Development of a small molecule that corrects misfolding and increases secretion of Z α1-antitrypsin

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)
Dear Prof. Lomas,

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now heard back from the three referees who agreed to evaluate your manuscript. As you will see from the reports below, while referee #1 and #2 are overall supporting publication of your work, referee #3 highlights the interest of the study but also raises a number of concerns that should be addressed in a major revision of the current manuscript. After a cross-commenting exercise it became clear that a control experiment with M alpha1-antitrypsin-expressing cells treated with GSK716 should be included, while no further in vivo experiments are required. However, addressing the reviewers' concerns in full, experimentally or in writing, will be necessary for consideration of your manuscript in our journal. Particularly, you should provide a detailed response to the referee #3 criticism of the mouse model, and in vivo experiments and discuss the limitations of the study in that regard. Also, PDB validation reports should be made available as suggested by the referee #1.

Please be aware that the acceptance of the manuscript will entail a second round of review. Please note that EMBO Molecular Medicine encourages a single round of revision only and therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. For this reason, and to save you from any frustrations in the end, I would strongly advise against returning an incomplete revision.

We would welcome the submission of a revised version within three months for further consideration. However, we realize that the current situation is exceptional on the account of the COVID-19/SARS-CoV-2 pandemic. Please let us know if you require longer to complete the revision.

I look forward to receiving your revised manuscript.

***** Reviewer's comments *****

Referee #1 (Remarks for Author):

This paper represents a triumphant progress, led by the first author over a period of nearly 30 years,
from the initial proposal of a conformational basis for a common inherited disorder to, here, the
selection and design of a pharmaceutically effective counter. In terms of molecular diseases it is an
unique achievement: e.g. even after 70 years there is still no effective counter to the sickle-
haemoglobin mutation. The paper outlines and clearly describes careful and painstaking studies.
The deductions and conclusions are data-based and frank (no hype!). There are some caveats that
do not affect the strength of the paper but to which the authors should respond and modify as
appropriate.
Over the period of thirty years there has inevitably been relevant refinements as to the underlying
mechanism of polymerisation. There is now totally persuasive evidence that the fundamental
defect is the aberration of the bonding centred on Glu342 that pinions strand 5a in place and
hence guides the final incorporation of the C-terminus into the body of the antitrypsin molecule
and subsequently hinders its aberrant release. The supporting evidence is summarised in the paper of
Huang et al (JBC 2016: 291,15674) on the 'Molecular mechanism of Z alpha-1-antitrypsin
deficiency', which includes the crystal structure of Z antitrypsin. It should be cited. The Huang paper
in no way pre-empts the current submission and indeed greatly strengthens it, almost as a
preliminary study. The two papers are complementary, with satisfying agreements as to the drug-
binding site. The very real advance in the submitted paper being the confirmation structurally of
what is otherwise a deduced binding-site and in the design of a ligand that preferentially binds to
the Z variant rather than the M (normal wild-type).
The submitted paper describes new and important findings but it presents these in what is in
places a dated context. The findings here precisely fit with the consensus mechanism that is now
supported by a whole body of studies in the field. It is important to get this right which could be
achieved with some minor rewriting. As it is, the Discussion can be misleading, as in 'The finding that
GSK716 mediates its action by binding to a cryptic pocket implies that intrahepatic polymers form a
near native or native conformation, rather than a more extended intermediate.' This is not so, and
should be removed. We are looking at the stabilisation of equilibria, be it in the partially folded form
or the fully-folded but labile aberrant native form.
An outstanding finding in the submitted paper is in the determination of the structure of the
antitrypsin-GSK17 complex. Although this structure seemed instinctively correct the data was
succinct and a backup expert crystallographic opinion was sought, as follows.
'The crystallography is almost certainly fine, judging from the overall statistics and the resolution,
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When a structure is deposited at the PDB, the authors receive a validation report that is intended
for potential reviewers. This should be provided with the manuscript to allow referees to assess the
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section, but it's blank. Similarly, in the Refinement section the line for Water under "No. atoms" is
blank, and both lines for Protein and Water under B-factors are blank. If numbers are given in these
two subsections for protein and water, they should also be given for the ligand. In particular, it's
important to be able to compare the mean B-factor of the ligand with that of the protein.
Whenever an important part of a structural paper is the binding of a ligand, it is accepted practice to
provide a figure showing electron density for the ligand. Ideally, this should be unbiased electron
density, computed either from a difference map before the ligand was added or after omitting the
ligand and carrying out some refinement.
One comment: the paper reports that the structure was solved by molecular replacement, using a
model that had originally been obtained from PDB entry 2qug. Since 2qug seems to be isomorphous
to this structure, it would have been simpler to carry out rigid-body refinement than to use
molecular replacement, which will give a structure with a different choice of origin and symmetry
copy; this makes structure comparison less convenient to users of the structures than the rigid-
body refinement option.'
The authors should respond to these points and the Editor/Journal note the desirability of making PDB validation reports available to reviewing referees.

Referee #2 (Comments on Novelty/Model System for Author):

The models are well-suited to this proof of principle analysis.

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The paper presents an analysis of a small molecule corrector of AAT folding that interdicts polymerization of Z-type AAT. The candidate molecule was identified by high throughput screening from a large library. The paper from a distinguished group of investigators is well-written and compelling. The curious findings are that there is no reduction in intrahepatic polymers despite a 7-fold increase in circulating Z-type AAT and that the lower doses (10 and 30 mg/kg) exerted the same effect as the 100 mg/kg dose despite low free drug levels. The authors do offer conjecture to explain these findings. They also offer an explanation as to how the opposing effects of GSK716 to decrease serine proteinase inhibition of AAT vs. to enhance secretion of Z-type AAT might result in net benefit. Of course, definitively understanding the net effect of GSK716 will require further study. Within the context of a proof-of-principle analysis, including examining the effects in mice, the paper offers an important contribution.

Referee #3 (Comments on Novelty/Model System for Author):

Cell line models are used to demonstrate that the lead compound, GSK716, reduces the intracellular accumulation of polymers and increases secretion of Z-AAT. Similar results were obtained using the PiZ mouse model. However, this study did not provide convincing evidence demonstrating the beneficial effects of abolished polymerization and increased secretion of Z-AAT in the cell- or mouse-models. In Fig. 2f, the authors show that the treatment with GSK716 modestly improved the viability of Z-AAT expressing cells following a "second insult" namely, tunicamycin treatment. However, M-AAT expressing cells + GSK716 control was notably missing. Furthermore, it is unclear why 13-15 week old, PiZ hemizygous mice were used in this study. At this age, the authors themselves report that there were no signs of liver fibrosis. Use of mice at an age with early signs of fibrosis may have been more informative in determining whether GSK716 treatment could prevent progression or reverse existing fibrosis.

Referee #3 (Remarks for Author):

It is widely accepted that the Z-mutant of AAT accumulates as polymers within the endoplasmic reticulum of hepatocytes and this is responsible for the liver disease associated with severe AAT-deficiency. It is also widely accepted that the accumulation of Z-AAT in the liver, significantly impairs secretion of AAT into the plasma. The reduced circulating AAT is thought to be responsible, as least in part, for the lung disease associated with AAT-deficiency. As such, a compound that abolishes Z-AAT polymer formation and consequently enhances secretion of AAT would have beneficial effects on both the liver and lung diseases associated with AAT-deficiency. In an effort to identify such a compound, the authors conducted a screen and identified a strong lead compound, GSK716, that blocked Z-polymer accumulation and increased secretion of Z-AAT in their cell line.
models. Further studies performed in mice showed an apparent increase in secretion as determined by an increase in circulating Z-AAT in the plasma. Unfortunately, the compound had no effect on preventing the accumulation of Z-AAT polymers in the mouse hepatocytes.

Although the in vitro data on the lead compound's ability to prevent polymerization is impressive, it is unclear, from this study, whether the decrease in accumulation of Z-AAT is actually beneficial to the cells in anyway. The authors tried to answer this question by generating a tunicamycin survival curve (Fig. 2f). Interestingly, GSK716 treatment modestly improved viability of Z-expressing cells, however, an important control, notably M-expressing cells treated with GSK716 was missing. Further studies are required to address this important question.

In some instances, there is inadequate information, in the figure legends and in the methods, to adequately understand how the experiments were conducted and what the results actually mean. The authors indicated that GSK716 "increased the secretion of Z a1-antitrypsin approximately 3-fold compared to vehicle control (mean pEC50 6.2 {plus minus} 0.23; n = 74) (Fig. 2b)," however, it is unclear how they came to that conclusion from the data provided. Fig. 2b show 100% secretion. Fig. 2d shows 3-fold secretion. What does 100% secretion mean? What does 3-fold secretion mean? More information should be included to assist the reader in determining the significance of the data.

The authors then provide data in the PiZ mouse model showing that GSK716 treatment significantly increased plasma levels of Z-AAT. They concluded that this was due to increased secretion of Z-AAT from the liver, however, it is unclear whether this is actually the case since the amount of Z-AAT in the hepatocytes remained unchanged in control- and GSK716-treated mice.

Although, GSK716 failed to alter Z-AAT polymer accumulation in the liver, one could argue that the 7-fold increase in secretion of Z-AAT would be beneficial to the lung disease associated with AAT-deficiency. Unfortunately, GSK716 binds to Z-AAT in such a way that it abolishes the anti-proteinase activity of Z-AAT. This is a critically important as it implies that increasing secretion of Z-AAT has no apparent physiological benefits.

The authors' conclusion that "This study provides proof-of-principle that 'mutation ameliorating' small molecules are a viable approach to treat protein conformational diseases" is not supported by the data presented in this manuscript.

Minor points:

GSK716 is sometimes referred to as GSK'716A. Is this a different analog or a typo?

Fig. 2g, bottom right, should be "extracellular" not intracellular.
Referee #1 (Remarks for Author):  

This paper represents a triumphant progress, led by the first author over a period of nearly 30 years, from the initial proposal of a conformational basis for a common inherited disorder to, here, the selection and design of a pharmaceutically effective counter. In terms of molecular diseases it is an unique achievement: e.g. even after 70 years there is still no effective counter to the sickle-haemoglobin mutation. The paper outlines and clearly describes careful and painstaking studies. The deductions and conclusions are data-based and frank (no hype!). There are some caveats that do not affect the strength of the paper but to which the authors should respond and modify as appropriate.

We are very grateful to the reviewer for his/her supportive comments.

Over the period of thirty years there has inevitably been relevant refinements as to the underlying mechanism of polymerisation. There is now totally persuasive evidence that the fundamental defect is the aberration of the bonding centred on Glu342 that pinions strand 5a in place and hence guides the final incorporation of the C-terminus into the body of the antitrypsin molecule and subsequently hinders its aberrant release. The supporting evidence is summarised in the paper of Huang et al (JBC 2016: 291,15674) on the 'Molecular mechanism of Z alpha-1-antitrypsin deficiency', which includes the crystal structure of Z antitrypsin. It should be cited. The Huang paper in no way pre-empts the current submission and indeed greatly strengthens it, almost as a preliminary study. The two papers are complementary, with satisfying agreements as to the drug-binding site. The very real advance in the submitted paper being the confirmation structurally of what is otherwise a deduced binding-site and in the design of a ligand that preferentially binds to the Z variant rather than the M (normal wild-type).

The submitted paper describes new and important findings but it presents these in what is in places a dated context. The findings here precisely fit with the consensus mechanism that is now supported by a whole body of studies in the field. It is important to get this right which could be achieved with some minor rewriting. As it is, the Discussion can be misleading, as in 'The finding that GSK716 mediates its action by binding to a cryptic pocket implies that intrahepatic polymers form a near native or native conformation, rather than a more extended intermediate.' This is not so, and should be removed. We are looking at the stabilisation of equilibria, be it in the partially folded form or the fully-folded but labile aberrant native form.

We fully agree with the reviewer and have added the reference to Huang et al, 2016. Indeed, our group has recently used cryo-EM to characterise isolated polymers from the liver tissue of Z α1-AT homozygotes (Glu342Lys) who had undergone liver transplantation. The data show that the inter-subunit linkage of Z α1-AT is best explained by a C-terminal domain swap between molecules of α1-AT (Faull et al, 2020). These data are consistent with a head-to-tail arrangement of subunits in heat-induced polymers revealed by a complex with the non-Z-selective antibody 2H2 (Laffranchi et al, 2020). This is in keeping with the view expressed by the reviewer. We have removed 'The finding that GSK716 mediates its action by binding to a cryptic pocket implies that intrahepatic polymers form a near native or native conformation, rather than a more extended intermediate.'

We have changed the introduction to read 'Polymerisation from this state involves insertion of the RCL into β-sheet A with a domain-swap of the C-terminal region providing the inter-subunit linkage (Faull et al, 2020; Huang et al, 2016; Laffranchi et al, 2020). The resulting polymer is deposited within hepatocytes.'
The text ‘polymerises from a near-native conformation late in the folding pathway (Motamedi-Shad et al, 2016; Ordóñez et al, 2015) and therefore’ has been deleted from the results section. ‘The finding that GSK716 mediates its action by binding to a cryptic pocket implies that intrahepatic polymers form from a near-native or native conformation, rather than a more extended intermediate’ has been deleted from the discussion and replaced with ‘GSK716 stabilises the partially folded $\alpha_1$-antitrypsin or the fully-folded but labile aberrant native form.’

An outstanding finding in the submitted paper is in the determination of the structure of the antitrypsin-GSK17 complex. Although this structure seemed instinctively correct the data was succinct and a backup expert crystallographic opinion was sought, as follows. ‘The crystallography is almost certainly fine, judging from the overall statistics and the resolution, but there are a few things that should be explained better. When a structure is deposited at the PDB, the authors receive a validation report that is intended for potential reviewers. This should be provided with the manuscript to allow referees to assess the quality of the structural work.

We have provided the validation report from the PDB submission (D_1292111290_val-report-full_P1.pdf). The PDB entry is 7EAL. Unfortunately, the submission has been given the wrong title. We have asked for this to be corrected but there is currently a technical issue with the platform. The data in the PDB are correct.

The authors have left some blanks in Table 1. There is a line for Resolution in the Data collection section, but it’s blank. Similarly, in the Refinement section the line for Water under "No. atoms" is blank, and both lines for Protein and Water under B-factors are blank. If numbers are given in these two subsections for protein and water, they should also be given for the ligand. In particular, it’s important to be able to compare the mean B-factor of the ligand with that of the protein.

We are sorry that this was missed from the paper. The full Table has been included in the paper and is reproduced below.
**Table 1. Data collection and refinement statistics**

| Description                              | Value                      |
|------------------------------------------|----------------------------|
| PDBFILE                                  | 7AEL                       |
| Temperature                              | 100K                       |
| Wavelength                               | 0.9763                     |
| Resolution range                         | 55.05 - 1.76 (1.823 - 1.76) |
| Space group                              | C 1 2 1                    |
| Unit cell                                | 113.954 39.592 90.517 90 104.961 90 |
| Total reflections                        | 127818 (12581)             |
| Unique reflections                       | 38772 (3853)               |
| Multiplicity                             | 3.3 (3.3)                  |
| Completeness (%)                         | 99.2 (99.2)                |
| Mean I/σ(I)                              | 19.61 (2.19)               |
| Wilson B-factor                          | 32.95                      |
| R-merge                                  | 0.02941 (0.5007)           |
| R-meas                                   | 0.03523 (0.5991)           |
| CC1/2                                    | 0.999 (0.805)              |
| CC*                                      | 1 (0.944)                  |
| Reflections used in refinement           | 38771 (3853)               |
| Reflections used for R-free              | 1908 (178)                 |
| R-work                                   | 0.1969 (0.3065)            |
| R-free                                   | 0.2259 (0.3204)            |
| CC(work)                                 | 0.958 (0.739)              |
| CC(free)                                 | 0.943 (0.699)              |
| Number of non-hydrogen atoms             | 3189                       |
| macromolecules                           | 2868                       |
| ligands                                  | 32                         |
| Protein residues                         | 357                        |
| RMS(bonds)                               | 0.005                      |
| RMS(angles)                              | 1.02                       |
| Ramachandran favored (%)                 | 99                         |
| Ramachandran allowed (%)                 | 1.1                        |
| Ramachandran outliers (%)                | 0                          |
| Rotamer outliers (%)                     | 0.62                       |
| Clashscore                               | 0.52                       |
| Average B-factor                         | 47.79                      |
| macromolecules                           | 46.77                      |
| ligands                                  | 36.49                      |
| solvent                                  | 59.23                      |

Statistics for the highest-resolution shell are shown in parentheses.

Whenever an important part of a structural paper is the binding of a ligand, it is accepted practice to provide a figure showing electron density for the ligand. Ideally, this should be unbiased electron density, computed either from a difference map before the ligand was added or after omitting the ligand and carrying out some refinement.
The OMIT density maps for the ligand is shown below and reproduced in Figs. 3F and G.

One comment: the paper reports that the structure was solved by molecular replacement, using a model that had originally been obtained from PDB entry 2qug. Since 2qug seems to be isomorphous to this structure, it would have been simpler to carry out rigid-body refinement than to use molecular replacement, which will give a structure with a different choice of origin and symmetry copy; this makes structure comparison less convenient to users of the structures than the rigid-body refinement option.

We have ensured the deposited structure, 7AEL.pdb, shares the same choice of origin as 2qug.pdb

The authors should respond to these points and the Editor/Journal note the desirability of making PDB validation reports available to reviewing referees.

The validation report has been included with the resubmitted manuscript.

Referee #2 (Comments on Novelty/Model System for Author):

The models are well-suited to this proof of principle analysis.

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The paper presents an analysis of a small molecule corrector of AAT folding that interdicts polymerization of Z-type AAT. The candidate molecule was identified by high throughput screening from a large library. The paper from a distinguished group of investigators is well-written and compelling. The curious findings are that there is no reduction in intrahepatic polymers despite a 7-fold increase in circulating Z-type AAT and that the lower doses (10 and 30 mg/kg) exerted the same effect as the 100 mg/kg dose despite low free drug levels. The authors do offer conjecture to explain these findings. They also offer an explanation as
to how the opposing effects of GSK716 to decrease serine proteinase inhibition of AAT vs. to enhance secretion of Z-type AAT might result in net benefit. Of course, definitively understanding the net effect of GSK716 will require further study. Within the context of a proof-of-principle analysis, including examining the effects in mice, the paper offers an important contribution.

We are very grateful to the reviewer for his/her supportive comments

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We are grateful to the reviewer for his/her comments. We have repeated the experiment with M α₁-A1 expressing cells + GSK716. The new figure is reproduced below along with the figure legend. Cells expressing M α₁-A1 were more resistant to tunicamycin than cells expressing Z α₁-A1. 716 increased the resistance of Z α₁-A1 cells to tunicamycin to that of M α₁-A1. It had no effect on the survival of cells expressing M α₁-A1. This is included as revised Fig. 2F.

(F) CHO inducible cells expressing either wildtype M or Z α₁-antitrypsin were induced with 0.5μg/mL doxycycline and treated with 10μM GSK716 or with 0.1% DMSO vehicle (NT, not treated). After induction for 48 h, cells were treated with various doses of tunicamycin for 36 h. Cell viability was measured by Cell Counting Kit-8. The results are shown as mean±SEM, n=4.

Furthermore, it is unclear why 13-15 week old, PiZ hemizygous mice were used in this study. At this age, the authors themselves report that there were no signs of liver fibrosis. Use of mice at an age with early signs of fibrosis may have been more informative in determining whether GSK716 treatment could prevent progression or reverse existing fibrosis.
The mouse model has been used widely by the community to test novel therapies for \( \alpha_1 \)-AT deficiency, two of which have now progressed to evaluation in humans (Guo et al. J Clin Invest. 2014;124:251-61; Hidvegi T et al, Science. 2010;329:229-32; Pastore et al., Hepatology. 2017; 65:1865-1874). The primary aim of the mouse experiments was to assess target engagement and in particular whether GSK716 could increase the secretion of Z \( \alpha_1 \)-AT. Having shown this, we then looked to evaluate whether there was an effect on hepatic inclusions. We selected younger, and hemizygous, rather than older mice, as our longevity studies showed that circulating levels of Z \( \alpha_1 \)-AT increase with age (to levels much higher than seen in patients) and these artificially high levels may act as a high affinity sink sequestering drug and preventing bioavailability at the site of action in the liver. Further, older mice have larger \( \alpha_1 \)-AT inclusions that may be more difficult to reverse and any changes would be more difficult to detect than in younger animals. As the reviewer comments, the conclusion from our studies is that GSK716 did not reduce PAS positive inclusions, following testing in animals where the compound had the best chance of impacting them, and would also therefore be unlikely to reverse fibrosis.

The following text has been added to the results section ‘Younger hemizygous, rather than older mice, were selected as our longevity studies showed that circulating levels of Z \( \alpha_1 \)-antitrypsin increase with age (to levels much higher than seen in patients) and these artificially high levels may act as a high affinity sink sequestering drug and preventing bioavailability at the site of action in the liver. Further, older mice have larger \( \alpha_1 \)-antitrypsin inclusions that may be more difficult to reverse and any changes would be more difficult to detect than in younger animals.’

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We have repeated the experiment in Fig 2F with M \( \alpha_1 \)-AT expressing cells + GSK716 as described in the section above

*In some instances, there is inadequate information, in the figure legends and in the methods, to adequately understand how the experiments were conducted and what the results actually mean. The authors indicated that GSK716 “increased the secretion of Z a1-
antitrypsin approximately 3-fold compared to vehicle control (mean pEC50 6.2 {plus minus} 0.23; n = 74) (Fig. 2b),” however, it is unclear how they came to that conclusion from the data provided. Fig. 2b show 100% secretion. Fig. 2d shows 3-fold secretion. What does 100% secretion mean? What does 3-fold secretion mean? More information should be included to assist the reader in determining the significance of the data.

We thank the reviewer for raising this point. We have changed the legend to Fig 2B and 2D to make it clear that data in Fig. 2B (CHO-TET-ON-Z-A1AT cell assay) are normalised, using vehicle and a control compound (from the GSK716 series) at saturating concentration as high/low controls, as is standard practise for screening assays. The actual concentrations of α1-AT in the supernatants were not determined in the same experiments, and we have removed the comment about 3-fold increase of secretion in this context. However, in the iPSC-hepatocyte assays (Fig 2D), calibration curves were used to determine the levels of α1-AT in the supernatant in each individual experiment, therefore the fold increase induced by GSK716, compared with vehicle control, could be calculated and is shown in Fig 2D. The statement in the text is consistent with these data.

The text has been changed to read (new text in red). In comparison with controls, GSK716 completely blocked the intracellular formation of Z α1-antitrypsin polymers, as measured by staining with the 2C1 anti-Z α1-antitrypsin polymer monoclonal antibody (mean pIC50 = 6.3 ± 0.23; n = 71) (Figs. 2A, B). It also increased the secretion of Z α1-antitrypsin (mean pEC50 6.2 ± 0.23; n = 74) (Fig. 2B). Similar potency between the effects on secretion and polymerisation was observed throughout members of the lead series supporting the hypothesis that these effects are caused by the same pharmacological mode of action. GSK716 had a similar effect on the secretion and polymerisation of constitutively expressed Z α1-antitrypsin in iPSC-derived human hepatocytes with the ZZ α1-antitrypsin genotype (Yusa et al., 2011). It inhibited polymerisation and increased secretion with a mean pIC50 of 6.4 ± 0.45 (n = 16) and mean pEC50 of 6.5 ± 0.37 (n = 14), respectively, inducing an approximately 3-fold increase of secreted levels of Z α1-antitrypsin (Figs 2C, D).

Figure 2. GSK716 inhibits polymerisation of Z α1-antitrypsin in cell models of disease. (A) GSK716 was added to CHO-TET-ON-Z-A1AT cells (Ordóñez et al., 2013) with simultaneous induction of Z α1-antitrypsin expression using doxycycline, and polymer load was quantified with the 2C1 monoclonal antibody that is specific to pathological polymers of α1-antitrypsin (Miranda et al., 2010). The parent cell line that did not express Z α1-antitrypsin provided a negative control. GSK716 completely prevented intracellular polymer formation. (B) Quantification of immunostained CHO-TET-ON-Z-A1AT cells showed that GSK716 reduced intracellular polymer formation and increased the secretion of Z α1-antitrypsin in a dose-dependent manner with similar potencies. Data were normalised to vehicle and a control compound from the GSK716 series at saturating concentration. (C) GSK716 was administered to iPSC-derived-hepatocytes and (D) inhibited polymerisation and increased secretion with a similar potency. It induced an approximately 3-fold increase in secreted levels of Z α1-antitrypsin compared with vehicle control. This was apparent even after polymers had been allowed to form.

We have also amended the abstract to read ‘The lead compound blocks Z α1-antitrypsin polymerisation in vitro, reduces intracellular polymerisation and increases the secretion of Z α1-antitrypsin three-fold in an iPSC model of disease’ and have corrected the text in the Discussion.

The authors then provide data in the PiZ mouse model showing that GSK716 treatment significantly increased plasma levels of Z-AAT. They concluded that this was due to increased secretion of Z-AAT from the liver, however, it is unclear whether this is actually the
case since the amount of Z-AAT in the hepatocytes remained unchanged in control- and GSK716-treated mice.

The conclusion that the increased secretion is from the liver is based on: (i) the pulse chase experiments that showed GSK716 increased the secretion of $\alpha_1$-AT from CHO cells and iPSC derived hepatocytes (Fig. 3). (ii) treatment with GSK716 increased the monomer measured in liver homogenate by approx. 4-fold in keeping with the changes seen in blood and (iii) liver is the only tissue that can produce sufficient $\alpha_1$-AT to cause a 7 fold increase in circulating protein.

Although, GSK716 failed to alter Z-AAT polymer accumulation in the liver, one could argue that the 7-fold increase in secretion of Z-AAT would be beneficial to the lung disease associated with AAT-deficiency. Unfortunately, GSK716 binds to Z-AAT in such a way that it abolishes the anti-proteinase activity of Z-AAT. This is a critically important as it implies that increasing secretion of Z-AAT has no apparent physiological benefits.

The authors' conclusion that "This study provides proof-of-principle that 'mutation ameliorating' small molecules are a viable approach to treat protein conformational diseases" is not supported by the data presented in this manuscript.

We are grateful to the reviewer for raising this issue. The statement referred to our finding that GSK716 can bind to a site close to the mutation in Z-AAT and change its polymerisation behaviour to approximate that of M-AAT. We apologise for the confusion and have amended this sentence in the abstract and the impact section to read 'can block the aberrant polymerisation that underlies Z $\alpha_1$-antitrypsin deficiency'.

Minor points:

GSK716 is sometimes referred to as GSK’716A. Is this a different analog or a typo?

GSK716 and GSK’716 are the same. We have amended Fig. 2 to only refer to GSK716.

Fig. 2G, bottom right, should be "extracellular" not intracellular.

This has been corrected in the revised manuscript.
1st Dec 2020

Dear Prof. Lomas,

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. I am pleased to inform you that we will be able to accept your manuscript pending the following final amendments:

1) Figures: Please remove all figures from the main manuscript file and upload 1 file per figure. Figure legends and EV figure legend should stay in the manuscript text file.
2) Tables: Please upload Table EV1 as individual file. The legend for Table EV1 should be removed from the manuscript text file and added to the Table EV1 file in a separate tab.
3) In the main manuscript file, please do the following:
   - Correct/answer the track changes suggested by our data editors by working from the attached/uploaded document.

***** Reviewer's comments *****

Referee #1 (Remarks for Author):

I have carefully reviewed the paper and now unequivocally recommend publication.

Referee #3 (Remarks for Author):

The authors have addressed the concerns raised in the initial review. As such I recommend it for publication in the EMBO Molecular Medicine.
The authors performed the requested changes.
10th Dec 2020

Dear Prof. Lomas,

We are pleased to inform you that your manuscript is accepted for publication.
| Question                                                                 | Answer |
|-------------------------------------------------------------------------|--------|
| Is there an estimate of variation within each group of data?             | No     |
| Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. | No, non-parametric tests were used. |
| Are any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g., blinding of the investigator)? If yes, please describe. | To minimize bias, if there were animal that had an initial smaller tumor size, they were equally distributed to each treatment group. There was no subjective bias in animal selection. |
| For animal studies, include a statement about randomization even if no randomization was used. | Tumor-bearing animals prior to particular treatment were randomly selected. There was no subjective bias in animal selection. |
| Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g., randomization procedure)? If yes, please describe. | Tumor-bearing animals prior to treatment of different antibodies (which are being compared to each other) were randomly selected. To minimize bias, if animals had an initial smaller tumor size, they were equally distributed to each treatment group. There was no subjective bias in animal selection. |
| Were any steps taken to minimize the effects of subjective bias when determining how many times the experiment shown was independently replicated in the laboratory? | Tumor-bearing animals prior to particular treatment were randomly selected. There was no subjective bias in animal selection. |
| Does the data show the exact sample size (n) for each experimental group? | Yes     |
| Does the data show the exact sample size (n) for each condition?          | Yes     |
| Does the data show the exact sample size (n) for each sample?             | Yes     |
| Does the data show the exact sample size (n) for each sample or group?   | Yes     |
| Does the data show the exact sample size (n) for each experimental group?| Yes     |
| Is there an estimate of variation within each group of data?              | Yes    |
| Do the data meet the assumptions of the tests (e.g., normal distribution)? | Yes |
| Are any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g., blinding of the investigator)? If yes, please describe. | To minimize bias, if there were animal that had an initial smaller tumor size, they were equally distributed to each treatment group. |
| For animal studies, include a statement about randomization even if no randomization was used. | Tumor-bearing animals prior to particular treatment were randomly selected. |
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| Does the data show the exact sample size (n) for each experimental group? | Yes     |
| Does the data show the exact sample size (n) for each condition?          | Yes     |
| Does the data show the exact sample size (n) for each sample?             | Yes     |
| Does the data show the exact sample size (n) for each sample or group?   | Yes     |
| Does the data show the exact sample size (n) for each experimental group?| Yes     |
| Is there an estimate of variation within each group of data?              | Yes    |
| Do the data meet the assumptions of the tests (e.g., normal distribution)? | Yes |
| Are any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g., blinding of the investigator)? If yes, please describe. | To minimize bias, if there were animal that had an initial smaller tumor size, they were equally distributed to each treatment group. |
| For animal studies, include a statement about randomization even if no randomization was used. | Tumor-bearing animals prior to particular treatment were randomly selected. |
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| Does the data show the exact sample size (n) for each experimental group? | Yes     |
| Does the data show the exact sample size (n) for each condition?          | Yes     |
| Does the data show the exact sample size (n) for each sample?             | Yes     |
| Does the data show the exact sample size (n) for each sample or group?   | Yes     |
| Does the data show the exact sample size (n) for each experimental group?| Yes     |
22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) for experiments where treatment results in significant differences in tumor size, the follow-up analysis was carried out by size match to avoid variation before statistical comparison.

23. We recommend consulting the ARRIVE guidelines (see link list at top right) (Pitts et al., 2009) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under ‘Reporting Guidelines’, for additional recommendations. Please confirm compliance.

24. For tumour marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under ‘Reporting Guidelines’. Please confirm you have submitted this list.

25. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.

26. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under ‘Reporting Guidelines’. Please confirm you have submitted this list.

27. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under ‘Reporting Guidelines’. Please confirm you have followed these guidelines.