

## Small Molecules Block the Polymerization of Z $\alpha_1$ -Antitrypsin and Increase the Clearance of Intracellular Aggregates

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The Z mutant of  $\alpha_1$ -antitrypsin (Glu342Lys) causes a domain swap and the formation of intrahepatic polymers that aggregate as inclusions and predispose the homozygote to cirrhosis. We have identified an allosteric cavity that is distinct from the interface involved in polymerization for rational structure-based drug design to block polymer formation. Virtual ligand screening was performed on 1.2 million small molecules and 6 compounds were identified that reduced polymer formation *in vitro*. Modeling the effects of ligand binding on the cavity and re-screening the library identified an additional 10 compounds that completely blocked polymerization. The best antagonists were effective at ratios of compound to Z  $\alpha_1$ -antitrypsin of 2.5:1 and reduced the intracellular accumulation of Z  $\alpha_1$ -antitrypsin by 70% in a cell model of disease. Identifying small molecules provides a novel therapy for the treatment of liver disease associated with the Z allele of  $\alpha_1$ -antitrypsin.

### Introduction

$\alpha_1$ -Antitrypsin is synthesized in the liver and released into the plasma where it is the most abundant circulating protease inhibitor. Most individuals carry the normal M allele of  $\alpha_1$ -antitrypsin but 4% of the Northern European population are heterozygous for the severe Z deficiency variant (Glu342Lys). The Z mutation perturbs the relationship between  $\beta$ -sheet A and the exposed mobile reactive loop that binds to the target proteinase (Figure 1).<sup>1</sup> The resulting unstable intermediates<sup>2–4</sup> then link sequentially to form loop-sheet polymers in which the reactive center loop of one  $\alpha_1$ -antitrypsin molecule inserts as strand 4 of  $\beta$ -sheet A of another.<sup>2,3,5–7</sup> It is this accumulation of polymers within the endoplasmic reticulum of hepatocytes that underlies the Periodic acid Schiff positive inclusions that are the hallmark of Z  $\alpha_1$ -antitrypsin deficiency.<sup>5,8–10</sup> These inclusions predispose the Z  $\alpha_1$ -antitrypsin homozygote to hepatitis,<sup>11</sup> cirrhosis, and hepatocellular carcinoma.<sup>12</sup> The resulting lack of circulating  $\alpha_1$ -antitrypsin allows uncontrolled proteolytic digestion within the lung and early onset panlobular emphysema (see ref 13 for review).

$\alpha_1$ -Antitrypsin is the archetypal member of the serine proteinase inhibitor or serpin superfamily. The process of polymer formation is now recognized to underlie diseases associated with other members of this family. For example, mutants of antithrombin,<sup>14,15</sup> C1-inhibitor,<sup>16,17</sup> and  $\alpha_1$ -antichymotrypsin<sup>3,18</sup> cause the protein to form polymers that are retained

within the liver, thereby causing a plasma deficiency that results in thrombosis, angioedema, and emphysema, respectively. Perhaps most striking is our description of mutants of a neurone-specific protein, neuroserpin, that polymerize to cause a novel inclusion body dementia that we have called familial encephalopathy with neuroserpin inclusion bodies or FENIB.<sup>19,20</sup> In view of the common mechanism that underlies these disorders, we have grouped them together as a new class of disease, the “serpinopathies”.<sup>21</sup>

The only curative treatment currently available for the cirrhosis associated with  $\alpha_1$ -antitrypsin deficiency is liver transplantation. Thus, it is essential to develop effective strategies to block polymerization to treat the associated disease.<sup>22</sup> Previous approaches have focused on chemical chaperones<sup>23,24</sup> that stabilize the mutant protein and reactive loop peptides that can bind as strand 4 of  $\beta$ -sheet A of Z  $\alpha_1$ -antitrypsin and so prevent the acceptance of an exogenous reactive loop.<sup>4,25</sup> However, neither strategy has yet been proven to be a viable therapeutic option in man.<sup>26</sup> Finding a small drug that can bind to  $\alpha_1$ -antitrypsin and prevent polymerization *in vitro* and *in vivo* is particularly challenging. Ideally, this small molecule should bind to a region of the protein that is distinct from the strand 4 position of  $\beta$ -sheet A for although this is the binding site of exogenous reactive loop peptides to form polymers (Figure 1a), it is also critical for the inhibitory function of  $\alpha_1$ -antitrypsin.<sup>27</sup>

A lateral hydrophobic pocket was identified in crystal structures of  $\alpha_1$ -antitrypsin.<sup>1,28</sup> This pocket is lined by strand 2 of  $\beta$ -sheet A and  $\alpha$ -helices D and E (Figure 1a). It is patent in the native protein but is occupied as  $\beta$ -sheet A accepts an exogenous reactive loop peptide during polymer formation.<sup>1</sup> The potential utility of the cavity as a target for rational drug design was demonstrated by the introduction of the “cavity-filling” mutation Thr114Phe.<sup>29</sup> This mutation retarded polymer formation and increased the secretion of Z  $\alpha_1$ -antitrypsin from a *Xenopus* oocyte expression system.<sup>29</sup> However, it is unknown whether this pocket could be targeted with sufficient affinity by a small molecule ligand. We report here the use of structure-

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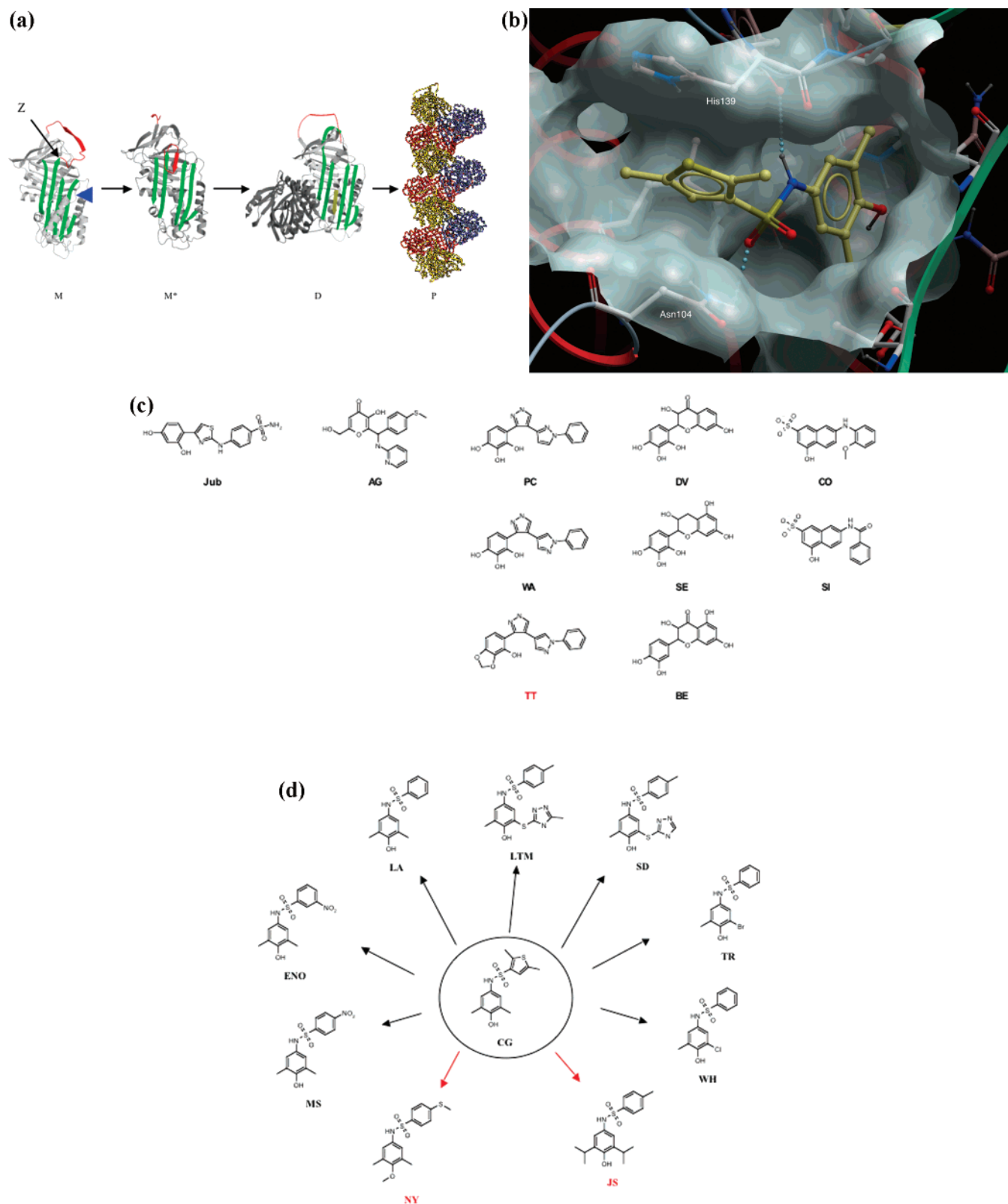
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**Figure 1.** Rational drug design to prevent the polymerization of  $\alpha_1$ -antitrypsin. (a) Pathway of the polymerization of  $\alpha_1$ -antitrypsin. The Z mutation of  $\alpha_1$ -antitrypsin (Glu342Lys at P17; arrowed) perturbs the structure of  $\beta$ -sheet A (green) and the mobile reactive center loop (red) to form the intermediate  $M^*$ . The patent  $\beta$ -sheet A can then accept the loop of another molecule (as strand 4) to form a dimer (D) which then extends into polymers (P).<sup>2-4</sup> It is these polymers that accumulate within hepatocytes to cause liver disease.<sup>5</sup> The position of the lateral hydrophobic pocket that is the target of rational drug design is shown with a blue arrowhead. Note the change in conformation in this region of the molecule as it forms  $M^*$  and then dimers and polymers. (b) The predicted binding pose of CG to the lateral hydrophobic cavity with Asn104 in an alternate conformation. The residues that most effect the dimensions of the pocket, Asn104 and His139, are highlighted. (c) The parent compounds Jub, AG, PC, DV, and CO and their analogues that also blocked polymerization. Compound TT (red) was designed as a control that did not block polymer formation. (d) The parent compound CG and its analogues. Compounds labeled in red (NY and JS) are designed not to block polymerization.

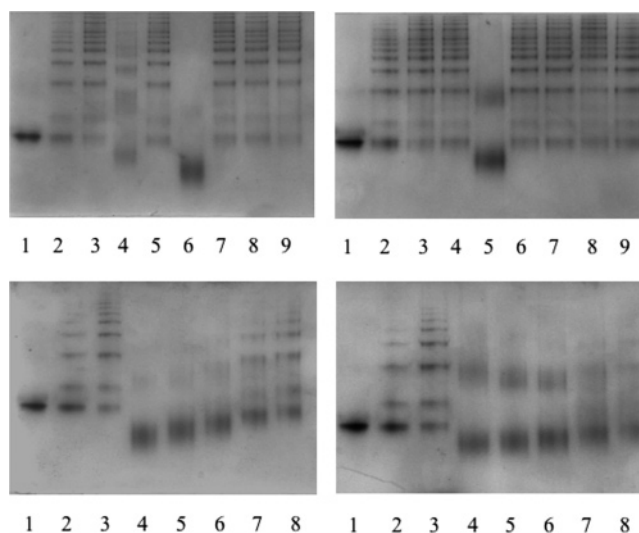
based drug discovery to identify a lead small molecule antagonist that can bind to the lateral hydrophobic pocket of Z  $\alpha_1$ -antitrypsin and block polymerization in vitro and in a cell model of disease.

## Results and Discussion

**In Silico Screening To Identify Small Molecules That Antagonize the Polymerization of  $\alpha_1$ -Antitrypsin.** The ICM PocketFinder analysis tool<sup>30</sup> was used to determine if either of the two high-resolution structures of native  $\alpha_1$ -antitrypsin (PDB codes: 1QLP<sup>1</sup> and 1HP7<sup>28</sup>) would be suitable for virtual screening. The lateral hydrophobic cavity was identified as being “druggable” in both structures but the cavity in structure 1QLP was better suited to binding small molecules. Virtual screening with the ICM software suite (Molsoft L.L.C.) against a non-redundant library of approximately 1.2 million commercial druglike compounds was performed on the original crystallographic coordinates (PDB 1QLP) of the lateral hydrophobic pocket. However, compounds nominated from screening the deposited structure were not effective in preventing the polymerization of Z  $\alpha_1$ -antitrypsin. As a consequence, side chain simulation analysis (see Experimental Section) was performed to identify the most flexible residues that line the cavity. Asn104 was highlighted by computational analysis as the most flexible residue. Visual inspection confirmed that this residue had the most significant effects on the dimensions and drugability of the cavity. An alternate conformation for this target site was generated by modeling low-energy conformations for Asn104 through internal coordinate Monte Carlo side chain simulations.<sup>31</sup> The resulting conformation differs from the PDB structure only in the position of the Asn104 side chain by 0.5 Å root-mean-square deviation (rmsd) after optimal superposition.

Virtual screening with the non-redundant library of 1.2 million commercial druglike compounds was then performed with this alternate conformation of  $\alpha_1$ -antitrypsin. Sixty-eight compounds from the initial screen of 1.2 million small molecules were selected for further characterization in vitro.

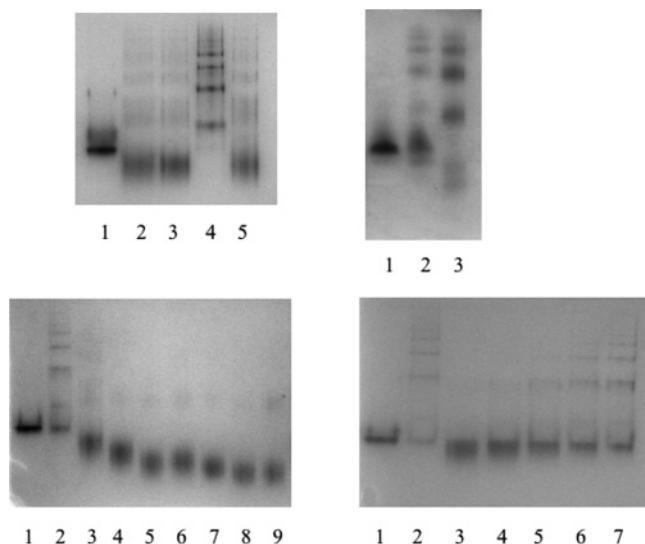
**Assessment of the Effect of Small Molecules on Polymerization, Structure, and Function of  $\alpha_1$ -Antitrypsin.** Most compounds nominated from the screen had no or negligible effects on polymerization (Figure 2) while some even accelerated polymer formation. Four compounds (denoted Jub, AG, CO, and DV; Figure 1c) reduced the rate of polymerization, one compound (PC; Figure 1c) prevented the formation of higher molecular mass polymers, and one compound (CG; Figure 1d) completely blocked polymerization (Figures 2a and 2b). CG (see Figure 1b for predicted binding pose) completely blocked the polymerization of Z  $\alpha_1$ -antitrypsin at 50  $\mu$ M (25-fold molar excess) but was still effective at reducing polymerization at 20  $\mu$ M (10-fold molar excess) (Figure 2c). PC effectively reduced polymerization at 10  $\mu$ M (5-fold molar excess), although a dimer band was still present (Figure 2d). CG and PC, and indeed all the compounds that reduced polymerization of Z  $\alpha_1$ -antitrypsin, caused an anodal band shift in the residual monomeric protein when analyzed by nondenaturing polyacrylamide gel electrophoresis (PAGE). Further analysis demonstrated that CG induced the band shift of Z  $\alpha_1$ -antitrypsin between 8 and 18 h when incubated in 100-fold molar excess (data not shown). The band shift was not due to cleavage of the protein (as assessed by N-terminal sequencing analysis), denaturation (as assessed by far UV circular dichroism), or covalent linkage as CG is chemically inert. However, the Z  $\alpha_1$ -antitrypsin:CG complex was remarkably stable with a melting temperature in excess of



**Figure 2.** 7.5% (w/v) nondenaturing PAGE to assess the effect of compounds identified in silico on the polymerization of Z  $\alpha_1$ -antitrypsin. Each lane contains 2  $\mu$ g of protein. (a) (top left): Lane 1, monomeric Z  $\alpha_1$ -antitrypsin; lane 2, Z  $\alpha_1$ -antitrypsin heated at 0.1 mg/mL and 41 °C for 7 days; lane 3, Z  $\alpha_1$ -antitrypsin heated at 0.1 mg/mL and 41 °C for 7 days in the presence of 4.75% (v/v) DMSO; lanes 4–9, Z  $\alpha_1$ -antitrypsin heated at 0.1 mg/mL and 41 °C for 7 days with 100-fold molar excess of AG, BS, CG, EC, FP, and GP, respectively, all with a final concentration of 4.75% (v/v) DMSO. (b) (top right): Lane 1, monomeric Z  $\alpha_1$ -antitrypsin; lane 2, Z  $\alpha_1$ -antitrypsin heated at 0.1 mg/mL and 41 °C for 7 days; lane 3, Z  $\alpha_1$ -antitrypsin heated at 0.1 mg/mL and 41 °C for 7 days in the presence of 4.75% (v/v) DMSO; lanes 4–9, Z  $\alpha_1$ -antitrypsin heated at 0.1 mg/mL and 41 °C for 7 days with 100-fold molar excess of OC, PC, RS, SK, TCR, and UX, respectively, all with a final concentration of 4.75% (v/v) DMSO. (c) (bottom left): 7.5% (w/v) nondenaturing PAGE to assess the effect of decreasing concentrations of CG on the polymerization of Z  $\alpha_1$ -antitrypsin. Lane 1, monomeric Z  $\alpha_1$ -antitrypsin; lane 2, Z  $\alpha_1$ -antitrypsin heated at 0.1 mg/mL and 41 °C for 3 days; lane 3, Z  $\alpha_1$ -antitrypsin heated at 0.1 mg/mL and 41 °C for 3 days in the presence of 4.75% (v/v) DMSO; lanes 4–8, Z  $\alpha_1$ -antitrypsin heated at 0.1 mg/mL and 41 °C for 3 days with 200, 100, 50, 20, and 10  $\mu$ M CG, respectively, all with a final concentration of 4.75% (v/v) DMSO. (d) (bottom right): 7.5% (w/v) nondenaturing PAGE to assess the effect of decreasing concentrations of PC on the polymerization of Z  $\alpha_1$ -antitrypsin. Lane 1, monomeric Z  $\alpha_1$ -antitrypsin; lane 2, Z  $\alpha_1$ -antitrypsin heated at 0.1 mg/mL and 41 °C for 3 days; lane 3, Z  $\alpha_1$ -antitrypsin heated at 0.1 mg/mL and 41 °C for 3 days in the presence of 4.75% (v/v) DMSO; lanes 4–8, Z  $\alpha_1$ -antitrypsin heated at 0.1 mg/mL and 41 °C for 3 days with 200, 100, 50, 20, and 10  $\mu$ M PC, respectively, all with a final concentration of 4.75% (v/v) DMSO.

100 °C (which compares to a  $T_m$  of 54.1 °C in 3.7% v/v ethanol in the absence of CG) and was inactive as a proteinase inhibitor. It was not possible to determine a value for the  $K_d$  between CG and Z  $\alpha_1$ -antitrypsin using intrinsic tryptophan fluorescence. We therefore used dialysis to determine whether this interaction with the compound led to an irreversible transition. Z  $\alpha_1$ -antitrypsin was incubated with a 100-fold molar excess of CG at 37 °C for 3 days and then extensively dialyzed ( $3 \times 1$  L) against PBS. The anodal band shift remained after dialysis (Figure 3a) with the complex being resistant to polymerization when heated at 0.1 mg/mL for a further 3 h at 60 °C. Thus, the interaction between CG and Z  $\alpha_1$ -antitrypsin, or the conformational transition that ensues, was resistant to dialysis, leading to a permanent nonpolymerizable state.

Mass spectrometry was used to assess the binding of CG to Z  $\alpha_1$ -antitrypsin. Identical digest products were obtained for both Z  $\alpha_1$ -antitrypsin and the Z  $\alpha_1$ -antitrypsin:CG complex. There

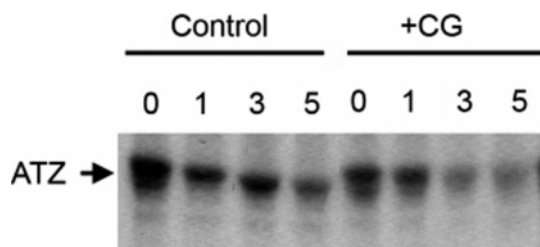


**Figure 3.** Binding of CG and its analogues to Z  $\alpha_1$ -antitrypsin and other serpins. (a) (top left): 7.5% (w/v) nondenaturing PAGE with each lane containing 2  $\mu$ g of protein. The interaction between Z  $\alpha_1$ -antitrypsin and CG leads to an irreversible transition. Lane 1, Z  $\alpha_1$ -antitrypsin; lane 2, Z  $\alpha_1$ -antitrypsin incubated with CG at 37 °C for 3 days; lane 3, Z  $\alpha_1$ -antitrypsin incubated with CG at 37 °C for 3 days and then dialyzed in 3  $\times$  1 L of PBS; lane 4, Z  $\alpha_1$ -antitrypsin heated at 0.1 mg/mL and 60 °C for 3 h; lane 5, Z  $\alpha_1$ -antitrypsin incubated with CG at 37 °C for 3 days, dialyzed in 3  $\times$  1 L of PBS and then heated at 0.1 mg/mL and 60 °C for 3 h. (b) (top right): CG does not inhibit the polymerization of  $\alpha_1$ -antichymotrypsin. 7.5% (w/v) nondenaturing PAGE with each lane containing 2  $\mu$ g of protein. Lane 1,  $\alpha_1$ -antichymotrypsin; lane 2,  $\alpha_1$ -antichymotrypsin heated at 0.1 mg/mL and 45 °C for 3 days in 3.7% (v/v) ethanol; lane 3,  $\alpha_1$ -antichymotrypsin incubated at 0.1 mg/mL and 45 °C for 3 days with CG in 3.7% (v/v) ethanol. (c) (bottom left): Effect of the analogues of CG on the polymerization of Z  $\alpha_1$ -antitrypsin. Lane 1, Z  $\alpha_1$ -antitrypsin; lane 2, Z  $\alpha_1$ -antitrypsin heated at 0.1 mg/mL and 37 °C for 3 days in 3.7% (v/v) ethanol; lanes 3–9, Z  $\alpha_1$ -antitrypsin heated at 0.1 mg/mL and 37 °C for 3 days in 100-fold molar excess of CG, LA, ENO, MS, SD, TR, and WH, respectively, all with a final concentration of 3.7% (v/v) ethanol. (d) (bottom right): Effect of the LTM on the polymerization of Z  $\alpha_1$ -antitrypsin. Lane 1, Z  $\alpha_1$ -antitrypsin; lane 2, Z  $\alpha_1$ -antitrypsin heated at 0.1 mg/mL and 37 °C for 3 days in 3.7% (v/v) ethanol; lanes 3–7, Z  $\alpha_1$ -antitrypsin heated at 0.1 mg/mL and 37 °C for 3 days in 10, 7.5, 5, 2.5, and 1  $\mu$ M of LTM, respectively, all with a final concentration of 3.7% (v/v) ethanol.

was 70% coverage of the protein sequence but CG was not associated with any of the identified fragments.

**Assessment of the Effect of Small Molecules on Polymerization of Other Members of the Serpin Superfamily.** Members of the serine proteinase inhibitor or serpin superfamily are structurally homologous, but the lateral hydrophobic pocket is not present in antithrombin or  $\alpha_1$ -antichymotrypsin.<sup>1</sup> There is currently no crystal structure of native neuroserpin. CG caused an anodal migration of  $\alpha_1$ -antichymotrypsin, antithrombin, and wildtype and mutant Syracuse (Ser49Pro)<sup>32</sup> neuroserpin but did not prevent their polymerization (Figure 3b and data not shown).

**In Silico Screening Following Modeling of Ligand-Induced Changes in the Conformation of Z  $\alpha_1$ -Antitrypsin.** Even small ligand-induced changes in the conformation of Z  $\alpha_1$ -antitrypsin can have major effects on binding affinity. These ligand-induced changes were modeled for the best compounds, CG and PC, to target more relevant induced-fit pocket conformations for the second round of virtual screening. This was achieved by performing internal coordinate Monte Carlo side chain simulations in the presence of the ligand in its predicted binding pose.<sup>31</sup> The major difference between the resulting two conformations, selected for follow-up virtual screening, and the PDB structure



**Figure 4.** Effect of CG on the secretion of Z  $\alpha_1$ -antitrypsin. Hepala cells were transiently transfected with Z  $\alpha_1$ -antitrypsin in the presence or absence of CG. Lanes 1–4, Z  $\alpha_1$ -antitrypsin radiolabeled with [<sup>35</sup>S]-methionine and chased up to 5 h. Immunoprecipitation experiments demonstrate that the half-life of the Z variant is between 3 and 5 h. Lanes 5–8, transfected Hepala cells were treated with 100  $\mu$ M CG for 16 h prior to the pulse-chase experiment. Z  $\alpha_1$ -antitrypsin was radiolabeled with [<sup>35</sup>S]-methionine in the presence of CG and chased up to 5 h. Glycan trimming of Z  $\alpha_1$ -antitrypsin was not detected in association with an increased rate of intracellular clearance.

are in the position of Asn104 and His139. Comparison of these conformations to the PDB structure show an rmsd of 0.77 and 1.0 Å, respectively.

Virtual screening with the non-redundant library of 1.2 million commercial druglike compounds against these newly derived conformations identified a further 19 compounds for in vitro testing. Ten of these compounds were structurally similar to the original active compounds, CG or PC. The PC analogue WA (Figure 1c) had an effect similar to PC by allowing only the formation of dimers. BE and SE reduced polymer formation to the same extent as the parent molecule DV but the analogues of CO (e.g., SI) had little effect (Figure 1c). The most potent blockers of polymerization were analogues of CG: LA, ENO, MS, SD, TR, WH, and LTM (Figures 1d and 3c). LA completely blocked the polymerization of Z  $\alpha_1$ -antitrypsin at 50  $\mu$ M (25-fold excess) and reduced polymerization at 10  $\mu$ M (5-fold excess). WA, ENO, MS, SD, TR, WH, and LTM completely blocked polymerization at 10  $\mu$ M (5-fold excess) while WA and WH still had a modest effect at 7.5  $\mu$ M. SD, TR, and LTM were effective at concentrations as low as 5  $\mu$ M (2.5-fold excess) (Figure 3d and data not shown). Like the parent compounds, all analogues that blocked polymerization caused an anodal band shift on nondenaturing PAGE and inactivated Z  $\alpha_1$ -antitrypsin as a proteinase inhibitor. The specificity of the compounds to the lateral hydrophobic pocket was assessed by selecting close analogues predicted not to bind the lateral hydrophobic pocket. None of these compounds (TT, NY, and JS; Figure 1c and 1d) had any demonstrable effect on the polymerization of Z  $\alpha_1$ -antitrypsin.

**Characterization of the Effect of the Compounds in a Cell Model of Z  $\alpha_1$ -Antitrypsin Deficiency.** The effect of the small molecules on the intracellular fate of Z  $\alpha_1$ -antitrypsin was then assessed in murine hepatoma cells, Hepa1a. CG reduced the intracellular retention of Z  $\alpha_1$ -antitrypsin by 70% (Figure 4) in keeping with an accelerated rate of clearance when compared to control cells. The majority of Z  $\alpha_1$ -antitrypsin was cleared 1–3 h after pulse labeling compared to 5 h for Z  $\alpha_1$ -antitrypsin that was not treated with CG. There was no change in the electrophoretic mobility of Z  $\alpha_1$ -antitrypsin when isolated from cells incubated with CG. No increase in the secretion of Z  $\alpha_1$ -antitrypsin was detected (data not shown). Neither NY nor LTM accelerated the degradation nor increased secretion of Z  $\alpha_1$ -antitrypsin in this cell model of disease.

Polymers of Z  $\alpha_1$ -antitrypsin are retained within hepatocytes where they aggregate to form the inclusions that underlie the associated liver disease. The pathways by which polymers are

handled within hepatocytes are now being elucidated. Trimming of asparagine-linked oligosaccharides target Z  $\alpha_1$ -antitrypsin polymers into an efficient nonproteasomal disposal pathway within hepatocytes.<sup>33–35</sup> However, the proteasome has an important role in metabolizing Z  $\alpha_1$ -antitrypsin in some hepatic<sup>36</sup> and extrahepatic<sup>37,38</sup> mammalian cell lines. Moreover, there is increasing evidence that the retained Z  $\alpha_1$ -antitrypsin stimulates an autophagic response within the hepatocyte.<sup>39–41</sup> Our data demonstrate that at least one of the small molecules (CG) is able to increase the clearance of Z  $\alpha_1$ -antitrypsin in a cell model of disease. Further studies are required to define the disposal pathways that clear the Z  $\alpha_1$ -antitrypsin:CG complex. However, the resulting Z  $\alpha_1$ -antitrypsin had a normal electrophoretic mobility when isolated from cells which implies that treatment with CG either ablated the trimming of asparagine-linked oligosaccharides or that molecules were more rapidly degraded in response to the accelerated rate at which oligosaccharides were modified, such that only the unmodified population remained. The latter conclusion is most likely correct as cleavage of the appendages is an obligatory step in the intracellular degradation process. In either case the accelerated clearance of mutant protein provides strong support for the likely success of these compounds (or their derivatives) in vivo. The ability of CG to target the mutant protein for degradation would reduce the protein overload and therefore attenuate the hepatic toxicity associated with the accumulation of polymers of Z  $\alpha_1$ -antitrypsin.

**In Vitro Selectivity of CG.** The Molecular Libraries Screening Center Network (MLSCN) is a consortium of academic laboratories responsible for screening compound libraries against cell and cell-free in vitro assays (<http://grants1.nih.gov/grants/guide/rfa-files/RFA-RM-04-017.html>). As of June 2007, the compound CG (PubChem ID: MLS000521559) has tested negative (between 5 and 10  $\mu$ M) in all 30 MLSCN screens, including 9 cell (PubChem Assay ID (AID): 598, 602, 620, 648, 710, 719, 729, 731, 736) and 21 cell-free (AID: 583, 568, 618, 619, 629, 631, 632, 633, 639, 640, 687, 693, 690, 696, 697, 701, 704, 702, 717, 720, 722) assays. The targets in these assays include traditional enzyme active sites (e.g., protein kinase, tyrosine phosphatase, and matrix metallo-proteinase), protein–protein and protein–RNA binding sites, as well as cytotoxic and reporter-based cell screens. This wide panel of screens shows that CG is not a promiscuous compound and is therefore less likely to have unwanted off-target effects.

## Conclusion

Taken together, our data demonstrate the successful use of structure-based drug design to target a cavity that is distinct from the interface involved in polymerization of mutant  $\alpha_1$ -antitrypsin. The discovered compounds are effective at reducing the polymerization of mutant Z  $\alpha_1$ -antitrypsin in vitro while the lead compound CG reduces aggregates of Z  $\alpha_1$ -antitrypsin in a cell model of disease. CG is highly selective as it is inactive in a diverse range of 30 cell-based and cell-free in vitro assays. Our strategy represents a novel and highly effective approach to the treatment of the liver disease associated with homozygosity of the Z allele of  $\alpha_1$ -antitrypsin.

## Experimental Section

**In Silico Screening To Identify Small Molecules That Antagonize the Polymerization of  $\alpha_1$ -Antitrypsin.** Virtual screening was performed with the original crystallographic coordinates of the lateral hydrophobic pocket of 1QLP<sup>1</sup> against a non-redundant library of approximately 1.2 million commercial druglike compounds from 10 vendors: Asinex (Russia), BioNet (UK), Chem-

bridge (USA), Chemical Diversity (USA), IBS (Russia), Maybridge (USA), Sigma-Aldrich (USA), Specs (Netherlands), Tripos (USA), and TimTec (Russia). The screen was repeated following an assessment of the flexibility of the side chains of the pocket and modeling the effect of ligand-induced changes. In each case the virtual screening results were ranked by their ICM score and the following conditions were imposed to nominate compounds for biological testing: (i) a permissive cut-off score that resulted in only the top 1% (approximately 400) of top-scoring compounds being retained, (ii) the location of the ligand in the pocket, and (iii) the ligand should make at least one hydrogen bond with the protein.

**Side Chain Flexibility Analysis.** Analysis of the local flexibility around the lateral hydrophobic pocket was based on an ICM biased probability Monte Carlo simulation<sup>31</sup> of the 17 side chains surrounding the pocket (N104, L110, Q105, H139, Q109, Q111, S140, L103, S56, L112, N116, L100, T114, T59, N186, I188, and Y138). Only the lowest energy representatives were selected for conformations closer than 15° in side chain torsion rmsd.<sup>42</sup> The rmsd of atoms with Boltzmann-weighted contributions from these filtered low-energy conformations were then calculated. The side chain of Asn104 exhibited the maximal possible deviation (3.6 Å).

**Assessment of the Effect of Small Molecules on Polymerization, Structure, and Function of  $\alpha_1$ -Antitrypsin.** The lead compounds or analogues were solubilized to 2 mg/mL in dimethylsulfoxide (DMSO) or 1 mg/mL in 50% (v/v) ethanol before being diluted to 0.4 mg/mL with PBS.  $\alpha_1$ -Antitrypsin was purified from the plasma of either PiM or PiZ homozygotes<sup>43</sup> and the effect of compounds on polymerization was assessed by incubation in 100-fold molar excess with 2  $\mu$ g of Z or M  $\alpha_1$ -antitrypsin (200  $\mu$ M compound to 2  $\mu$ M  $\alpha_1$ -antitrypsin). The final concentration of DMSO or ethanol was the same for each sample and the controls (4.75% (v/v) and 3.7% (v/v), respectively). Circular dichroism (CD) spectroscopy and thermal unfolding experiments were performed as detailed previously.<sup>29</sup> The effect of the compounds on the activity of  $\alpha_1$ -antitrypsin was assessed using an ELISA assay.

**Mass Spectrometry.** The molecular mass of native Z  $\alpha_1$ -antitrypsin or Z  $\alpha_1$ -antitrypsin incubated with the small molecule was determined by MALDI. Assessment of binding of the small molecule to a domain of  $\alpha_1$ -antitrypsin was undertaken by precipitating native Z  $\alpha_1$ -antitrypsin or Z  $\alpha_1$ -antitrypsin incubated with the small molecule with acetone and then treating with cyanogen bromide and digesting with trypsin in 4 M urea. This was performed with or without treatment with PNGase F to remove the glycans.

**Effect of the Compounds on Other Members of the Serpin Superfamily.** Plasma antithrombin was from Dan Johnson, Department of Haematology, University of Cambridge, plasma  $\alpha_1$ -antichymotrypsin was from Sigma, and wild-type neuroserpin and the Syracuse mutant of neuroserpin that causes FENIB (Ser49Pro) were purified as described previously.<sup>32</sup> These serpins were incubated at 0.1 mg/mL in PBS at 37 or 45 °C for 3 or 7 days in the presence or absence of 200  $\mu$ M compound and 3.7% (v/v) ethanol. The effect on polymerization was then assessed by 7.5% (w/v) nondenaturing PAGE.

**Characterization of the Effect of the Compounds in a Cell Model of Z  $\alpha_1$ -Antitrypsin Deficiency.** The murine hepatoma cell line, Hepala, was grown as monolayers in a standard growth medium and split into 100 mm dishes at a density of 70% confluence. The following day cells were transfected with hATZ/pcDNA3.1 Zeo(+) (Z  $\alpha_1$ -antitrypsin subcloned into the unique EcoRI site of pcDNA3.1 Zeo(+)) using the Lipofectamine 2000 protocol. Twenty-four hours post-transfection the cells were collected and split at the same density into 60 mm dishes. The small molecules (CG, NY, and LTM) were administered 48 h post-transfection at a concentration of 100  $\mu$ M with the scientists being blind to the in vitro efficacy of the compounds that were being assessed. The cells were incubated with the small molecule for 16 h and then subjected to methionine starvation in a methionine-free medium supplemented with the small molecule for

30 min. [<sup>35</sup>S]methionine was added [0.075 mCi (1 Ci = 37 GBq) per 60 mm dish] during a 20 min pulse followed by up to 5-h chase in serum-free DMEM (Gibco/BRL) containing 0.2 mM unlabeled methionine with the small molecule. The cells were lysed with buffered Nonidet P-40 detergent at designed time points and immunoprecipitates were resolved by SDS-PAGE and then detected by fluorography.

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