Molecular mousetraps, α_1-antitrypsin deficiency and the serpinopathies

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ABSTRACT – Point mutations in members of the serine proteinase inhibitor or serpin superfamily cause them to change shape, polymerise and be deposited in the tissues. This process is best seen in mutants of α_1-antitrypsin within hepatocytes to cause periodic acid-Schiff (PAS) positive inclusions and cirrhosis. An identical process underlies the PAS positive inclusions of mutants of neuroserpin within neurones to cause a dementia that we have called familial encephalopathy with neuroserpin inclusion bodies (FENIB). In both cases, there is a direct correlation between the molecular instability, the rate of intracellular polymer formation and the severity of disease. This process of polymerisation also explains the failure to secrete mutants of other members of the serpin superfamily – antithrombin, C1 inhibitor and α_1-antichymotrypsin – to cause thrombosis, angio-oedema and emphysema, respectively. In view of the common mechanism underlying these conditions, we have grouped them together as the serpinopathies.

KEY WORDS: α_1-antitrypsin, conformational disease, neuroserpin, polymerisation, serpins

The serine proteinase inhibitor or serpin superfamily

The serine proteinase inhibitors or serpins are important inhibitors of a wide range of proteolytic cascades. Members of this family include α_1-antitrypsin, C1 inhibitor, antithrombin and plasminogen activator inhibitor-1 which play important roles in the control of proteinases involved in the inflammatory, complement, coagulation and fibrinolytic pathways, respectively.[1] The superfamily is defined by more than 30% sequence homology with the archetypal member α_1-antitrypsin and conservation of tertiary structure. This structure is composed of three β-sheets (A–C) and an exposed mobile reactive loop (Fig 1a).[2–8] The reactive loop presents a peptide sequence as a pseudosubstrate for the target proteinase. After docking, the enzyme cleaves the P1–P1' peptide bond of the serpin[9] and the proteinase is inactivated by a dramatic conformational transition that swings it 70 Å from the upper to the lower pole of the protein in association with the insertion of the reactive loop as an extra strand (s4A) in β-sheet A (Fig 1a).[10–14] The altered conformation of α_1-antitrypsin bound to its target enzyme is then recognised by hepatic receptors and cleared from the circulation.[15–17] This remarkable conformational transition can be likened to the function of a mousetrap and is central to the inhibitory activity of the serpins. However, as with most sophisticated mechanisms, the mobile domains are vulnerable to dysfunction. In the case of the serpins, mutations cause aberrant conformational transitions that result in the retention of the serpin within the cell of synthesis. This gives rise to clinical conditions that result from either:

(i) protein overload and death of the cell in which the serpin is synthesised (toxic gain of function) such as Z α_1-antitrypsin related cirrhosis and the dementia familial encephalopathy with neuroserpin inclusion bodies (FENIB), or

(ii) plasma deficiency (loss of function) such as deficiency of plasma antithrombin, C1-inhibitor or α_1-antichymotrypsin. These can be manifest as diseases as diverse as thrombosis, angio-oedema and emphysema respectively.

We have shown that there is a common mechanism underlying these conditions and so have grouped them together as a new class of disease, the serpinopathies.[18–20]

Polymerisation of mutants of α_1-antitrypsin causes cirrhosis and plasma deficiency

Alpha-1-antitrypsin is an acute phase glycoprotein that is synthesised and secreted by the liver. The primary role of α_1-antitrypsin is to inhibit the enzyme neutrophil elastase. Most individuals carry two normal M alleles that result in plasma concentrations of 1.5–3.5 g/l. The most important deficiency mutation is the Z allele (Glu342Lys). Approximately 4% of Northern Europeans are heterozygous for the Z allele (PI*MZ) with approximately 1 in 2,000 being homozygotes (PI*Z). The Z allele results in the retention of synthesised α_1-antitrypsin within the endo-

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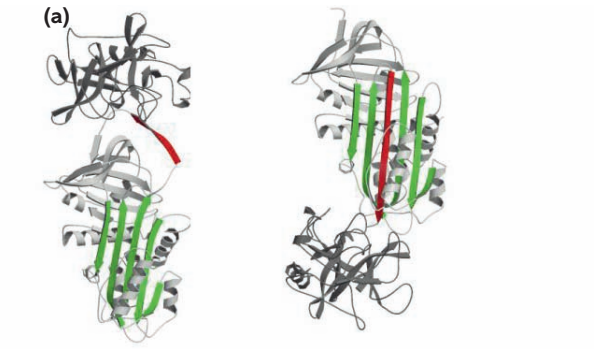


Fig 1a. Inhibition of neutrophil elastase by α_1-antitrypsin. Following docking (left), the neutrophil elastase (grey) is inactivated by movement from the upper to the lower pole of the protein (right). This is associated with insertion of the reactive loop (red) as an extra strand into β-sheet A (green). (Reproduced from Ref 18 with permission.)

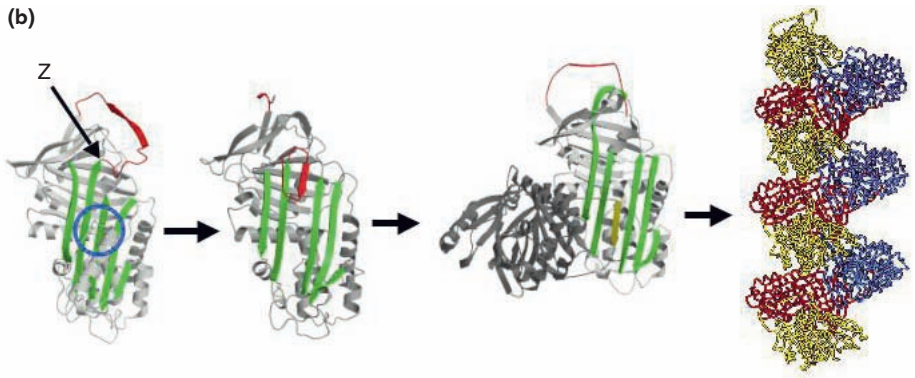


Fig 1b. The structure of α_1-antitrypsin is centred on β-sheet A (green) and the mobile reactive centre loop (red). Polymer formation results from the Z variant of α_1-antitrypsin (Glu342Lys at P_{17}; arrowed) or mutations in the shutter domain (blue circle) that open β-sheet A to favour partial loop insertion and the formation of an unstable intermediate (M*). The patent β-sheet A can accept the loop of another molecule to form a dimer (D) which then extends into polymers (P). The individual molecules of α_1-antitrypsin within the polymer, although identical, are coloured red, yellow and blue for clarity. (Adapted from Ref 77 with permission.)

plasmic reticulum of hepatocytes. The accumulation of abnormal protein starts *in utero*[21] and is characterised by the formation of diastase-resistant, periodic acid-Schiff positive inclusions of α_1-antitrypsin in the periportal cells[22,23] (Fig 2). Seventy-three per cent of Z α_1-antitrypsin homozygote infants have a raised serum alanine aminotransferase in the first year of life but in only 15% of children is it still abnormal by 12 years of age.[24–27] Similarly, serum bilirubin is raised in 11% of PI*Z infants in the first 2–4 months but falls to normal by six months of age. One in 10 infants develops cholestatic jaundice and 6% develop clinical evidence of liver disease without jaundice. These symptoms usually resolve by the second year of life but approximately 15% of patients with cholestatic jaundice progress to juvenile cirrhosis. The overall risk of death from liver disease in PI*Z children during childhood is 2–3%, with boys being at greater risk than girls. All adults with the Z allele of α_1-antitrypsin have slowly progressive hepatic damage that is often subclinical and only evident as a minor degree of portal fibrosis. However, up to 50% of Z α_1-antitrypsin homozygotes present with clinically evident cirrhosis and occasionally with hepatocellular carcinoma.[28] The lack of circulating plasma α_1-antitrypsin leaves the lungs exposed to enzymatic damage that is thought to underlie the adult onset emphysema (see later).

We have shown that the Z variant of α_1-antitrypsin is retained within hepatocytes as it causes a unique conformational transition and protein–protein interaction. The mutation distorts the relationship between the reactive centre loop and β-sheet A (Fig 1b). The consequent perturbation in structure allows the reactive centre loop of one α_1-antitrypsin molecule to lock into the A sheet of a second to form a dimer which then extends to form chains of loop-sheet polymers.[29–34] These polymers accumulate within the endoplasmic reticulum of hepatocytes to form the PAS positive inclusions that are the hallmark of Z α_1-antitrypsin liver disease.[29,35,36] Although many α_1-antitrypsin deficiency variants have been described, only two other mutants of α_1-antitrypsin have similarly been associated with profound plasma deficiency and hepatic inclusions: α_1-antitrypsin Siiyama (Ser53Phe)[37,38] and Mmalton[39] (deletion of phenylalanine at position 52, also known as Mnichinan[40] and Mcagliari[41]). Both of these mutants are in the shutter domain underlying the bifurcation of strands 3 and 5 of β-sheet A (Fig 1b). The mutations disrupt a hydrogen bond network that is based on 334His and bridges strands 3 and 5 of the A sheet,[42] causing it to part to allow the formation of folding intermediates[43] and loop-sheet polymers *in vivo*.[44,45]

Polymerisation also underlies the mild plasma deficiency of other variants that perturb the shutter domain: S (Glu264Val) and I (Arg39Cys) α_1-antitrypsin.[46,47] These point mutations cause less disruption to β-sheet A than does the Z variant. Thus, the rates of polymer formation are much slower than that of Z α_1-antitrypsin[31] and this results in less retention of protein within hepatocytes, milder plasma deficiency, and the lack of a clinical phenotype. However, if a mild, slowly polymerising I or S variant of α_1-antitrypsin is inherited with a rapidly polymerising Z variant, then the two can interact to form het-

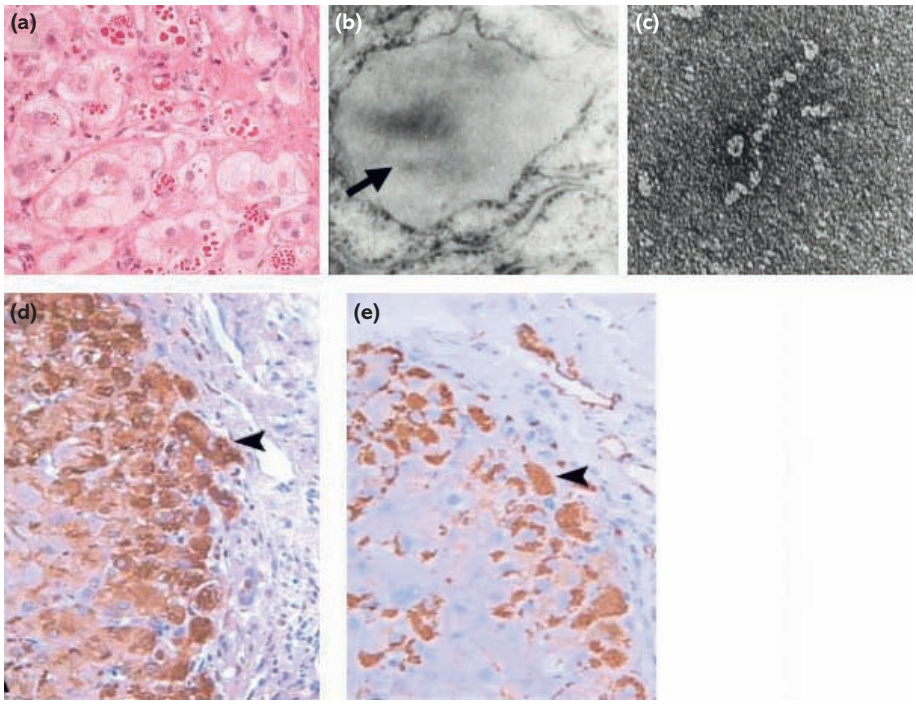


Fig 2. Z α_1-antitrypsin is retained within hepatocytes as intracellular inclusions. These inclusions are PAS positive and diastase resistant (Fig 2a) and are associated with neonatal hepatitis and hepatocellular carcinoma. Fig 2b: Electron micrograph of an hepatocyte from the liver of a patient with Z α_1-antitrypsin deficiency shows the accumulation of α_1-antitrypsin within the rough endoplasmic reticulum. These inclusions are composed of chains of α_1-antitrypsin polymers shown here from the plasma of a Siiyama α_1-antitrypsin homozygote (Fig 2c). More recently, polymers have been identified within PAS positive inclusions with a monoclonal anti-polymer α_1-antitrypsin antibody[30,36] (Fig 2e and f). Immunohistochemistry of liver from an individual with Z α_1-antitrypsin deficiency showing staining with an anti-α_1-antitrypsin polyclonal antibody (Fig 2e) and a monoclonal anti-polymer α_1-antitrypsin antibody (Fig 2f). It is these intracellular inclusions of polymers that are associated with neonatal hepatitis and hepatocellular carcinoma. (Figs 2a, b and c reproduced from Refs 96, 29 and 44, respectively. Figs 2d and e reproduced from Ref 36. All figures reproduced with permission.)

eropolymers within hepatocytes leading to inclusions and finally cirrhosis.[47–49] Thus, the severity of retention of mutants of α_1-antitrypsin within hepatocytes can be explained by the rate of polymer formation. Those mutants that cause the most rapid polymerisation cause the most retention of α_1-antitrypsin within the liver. This in turn correlates with the greatest risk of liver damage and cirrhosis, and the most severe plasma deficiency.

Polymerisation of Z α_1-antitrypsin and emphysema

Emphysema was noted in some of the first individuals who were reported to have an absence of the alpha-1 band on serum protein electrophoresis.[50] It was confirmed by family studies[51] and is now the only genetic factor that is widely accepted to predispose smokers to emphysema. The respiratory disease associated with α_1-antitrypsin deficiency usually presents with increasing dyspnoea with cor pulmonale and polycythaemia occurring late in the course of the disease. Chest radiographs typically show bilateral basal emphysema with paucity and pruning of the basal pulmonary vessels. Upper lobe vascularisation is relatively normal. Ventilation perfusion radioisotope scans and angiography also show abnormalities with a lower zone distribution.[52] Lung function tests are typical for emphysema with a reduced ratio of forced expiratory volume in 1 second to forced vital capacity (FEV_1/FVC), gas trapping (raised ratio of residual volume to total lung capacity) and low gas transfer factor. The onset of respiratory disease can be delayed to the sixth decade in never-smokers with PI*Z α_1-antitrypsin deficiency, and these individuals often have a normal lifespan.[53]

Key Points

- **The serine proteinase inhibitor or serpin superfamily includes proteins such as α_1-antitrypsin, antithrombin, C1 inhibitor, α_1-antichymotrypsin and neuroserpin**
- **These proteins inhibit their target proteinases by undergoing a remarkable conformational transition that can be likened to the movement of a mousetrap**
- **Mutations in mobile domains cause aberrant conformational transitions and a unique protein–protein linkage (polymerisation) that results in retention of the serpin in the cell of synthesis**
- **Protein overload and death of the cell in which the mutant serpin is synthesised (toxic gain of function) underlies Z α_1-antitrypsin related cirrhosis and the dementia familial encephalopathy with neuroserpin inclusion bodies (FENIB)**
- **Retention of polymerised mutant serpins causes plasma deficiency of antithrombin, C1-inhibitor, α_1-antichymotrypsin and heparin co-factor II in association with disease**
- **Understanding the pathways of polymerisation has allowed the development of novel therapeutic strategies**

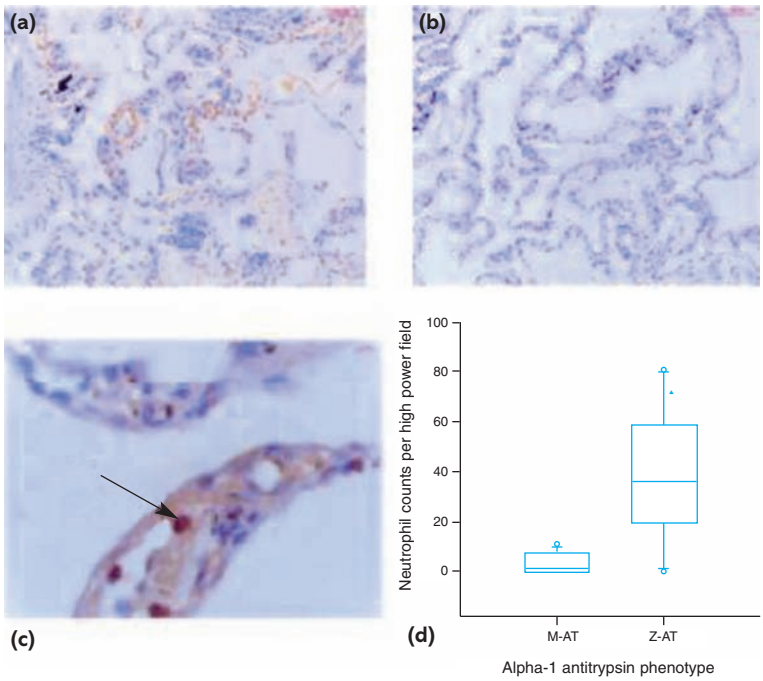


Fig 3. Polymers of α_1-antitrypsin can be detected in the emphysematous regions of the lungs from individuals with Z α_1-antitrypsin deficiency (a; brown staining), but not in regions of emphysema from M α_1-antitrypsin controls (b). The polymers co-localise with neutrophils in alveolar tissue (c; neutrophils in red and arrowed, polymers in brown). The chemoattractant properties of polymers are likely to be an important factor in the recruitment of excess neutrophils to the lungs of Z, rather than M α_1-antitrypsin homozygotes with emphysema (d). (Reproduced from Ref 65 with permission.)

Emphysema associated with plasma deficiency of α_1-antitrypsin is widely believed to be due the reduction in plasma levels of α_1-antitrypsin to 10–15% of normal. This in turn markedly reduces the α_1-antitrypsin that is available to protect the lungs against proteolytic attack by the enzyme neutrophil elastase.[54] The situation is exacerbated as the Z mutation reduces the association rate between α_1-antitrypsin and neutrophil elastase approximately five-fold.[55–58] Thus the α_1-antitrypsin available within the lung is not as effective as the normal M protein. This combination of α_1-antitrypsin deficiency, reduction in efficacy of the α_1-antitrypsin molecule and cigarette smoke can have a devastating effect on lung function,[59,60] probably by allowing the unopposed action of proteolytic enzymes. The inhibitory activity of Z α_1-antitrypsin can be further reduced as the Z mutation favours the spontaneous formation of α_1-antitrypsin loop-sheet polymers within the lung.[61] This conformational transition inactivates α_1-antitrypsin as a proteinase inhibitor, thereby further reducing the already depleted levels of α_1-antitrypsin that are available to protect the alveoli. Moreover, the conversion of α_1-antitrypsin from a monomer to a polymer converts it to a chemoattractant for human neutrophils.[62,63] The magnitude of the effect is similar to that of the chemoattractant C5a and present over a range of physiological concentrations (EC_{50} 4.5 ± 2 μg/ml). Polymers also induced neutrophil shape change and stimulated myeloperoxidase release and neutrophil adhesion.[62] The chemotactic properties of polymers were confirmed by one group[63] but refuted by another.[64] More recently, we have used a monoclonal antibody to demonstrate polymers in emphysematous tissue associated with Z α_1-antitrypsin deficiency (Fig 3a) but not in emphysema in individuals with normal levels of α_1-antitrypsin (Fig 3b). Neutrophils co-localised with polymers in the alveoli (Fig 3c). The pro-inflammatory properties of polymers were further confirmed by the demonstration that they caused a neutrophil influx when instilled into the lungs of mice.[65] Therefore the chemoattractant properties of polymers may explain the excess number of neutrophils in bronchoalveolar lavage[66] and in tissue sections of lung parenchyma (Fig 3d) from individuals with Z α_1-antitrypsin deficiency. Moreover, polymers may contribute to the excess inflammation that is apparent even in

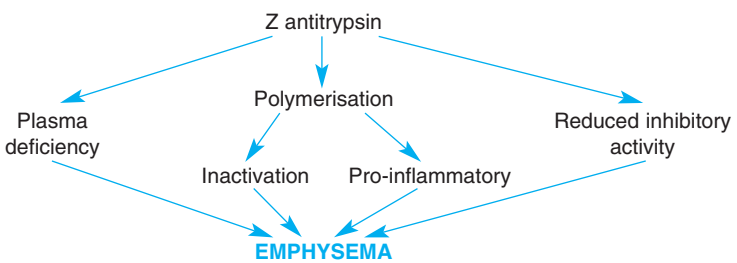


Fig 4. Proposed model for the pathogenesis of emphysema in patients with Z α_1-antitrypsin deficiency. The plasma deficiency and reduced inhibitory activity of Z α_1-antitrypsin may be exacerbated by the polymerisation of α_1-antitrypsin within the lungs. Polymerisation inactivates α_1-antitrypsin thereby further reducing the antiproteinase screen. Alpha$_1$-antitrypsin polymers may also act as a pro-inflammatory stimulus to attract and activate neutrophils thereby further increasing tissue damage. (Modified from Ref 19.)

individuals with Z α_1-antitrypsin deficiency with very early lung disease[67] and may drive the progressive inflammation that continues even after cessation of smoking. Any pro-inflammatory effect of polymers is likely to be exacerbated by inflammatory cytokines, cleaved or complexed α_1-antitrypsin,[68] elastin degradation products[69] and cigarette smoke, which themselves cause neutrophil recruitment. Thus our understanding of the biological properties of α_1-antitrypsin provides novel pathways for the pathogenesis of emphysema in individuals who are homozygous for the Z mutation (Fig 4).

For many years the emphysema associated with Z α_1-antitrypsin deficiency has been a paradigm for emphysema seen in smokers who have normal levels of α_1-antitrypsin. However, this is clearly an oversimplification as emphysema associated with Z α_1-antitrypsin deficiency has a different distribution (upper rather than lower lobe), different pathology (panlobular rather than centrilobular emphysema), the presence of pro-inflammatory lung polymers[61–63,65] and different patterns of gene expression.[70] It seems likely that more differences will become apparent as we dissect the pathways of inflammation and tissue damage in individuals with α_1-antitrypsin deficiency.

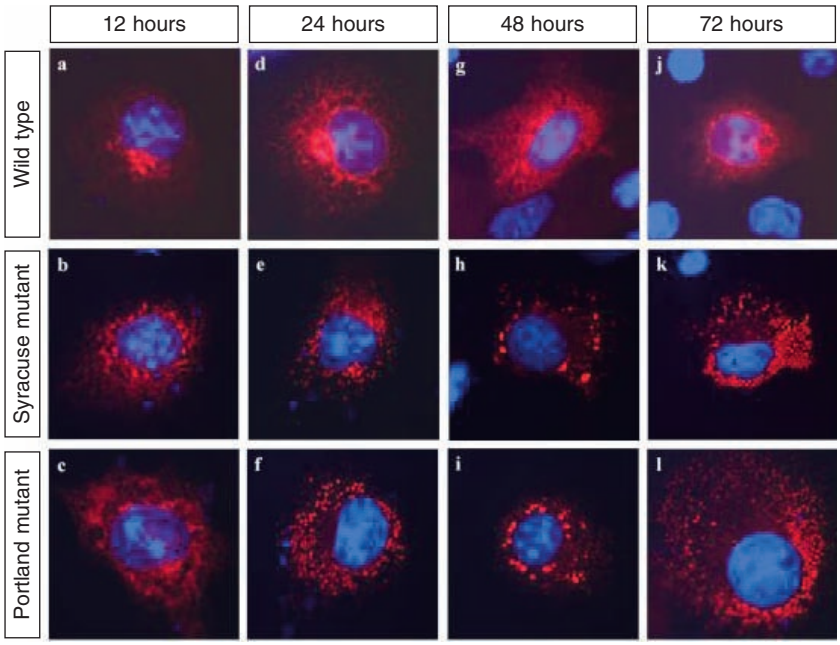


Fig 5a. Mutant Syracuse and Portland neuroserpin aggregate within COS-7 transfected cells. a-l: Immunocytochemistry with an anti-neuroserpin antibody showing the distribution of wildtype (a, d, g, j), Syracuse (b, e, h, k) and Portland (c, f, i, l) neuroserpin in COS-7 transfected cells. The nucleus appears blue due to DNA staining with DAPI. Over a three-day period, wildtype neuroserpin shows a normal endoplasmic reticulum staining pattern whereas the neuroserpin mutants form distinct protein aggregates after 24 hours of expression that persist for the three days of the experiment.

Polymerisation of mutants of antithrombin, C1 inhibitor, α_1-antichymotrypsin and heparin co-factor II causing liver retention and plasma deficiency

The phenomenon of loop-sheet polymerisation is not restricted to α_1-antitrypsin and has now been reported in mutants of other members of the serpin superfamily to cause disease. Naturally occurring mutations have been described in the shutter (Fig 1b) and other domains of the plasma proteins C1-inhibitor (Phe52Ser, Pro54Leu, Ala349Thr, Val366Met; Phe370Ser, Pro391Ser),[71,72] antithrombin (Pro54Thr, Asn158Asp, Phe229Leu)[73,74] and α_1-antichymotrypsin (Leu55Pro, Pro229Ala).[75–77] These mutations destabilise the serpin architecture to allow the formation of inactive reactive loop-β-sheet polymers that are also retained within hepatocytes. The associated plasma deficiency results in uncontrolled activation of proteolytic cascades and angio-oedema, thrombosis and chronic obstructive pulmonary disease respectively (see reviews[18–20]). More recently a mutation in heparin co-factor II (Glu428Lys) has been associated with plasma deficiency, but as yet this has not been shown to cause disease.[78] The mutation is of particular interest as it is the same as the Z allele that causes polymerisation and deficiency of α_1-antitrypsin. We have shown that this same mutation also causes temperature-dependent polymerisation and inactivation of the *Drosophila* serpin, necrotic.[79]

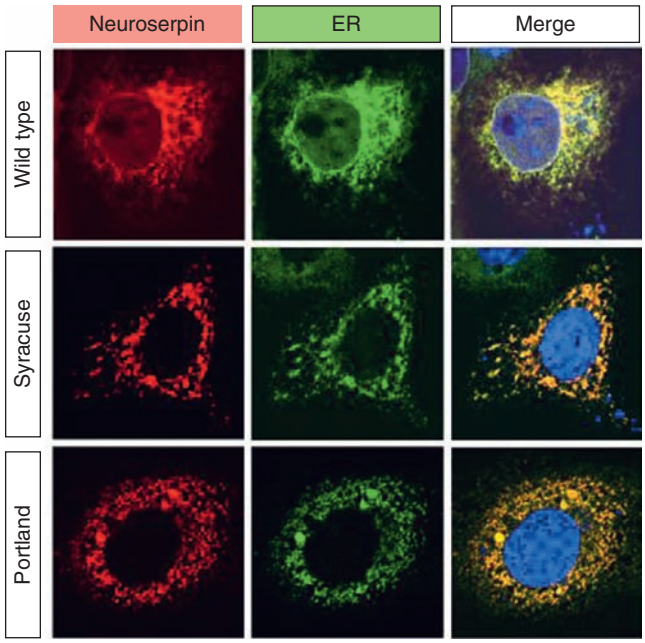


Fig 5b. Intracellular localization of wildtype, Syracuse and Portland neuroserpin in COS-7 transfected cells. Confocal microscopy of cells cultured for 24 h after transfection and stained for neuroserpin (labelled with Texas red) and an ER-resident protein, calreticulin (labelled with fluorescein). The merged image shows that the mutant protein is retained within the endoplasmic reticulum. The nucleus appears blue due to DNA staining with DAPI. (Figures reproduced from Ref 87 with permission.)

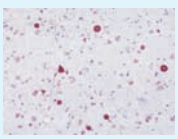
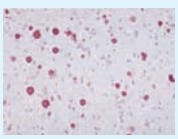
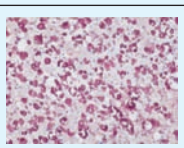
Polymers of neuroserpin and the dementia familial encephalopathy with neuroserpin inclusion bodies (FENIB)

Perhaps the most striking finding of polymer-associated disease is the inclusion body dementia, familial encephalopathy with neuroserpin inclusion bodies (FENIB).[80–82] This is an autosomal dominant dementia characterised by eosinophilic neuronal inclusions of neuroserpin (Collins' bodies) in the deeper layers of the cerebral cortex and the substantia nigra. The inclusions are PAS positive and diastase resistant and bear a striking resemblance to those of Z α_1-antitrypsin that form within the liver (Fig 2). The observation that FENIB was associated with a mutation Ser49Pro in the neuroserpin gene that was homologous to one in α_1-antitrypsin that causes cirrhosis (Ser53Phe)[44] strongly indicated a common molecular mechanism. This was confirmed by the finding that the neuronal inclusion bodies of FENIB were formed by entangled polymers of neuroserpin with identical morphology to those isolated from hepatocytes from an individual with Z α_1-antitrypsin related cirrhosis.[81]

Other families have now been identified with FENIB. These have allowed comparison of the severity of the mutation (as predicted by molecular modelling), the number of inclusions and the age of onset of dementia (Table 1). Affected members in the original family with Ser49Pro neuroserpin (neuroserpin Syracuse) had diffuse small intraneuronal inclusions of neuroserpin with an onset of dementia between the ages of 45 and 60 years.[80–82] A second family, with a conformationally more severe mutation (neuroserpin Portland; Ser52Arg), had larger inclusions and an onset of dementia in early adulthood, whilst a third family, with yet another mutation (His338Arg), had even more inclusions and the onset of dementia in adolescence. The most striking example was the family with the most 'polymerogenic' mutation of neuroserpin, Gly392Glu. This replacement of a consistently conserved residue in the shutter region resulted in large inclusions with affected family members dying by age 20 years.[83]

The role of polymerisation in disease is supported in our demonstration that recombinant Ser49Pro neuroserpin has a greatly accelerated rate of polymerisation when compared to the wild type protein,[84–86] and that Ser52Arg, which causes a more severe clinical phenotype, polymerises even more rapidly.[85] The cellular handling of neuroserpin has been assessed by transiently transfecting COS cells with wildtype neuroserpin and mutants of neuroserpin that cause FENIB (Fig 5). The most striking feature of the cell model is the retention of Syracuse (Ser49Pro) and Portland (Ser52Arg) neuroserpin as intracellular aggregates composed of polymers of mutant neuroserpin, similar to the loop-sheet polymers of mutant neuroserpin that can be isolated from the brains of individuals affected by FENIB.[87] Once again, Portland (Ser52Arg) neuroserpin accumulates more rapidly than the Syracuse (Ser49Pro) mutant, in keeping with the more severe clinical phenotype. Thus FENIB shows a clear genotype–phenotype correlation, with the severity of disease correlating closely with the propensity of the mutated neuroserpin to form polymers (Table 1).

Table 1. Correlation between the rate of polymerisation of mutants of neuroserpin, the number of inclusions and the severity of the associated dementia (based on data from Refs 81, 83–85, 87). There is a striking genotype-phenotype correlation that is explicable by the rate of polymer formation and hence the number and size of intracellular inclusions (shown in red).

Mutation	Histology of inclusions at post-mortem	Rate of polymerisation	Age of onset of symptoms	Clinical manifestations
Ser49Pro		+	48	Dementia, temor, seizures in terminal stages
Ser52Arg		++	24	Myoclonus, status epilepticus, dementia
His338Arg	N/A	+++	15	Myoclonic seizures, dementia, tremor, dysarthria
Gly392Glu		++++	13	Myoclonus, status epilepticus, dementia, chorea

Novel strategies to prevent polymer formation and disease

Our understanding of the serpinopathies has allowed the development of new strategies to attenuate polymerisation and so treat the associated disease. We have identified a hydrophobic pocket in α_1-antitrypsin that is bounded by strand 2A and helices D and E.[5,88] The cavity is patent in the native protein but is filled as β-sheet A accepts an exogenous reactive loop peptide during polymerisation.[5] The introduction of bulky residues into this pocket retards the polymerisation of M α_1-antitrypsin and increases the secretion of Z α_1-antitrypsin from a *Xenopus* oocyte expression system.[89] We are currently screening data bases for lead compounds that can bind to this cavity, stabilise β-sheet A and so ameliorate polymer formation.

An alternative approach is to block the aberrant reactive loop-β-sheet A linkage that underlies polymerisation. We have shown previously that the polymerisation of Z α_1-antitrypsin can be blocked by annealing of reactive loop peptides to β-sheet A.[29,90] However, such peptides were too long (11–13 amino acids in length) to be lead compounds for blocking mimetics and were non-specific, being able to bind to other members of the serpin superfamily.[90–92] More recently, we have designed a 6-mer peptide that specifically anneals to Z α_1-antitrypsin alone and blocks polymerisation.[93–95] The aim now is to convert these peptides into small drugs that can be used *in vivo*.

Acknowledgements

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