. This represents a significant unmet medical need and requires urgent attention.

To address this challenge, we rationalized that whilst the clinical manifestations of proteinopathies are often distinct from one another, the mechanisms underpinning them are

largely conserved⁷⁻⁹ and that the most efficient approach to identifying new medicines in ATZ would be to combine our prior expertise in chemical screening campaigns with a human-specific proteinopathy disease model. We accordingly used our patient-derived induced pluripotent stem cell (hiPSC) hepatocyte *in vitro* model of ATZ¹⁰⁻¹³ to screen a small molecule library of over 1,000 known annotated small molecules (provided by Janssen Research and Development in participation with the Phenomics Discovery Initiative) with the objective of identifying ATZ polymer lowering molecular targets. Hits from the initial screen were subsequently validated in additional patient-iPSC and cell models before one candidate was taken forwards for further mechanistic and efficacy testing *in vivo*, as described in the results sections below.

Results:

A high-throughput annotated screen identifies seven new targets for A1ATD

The goal of this study was to identify new therapeutics for patients with Alpha-1 antitrypsin deficiency (A1ATD). To this end, we hypothesized that compounds known to target protein degradation and ER stress would be efficacious in reducing alpha-1 polymer (ATZ) load^{4,14,15}. Accordingly, an annotated compound library containing molecules specific to these pathways was screened against our previously developed *in vitro* iPSC disease model derived from a patient who suffered from A1ATD related liver disease as a result of carrying the Z genetic mutation (PiZ, Fig. 1a)^{10,13}. The iPSC-hepatocyte model was miniaturized into a 384-well plate format with high ATZ polymer and albumin expression representing the disease and hepatic phenotypes, respectively (Supplementary Fig. 1a). The screen was designed to identify compounds capable of reducing ATZ polymer whilst preserving cell viability and phenotype. We tested each compound at 10 μ M concentration in duplicate for 24 hours. Efficacy was interpreted

as a reduction in immuno-fluorescence intensity following staining with the ATZ polymer-specific monoclonal antibody (2C1)¹⁶ and captured using high throughput imaging. The analysis pipeline involved automatic nucleus detection and counting by DAPI signal for ATZ polymer quantification (Fig. 1b). In brief, each detected DAPI+ object was used to generate a 2.5µm ring mask in the circumference which the signal intensity of ATZ polymer was quantified. Of the 1,041 compounds screened, 14 compounds with the lowest z-scores (<-1.92) in comparison to untreated PiZ iPSC-hepatocytes (Fig. 1c and Supplementary Fig. 1b) significantly shifted ATZ expression down towards the range of our healthy control (Supplementary Fig. 1c). We then refined our selection further by performing a set of confirmatory dose-dependent experiments with these 14 compounds (Supplementary Fig. 2), which in turn led us to pursue the following 7 targets: kinase insert domain receptor (KDR), mitogen-activated protein kinase 8 (MAPK8), cyclin-dependent kinase 2 (CDK2), fatty acid-binding protein 1 (FABP1), nuclear receptor subfamily 3 group C member 1 (NR3C1), nuclear receptor subfamily 1 group H member 2 (NR1H2) and leucine-rich repeat kinase 2 (LRRK2). To validate these targets, we purchased commercially available inhibitors (all of which were chemically different from those used in the initial screens) and tested them in a dose-dependent manner across three different PiZ patient lines (Supplementary Fig. 3).

Targeting LRRK2 reduces ATZ polymers across multiple disease models

Our initial experiments found that inhibiting both CDK2 and LRRK2 resulted in significant polymer reduction across all three PiZ patient lines (Supplementary Fig. 3 and Fig. 1d). Interestingly, LRRK2 kinase inhibitors and antisense oligonucleotides are being developed as potential new medicines for another protein misfolding disease; Parkinson's¹⁷. Therefore, the

combination of our experimental data and the biological relevance of this target led us to further investigate the potential of LRRK2 in A1ATD as below. To begin with, we crossed the LRRK2^{-/-} mouse (a mouse with a LRRK2 gene mutation resulting in a protein knockout) with the PiZ mouse¹⁸ (a mouse that expresses the mutant Z version of the human SERPINA1 gene) to generate a new mouse model (LRRK2^{-/-} x PiZ) that is both deficient in LRRK2 and pre-disposed to PiZ related liver pathology (Fig. 2a). These mice were born viable and appeared grossly healthy. Histological characterization of their livers at 8 weeks showed a marked reduction in ATZ polymer load (2C1 expression) (Fig. 2b, c) and by 28 weeks a substantially reduced level of fibrosis (Picrosirius red staining) (Fig. 2d, e). These data supported our hypothesis that LRRK2 was an important regulator of ATZ polymer biology and potential therapeutic target. We, therefore, decided to test the polymer reducing capability of commercially available LRRK2 inhibitors, whose chemical structures targeted different binding sites of the LRRK2 kinase. For these studies, we first employed the CHO cell model, a genetically modified Chinese Hamster Ovary cell which expresses the mutant Z human AAT protein following addition of Tetracycline; “Tet-ON CHO”¹⁹. Induced CHO cells were treated with one of a panel of LRRK2 inhibitors for 48 hours and their impact on polymeric ATZ levels (characterized by 2C1 immunofluorescence staining) assessed. A dose-dependent polymer reduction (without toxicity) was observed with all six LRRK2 inhibitors tested except CZC-541252 (Fig. 2f). Next, to confirm these findings in a more relevant human model, we repeated these studies in iPSC-hepatocytes. Here we again observed significant polymer reduction and retention of cell viability with all but one of the molecules tested (Fig. 2g, h).

CZC-25146 reduces polymer load and inflammation whilst restoring secretion of normal AAT.

Of the LRRK2 inhibitors tested, CZC-25146 displayed the most favourable polymer reduction and cell viability profile *in vitro*. This compound was also noted to have poor blood-brain barrier penetrance when previously studied *in vivo*²⁰. These two features led us to postulate that it would be an ideal inhibitor for use in A1ATD patients where the target organ is the liver and we accordingly proceeded with CZC-25146 in the next round of investigations.

We first observed that a 24-hour treatment with CZC-25146 resulted in a dose-dependent reduction of ATZ polymer load across three different patient-derived iPSC-hepatocyte models without compromising cell viability (Fig. 3a, b and Supplementary Fig.4). After 48 hours treatment, using higher doses, further reductions in ATZ polymer were seen (Fig. 3c) along with significant reductions in expression of the transcription factor nuclear factor kappa B (NFkB) (Fig. 3d), a molecule previously reported to be one of the most important inducers of inflammation-associated pathology in this setting²¹. Importantly, for the objective of treating both the lung (deficiency of normal AAT) and liver (toxicity of ATZ polymer accumulation) diseases known to occur with A1ATD in parallel, we observed treatment with CZC-25146 increased secretion of normal AAT at the same time as reducing intracellular ATZ polymer load (Fig. 3e).

Next, to interrogate the potential of CZC-25146 as a therapy for PiZ patients, we conducted a proof-of-concept study using the PiZ mouse model¹⁸. The PiZ mice used in our studies were genetically modified to accumulate human ATZ protein in the ER of mouse hepatocytes in a manner analogous to the pathology observed in human disease. (Notably, this animal model does not suffer from deficiency of circulating AAT due to endogenous production of murine AAT, thus it does not recapitulate the lung pathology of this disease. Nonetheless, it is a suitable animal model

to study the impact of CZC-25146 on liver pathology). Animals were dosed via oral gavage at 250mg/kg (or vehicle controls) for 14 days. We chose to use male mice aged 6 weeks of age for our study, as this is believed to capture peak polymer accumulation level in this model. At 250mg/kg, CZC-25146 reduced the polymer levels as evaluated by the density of PAS-positive, diastase-resistant (PASD) globules across all five liver lobes in all mice (Fig. 4a). Inspection under higher magnification revealed a reduction in polymeric containing globules within hepatocytes, quantitative assessment of which revealed globule reduction to be consistent across all five liver lobes (Fig. 4b top) with an overall reduction from 60% in the control group to 37% in the CZC-25146 treated group (Fig. 4b bottom). Using the Meso Scale Discovery inflammatory cytokine array, we also found that CZC-25146 administration led to a broad selection of hepatic pro-inflammatory cytokines being down-regulated and restored to healthy control levels (Fig. 4c). From a safety standpoint, no apparent drug-induced liver injury was observed following treatment, with the liver architecture remaining intact throughout and hepatocytes not demonstrating hyperproliferation (Ki67) or carcinogenesis (Fig. S5).

Lastly, although CZC-25146 was previously defined as a highly selective LRRK2 kinase inhibitor, activity against other kinases (PLK4, GAK, TNK1, CAMKK2, and PIP4K2C) has subsequently been reported to occur²⁰. To confirm that the polymer reduction observed following CZC-25146 treatment of PiZ mice was a consequence of LRRK2 kinase inhibition, we therefore harvested livers and purified both mRNA and protein for RT-qPCR and immunoblotting analysis. LRRK2 transcriptional (Fig. 4d) and protein expression level (Fig. 4e) remained unchanged following treatment but LRRK2 activity, as measured by autophosphorylation represented by pSer935-LRRK2, was reduced, quantification of which was consistent with our immunohistochemical analysis (Fig. S6).

CZC-25146 mediated polymer reduction is associated with autophagy induction

Dysregulated intracellular protein degradation pathways have been postulated to contribute to the liver disease associated with ATZ polymer formation⁴. Autophagic degradation has gained particular interest because deletion of the *Atg5* gene was shown to exacerbate ATZ polymer load accumulation in the ER lumen²². Since LRRK2 is reported to be a regulator of autophagy^{23–25}, we hypothesized that the mechanism of CZC-25146 ATZ polymer reduction could be via induction of autophagy. To test our hypothesis, we first validated the functional link between CZC-25146 and autophagy. Leveraging the LC3-GFP human fibroblast line, in which the autophagosome membrane is labelled with GFP, we found that CZC-25146 treatment-induced autophagosome formation at levels similar to that observed with Rapamycin, which is known to be a potent inducer of autophagy (Fig. 5a)²⁶. Time-course analysis revealed that autophagosome formation peaked at 2 hours post-exposure to both CZC-25146 and Rapamycin before gradually decreasing (Fig. 5b). After establishing this link between CZC-25146 and autophagy activity in fibroblasts, we next evaluated autophagy gene expression in CZC-25146 treated PiZ patient iPSC-hepatocyte lines. *VPS34*, which is critical for the initiation of autophagy, along with downstream *ATG* related genes such as *ATG3*, *ATG5*, *Calnexin*, *LC3A* and *LC3B* were all found to be significantly upregulated following treatment and in a dose-responsive manner (Fig. 5c and Supplementary Fig. 7). The LC3-II to LC3-I ratio, as detected by immunoblotting, also increased in response to CZC-25146 (and Rapamycin) treatments indicating a link between autophagosome formation and ATZ polymer reduction observed in the cells (Fig. 5d). To show the functional importance of this observation, we next treated cells with a combination of the autophagy inhibitor ammonium chloride, NH_4Cl ²⁷ + CZC-25146 (Fig. 5e) and as expected, found NH_4Cl partially reversed the

polymer lowering effect of CZC-25146. Finally, to investigate whether CZC-25146 had the same effect *in vivo*, we performed immunohistochemistry for LC3 on PiZ treated mice. These analyses showed that administration of CZC-25146 restored autophagy to levels seen in healthy control mice (Fig. 5f, g).

Discussion:

Using a patient-derived iPSC-hepatocyte model of Alpha-1 antitrypsin deficiency (A1ATD), we screened over 1,000 annotated small molecules for their ability to reduce ATZ polymer load. Seven hits from the initial screen were taken forward and tested across multiple concentrations using three different patient iPSC lines. Based on the data from these initial rounds of testing and because of its clinical relevance to other related protein misfolding diseases, LRRK2 was selected as our target for further validation. We began that validation by showing that the LRRK2^{-/-} x PiZ mouse has lower levels of ATZ polymer accumulation and less liver damage (fibrosis). Next, we demonstrated that a collection of commercially purchased LRRK2 chemical inhibitors could similarly (though to different degrees of efficiency) cause ATZ polymer reduction across both iPSC and CHO cell models. One of these molecules, with a favourable biodistribution profile for targeting the liver²⁰, CZC-25146, was selected for follow on studies in the PiZ mouse. Here it was found to reduce polymer load and inflammation after only 14 days of oral treatment. Finally, we confirmed the mechanism of action of CZC-25146 to be via inhibition of LRRK2 kinase activity and augmentation of autophagy. Cumulatively, these data support the idea that LRRK2 inhibitors activate autophagic clearance of ATZ polymers from hepatocytes and that CZC-25146 along with related LRRK2 inhibitors may represent new therapeutic approaches for the liver disease associated with alpha-1-antitrypsin (Z).

Liver disease represents an increasing global health challenge. ATZ associated liver disease is caused by gain-of-function toxicity arising from AAT polymer accumulation. Recent developments to suppress AAT protein production via RNAi²⁸ or replace missing AAT via donor plasma infusions²⁹ are promising and have progressed to clinical trials but are unlikely to represent a complete long-term solution for patients since these approaches cannot address both liver and lung pathologies concomitantly. Instead, a therapy that can reduce ATZ polymers and restore the balance of normal AAT secretion from the liver is most needed. Gene editing to correct underlying mutations in the *SERPINA1* gene or cell therapy could solve this problem but there are currently no technologies advanced enough to facilitate sufficient levels of genetic correction or cellular engraftment as would be required to achieve clinically relevant outcomes. In the interim, a small molecule approach to either block polymer formation³⁰ or increase polymer degradation represent two of the most promising, potentially complementary, alternative solutions. Targeting intracellular degradation is particularly appealing since the clinical variations in liver disease severity observed in patients harbouring the same genetic mutation (ranging from no/minimal disease to liver failure requiring organ replacement) may result from variable capacity in the patient's hepatocytes to degrade misfolded proteins. Although the autophagy pathway has been reported to play an important role in ATZ polymer degradation, the identity of the specific molecular players involved in this pathway have remained elusive³¹. This may partly explain why attempts to augment autophagic flux non-specifically have so far yielded clinical results with limited success (ClinicalTrials.gov identifier NCT01379469)¹⁵. Identifying a hepatocyte-specific target to accelerate polymer destruction and restore/maintain normal AAT production may solve this issue but until recently has been hampered by a lack of suitable research tools. Using iPSC technology to engineer hepatocytes from patients with clinically

documented ATZ polymer-associated liver disease allowed us for the first time, to the best of our knowledge, to robustly identify molecular targets for polymer degradation. This was performed with a small molecule screen in a cell model wherein both the genetic defect (Glu-Lys 342) and patient-specific protein degradation machinery are conserved. Previous disease models which employ overexpression of the mutated human gene in a non-human background (e.g. human ATZ in mouse, hamster or worm cells)³² cannot achieve these insights because they are limited by species differences in the host cell machinery. We, therefore, propose that our approach is the most robust currently available tool for identifying protein degradation targets relevant for patients. Indeed all 7 of our validated hits represent highly interesting molecular targets to follow up on. We chose, however, to focus on LRRK2, a seven domain, 285kDa cytosolic protein implicated in lysosomal degradation^{33,34}. Previous work by us and others suggests that patients harbouring Z-AAT mutations develop liver disease when their polymer disposal mechanisms (autophagy - lysosomal - degradation pathway; ALP) are overrun. LRRK2 may therefore be a critical but hitherto unrecognized regulator of hepatocyte ALP and by inhibiting LRRK2 kinase activity, we could drive ATZ polymer disposal and ameliorate the associated liver disease. Interestingly, genetic variation at the *LRRK2* locus has been identified as a key contributor to the risk of another protein misfolding disorder, Parkinson's disease³⁴. This association led to the development of LRRK2 kinase inhibitors and antisense oligonucleotides as potential new medicines for PD, with leading candidates expected to enter late-stage clinical trials this year¹⁷. Their mechanism of effect however remains incompletely understood. Several studies suggest LRRK2 acts upon different stages of the ALP, with conflicting results on how this is mediated and the net physiological direction. For example, in neurons derived from patients with PD, it was shown that Rab10 is a mediator of LRRK2 kinase activity that negatively regulates

lysosomal activity and clearance of alpha-synuclein³⁵ whilst in infected macrophages, LRRK2 regulates the ALP, this time via Rab8A interaction with CHMP4B³⁶. In contrast, LRRK2 overexpression in kidney and neuroendocrine cells induced autophagy through a Ca^{2+} /CaMKK/AMPK pathway³⁷. These studies support a paradigm whereby LRRK2's role in ALP is both cell- and disease-specific. Our data suggests the level of LRRK2 kinase activity is an important regulator of ATZ polymer handling via the ALP in hepatocytes and that targeting LRRK2 could lead to a therapeutic reduction in polymer load with downstream reversal of liver disease in A1ATD. At this time, however, the specific molecular players interacting with LRRK2 to regulate those observations are yet to be elucidated. Future studies are required to increase our understanding of how LRRK2 works in this context and could therefore help drive new treatment approaches for ATZ. Notably, this may also increase our understanding of the pathological mechanisms in other liver diseases such as NAFLD in which protein aggregates and ER stress are postulated to represent fundamental components of a multi-factorial pathology^{8,38–41}.

In the immediate future, our data support further investigation of the LRRK2 inhibitor, CZC-25146, in ATZ. This compound was previously developed as a therapeutic for Parkinson's disease but did not progress further because it was found to cross the blood-brain barrier poorly²⁰. In contrast to its original indication, such bio-distribution profile may be advantageous when seeking to target pathology restricted to the liver and therefore provides further impetus to evaluate this molecule in the context of ATZ therapy. More broadly, we propose LRRK2 inhibitors, especially those already shown to be safe in clinical trials of Parkinson's disease²³, be considered to not only help further our mechanistic understanding of ATZ biology in the laboratory but also for therapeutic use in the clinic.

Methods:

Cells lines

hiPSCs: One in-house developed hiPSC line (line 1) and two gifted hiPSC lines (line 2; PiZZ1 and line 3; PiZZ6 from Andrew Wilson Lab, Boston University) used in this study were reprogrammed from the PiZ patients with severe liver disease. hiPSCs were cultured in Essential 8 medium (Gibco), on Vitronectin (Sigma Aldrich) coated plates, and passaged using ReLeSR (STEMCELL Technologies) every 3-4 days. hiPSCs were differentiated into hiPSC-derived hepatocytes (iHeps) using our previously established protocol¹⁰. In brief, iHeps were differentiated until day 30 prior to use in all experiments, with plating into assay format taking place at day 21.

Tetracycline-inducible (Tet-ON) CHO-K1 cell expressing polymeric A1AT E324K (gift from Lomas Lab) were maintained as previously described⁴². CHO-K1 cells were co-treated using 1µg/mL doxycycline (Takara Bio Europe, Clontech Inc.) and the small molecules for experiments described in the main text.

LC3-GFP fibroblasts were gifted from the Carlton Lab and were analyzed using both the Operetta CLS for quantitative assessment, and the SP8 confocal (Leica) for qualitative assessment. Fibroblasts were cultured in DMEM (Gibco) +10% FBS (Sigma Aldrich) + 1% Penicillin & Streptomycin (Sigma Aldrich) and passaged once a week using Trypsin (sigma). For experimentation, fibroblasts were cultured in OptiMEM (ThermoFisher, 31985062) +/- test compounds, without FBS. Data analysis was conducted on Harmony.

Annotated Compound Screen

The National Phenotypic Screening Center conducted the annotated compound screen, using a compound library of known small molecules with annotated targets provided by Janssen

Research and Development. A day before cell seeding, 384-well plates were coated with laminin-411 (LN411-03, Biolamina) at $3.75\mu\text{g}/\text{cm}^2$. The A1ATD and healthy control iPSC-hepatocytes used in the screen were commercially sourced (Definigen), thawed in Def-HEP thawing media (Definigen), and plated at a density of 20,000 cells per well. Subsequently, the cells were maintained in Def-HEP Recovery & Maintenance Medium (Definigen) until day 13 post-seeding. JANUS G3 (Perkin Elmer) automated liquid handling system was used for media changes every two days until day 13, then for test compound dispensing. A library of 1,041 annotated small molecules were all dissolved in DMSO and tested at a final concentration of $10\mu\text{M}$ for each compound. The test compounds were left for 24 hours, with each compound being tested in duplicate in separate plates. Subsequently, the wells were washed, fixed with 4% paraformaldehyde, and permeabilized with 0.5% Triton X for 5 minutes. The samples were then blocked in 3% donkey serum in 1% BSA for 1 hour and incubated with primary antibodies at 4°C overnight. Polymeric alpha-1 antitrypsin (A1AT) was assessed using the 2C1 monoclonal antibody (1:250 dilution; gifted by the Lomas lab) and Alexa-488 donkey anti-mouse secondary antibody (1:500 dilution; A21202, Life Technologies). Image acquisition was performed on an In Cell 6000 Analyzer (Cytiva), and image analysis was performed with Columbus software (Perkin Elmer). The 2C1 signal was quantified by detection of the nuclei (DAPI⁺ objects), then generating a ring-like structure by shape dilation by $2.5\mu\text{m}$. The signal intensity was measured in the ring region to obtain the average polymer load measurement per cell in the well. An average of all cells per well was used in the downstream analyses – fold-difference to the untreated A1ATD cells and z-score calculation for each well. The albumin expression was assessed for quality control purposes and was measured using the anti-albumin goat monoclonal antibody (1:500 dilution; A80-129A, Bethyl) and Alexa-647 donkey anti-goat secondary antibody (1:500

dilution; A21447, LifeTechnologies). Compounds that led to cell death, determined by a dramatic reduction in DAPI⁺ objects, or loss of albumin expression, were removed and excluded from the analysis. Hits were defined as compounds with a 2C1 z-score in the vicinity or lower than 1.92 (95th percentile of healthy control cells) were selected for a confirmatory screen. Fourteen hits selected in the primary screen were advanced onto confirmatory, dose-dependent studies. The compounds were tested in triplicates at two-fold serial dilution doses ranging from 20μM to 156nM. This secondary screen was performed on 384-well plates using the immunofluorescence methodology used in the primary screen. The 2C1 readouts were normalized to the DMSO-treated A1ATD cells and the seven compounds showing dose-response and no severe cytotoxicity (<75%) were selected for further validation.

Validation of Compound Screen

CZC-25146 (Tocris, 6071), Flurandrenolide (Sigma, 1284000), Fenofibrate (Sigma, F6020), NU6140 (Sigma, SML1323), 4-Chloro-2-Methylthiopyrimidine (Sigma, 145289), GW3965 (Sigma, G6295), 1-9-Pyrazoloanthrone (Sigma, S5567), CZC-542525 (Sigma, S6534), MLI-2 (Sigma, S9694), GNE-9605 (Sigma, S7368), GNE-0877 (Sigma, S7367) and GNE-7915 (Sigma, S7528) were tested on day 30 ATZ iHeps for either 24 or 48 hours, with polymeric A1AT assessed using the polymer-specific antibody 2c1 (gifted by the Lomas Lab) and viability assessed using the Presto Blue Viability Assay (ThermoFisher), conducted according to manufacturer's instructions. The appropriate dose-response starting point for each compound was determined using the manufacturer's solubility data and calculating the highest compound concentration that could be used without exceeding 0.1% DMSO in the culture. Antibodies used include: LRRK2 (Abcam, ab133474), total hA1AT (Abcam, ab9373), human ALB (Bethyl Laboratories, A80-129A). A1AT ELISA (Abcam, ab108798) was used according to the

manufacturer's instructions. High-content imaging was conducted using the Operetta CLS (Perkin Elmer) and data was analysed using Harmony software (Perkin Elmer). Differential gene expression was detected by quantitative reverse transcription polymerase chain reaction (RT-qPCR). RNA was extracted using RNeasy mini kit (Qiagen, 74104) according to the manufacturer's instructions. Subsequently, qPCR was conducted using CFX Connect Real-Time PCR Detection System (BioRad). All qPCR data was normalized to GAPDH and RPL13 which are considered housekeeping genes. Table S1 contains all the primer sequences used during this study.

Pre-clinical mouse study

Ethical compliance was obtained prior to working with all mouse models. The PiZ mouse was administered CZC-25146 at 250mg/kg dissolved in pure DMSO, administered for 14 days consecutively by oral gavage. Mice were culled after 14 days and each lobe of the liver was separated independently. For IHC characterization, all samples were washed thrice with PBS, followed by a 24-hour fixation with 10% formalin. Samples were then rewashed three times with PBS, before being processed, embedded into paraffin, sliced, and stained. The antibodies/stains used were: PASD (Sigma Aldrich, 395B-1KT), LC3 (Abcam, ab48394), Picrosirius Red (Abcam, ab246832), Ki67 (Abcam, ab15580). *In vivo* image analysis was conducted by taking three 5x shots of each liver lobe, equally distributed across each lobe (15 images per mouse). The images were analysed using ImageJ. Western Blot analysis was prepared by homogenizing the frozen liver tissue in Cell Lysis Buffer (Cell Signaling Technology, 9803) supplemented with phosphatase inhibitor (#78427, ThermoFisher) and protease inhibitor (ThermoFisher, 78429) using TissueLyzer II (Qiagen) for 30 min. Protein concentrations were normalized using a Pierce™ BCA protein assay kit (ThermoFisher) according to the manufacturer's instructions. Processed proteins

samples were run on 3–8% NuPAGE Tris-Acetate 1.5mM pre-cast gels (ThermoFisher, EA0378BOX) and primary antibodies used were: anti-phospho-Ser935 LRRK2 [UDD2 10(12)] (Abcam, ab133450) or anti-LRRK2 [MJFF2] (Abcam, ab133474). The secondary antibody was IR Dye 680RD (Licor, 962-68073). Blots were imaged using ChemiDoc MP (Biorad). Quantitation of the bands was performed using Image Studio (Licor).

Statistics

Statistical analysis involved a Kolmogorov-Smirnov test for normality, followed by an analysis of variance. When more than two groups were compared, the normality and variance analysis was followed by either an Ordinary One-way ANOVA (for parametric datasets) or Kruskal-Wallis test (for non-parametric datasets), followed by a Dunn's (non-parametric) or Dunnett's (parametric) multiple comparison test. If two groups were compared, the normality and variance testing was followed by either a T-test (parametric data) or a Mann-Whitney Test (non-parametric data). $*=p<0.05$, $**=p<0.01$, $***=p<0.001$, $****=p<0.0001$. All statistics were done on Graphpad Prism.

Study approval

All animal experiments were performed in accordance with UK Home Office regulations (UK Home Office Project License number PPL P26C63193).

Author contributions:

Conceptualization: STR, CM, DK, SSN

Methodology: STR, SS, DE, DK, SSN

Investigation: STR, CM

Visualization: DK, SSN, AMS,

Funding acquisition: STR, CM

Project administration: DK, SSN

Supervision: STR, CM, DD, DE,

Writing – original draft: SSN, DK

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Figures

Fig 1. High throughput annotated compound screen in patient-derived iPSC-hepatocytes identifies new targets for reducing alpha-1 antitrypsin Z (ATZ) polymers. **A** Schematic of the overall approach. **B** Image analysis pipeline involved nucleus detection and counting by DAPI signal. Each detected DAPI+ object was used to draw a 2.5μm ring mask for quantification of 2C1 signal (polymeric ATZ) in each cell. **C** Average polymer signal/cell was recorded and plotted following addition of each compound in triplicate. Reduction in polymer load (2C1) was used as a marker of therapeutic efficacy. Compounds with z-score lower than -1.92 (95th percentile of healthy control cells) were selected for follow up (shown in red). Untreated PiZ cells shown in black. Each dot represents the average of two measurements taken for each compound. N=1,041. **(D)** ID 22380519 targeting LRRK2 was identified as the top hit following the initial screen and the confirmatory dose-dependent study. It shows dose-dependent response in ATZ polymer reduction (pink) and no severe cytotoxic effects (black).

Fig. 2. Targeting LRRK2 reduces ATZ polymer across multiple models. **A** LRRK2^{-/-} PiZ mice were generated by crossing PiZ/C57B6 (expressing human ATZ polymer in liver) mice and LRRK2^{-/-}/C57B6 mice. **B** Representative images showing ATZ polymer levels in the livers of LRRK2^{-/-} x PiZ (right), PiZ (middle) and healthy controls (left). Scale bar, 200µm. **c** Semi-quantitative analysis of samples from **b** shows reduced ATZ polymer in LRRK2^{-/-} x PiZ (green bars) compared to PiZ (pink bars) mice. N=3 animals. **D** Representative images showing fibrosis levels (Picrosirius red staining) in the livers of LRRK2^{-/-} x PiZ (right), PiZ (middle) and healthy controls (left). Scale bar, 200µm. **e** Semi-quantitative analysis of samples in **d** shows reduced fibrosis in LRRK2^{-/-} x PiZ (green bars) compared to PiZ (pink bars) mice. N= 4 animals. Statistical analysis by ordinary ANOVA test followed by Dunnett's multiple comparison test. **F** A panel of LRRK2 inhibitors demonstrate dose-dependent polymer reduction (pink curve, inhibition) and viability (black curve, cytotoxicity) following 48 hours treatment of Tetracycline-inducible (Tet-ON) CHO-K1 cells. N=3. Nonlinear regression by four-parameter curve fit. **G** Representative confocal images showing polymeric ATZ expression (green) in patient-derived iPSC- hepatocytes treated with same panel of LRRK2 inhibitors as indicated. Scale bar, 150µm. **h** Quantitative assessment of confocal images from **g** demonstrates significant reduction of polymer (2C1) (left) following 48h treatment without reducing cell number (viability) (right). Each dot represents a biological replicate. Statistical analysis by ordinary ANOVA test followed by Dunnett's multiple comparison test against the control. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Fig. 3. CZC-25146 reduces ATZ polymer and restores AAT secretion *in vitro*, without compromising cell viability. **a** Representative images demonstrating ATZ polymer reduction in PiZ patient-derived iPSC-hepatocytes (middle row, green) with preserved total AAT (top row, red) after 24 hour treatment with CZC-25146 (middle column) compared to vehicle (left column) and health donor (right column) controls. Scale bar, 100μm. **b** Quantification of polymer load (top graph), number of cells (middle graph) and cell viability (bottom graph) following increasing doses of CZC-25146 (x axis) administered for 24 hours across each of three different PiZ patient iPSC lines. 48 hour treatment of patient-derived iPSC-hepatocytes with CZC-25146

at high (purple bars) and lower (green bars) doses reduces intrahepatic polymeric ATZ **c**, nuclear NFkB **d** and increases healthy AAT secretion **e** compared to untreated (grey bars) or vehicle controls (pink bars). N=10 replicates. Statistical analysis by Kruskal-Wallis test, followed by a Dunn's multiple comparison test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Fig. 4. CZC-25146 reduces ATZ polymer and decreases inflammation in the PiZ mouse liver. **a** Representative images of polymer load (PASD staining) in PiZ mouse livers following treatment with CZC-25146 (CZC, middle) or vehicle control DMSO (left). Healthy C57BL/6 mouse liver (right) shown as control. LL = left lateral lobe, LM = left medial lobe, CL = caudate lobe, RM = right medial lobe, RL = right lateral lobe. Scale bar, 150 μ m. **b** Quantification of staining from **a**

showing reduction in each of the five liver lobules (top) and in aggregate (bottom) following treatment with CZC (pink bar), vehicle control (grey bar) or with no treatment (purple bar). **c** Levels of inflammatory cytokines in liver tissue were reduced in animals treated with CZC (pink bar) compared to vehicle control (grey bar) returning to levels seen in the no treatment (green bar) group. N=5 mice per group. **d** RT-qPCR shows CZC-25146 treatment has no impact on LRRK2 gene expression of PiZ liver. **e** Immunoblotting analysis of purified LRRK2 from each of the three CZC-25146 treated PiZ mice compared to vehicle only and untreated (WT - healthy mouse) controls showed reduction in total LRRK2 protein level (row 1) and LRRK2 phosphorylation status (row3). Statistical analysis by unpaired Student t-test. N=3 mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Fig. 5. CZC-24156 induces autophagy *in vitro* and *in vivo*. **a** Representative images of LC3-GFP fibroblasts, following treatment with CZC-25146 (CZC, middle), vehicle control (left) or positive control Rapamycin (right). Images taken 2 hours after exposure. Scale bar, 50µm **b** Time course analysis following CZC (pink) or Rapamycin (green) treatment shows significant upregulation of LC3 expression from 2-10 hours compared to vehicle controls (grey). **c** RT-qPCR shows dose responsive increased expression of genes involved in the autophagy pathway following addition of CZC to patient-derived iPSC-hepatocytes at Low (pink) = 14.3µM or High (green) = 28.6µM concentrations. N=4 replicates. **d** Representative immunoblotting images (left) and quantification (right) of LC3 isoforms from PiZ patient iPS-Hepatocytes following treatment with CZC-25146 (CZC, middle), vehicle control (left) or positive control Rapamycin (right). N=3. **e** Representative images (left) and semi-quantitative analysis (right) showing the effect of treatment with CZC alone (left middle), CZC + autophagy inhibitor, NH₄Cl (right middle), NH₄Cl alone (far right) or vehicle control (far left) on ATZ polymer (2C1 expression, green) in patient-derived iPSC-hepatocytes. Scale bar, 100µm. **f** Representative macroscopic (top) or zoomed in (bottom) images of LC3 staining in livers of PiZ (far left), healthy mice (far right), CZC-25146 treated (right middle) or vehicle control (left middle). **g** Quantification of images from **f** separately analysed by each liver lobe (left) or pooled (right). Quantification was conducted using ImageJ. N=5 mice per group. Statistical analysis by Ordinary ANOVA test, followed by Dunnet's multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

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