



Invited Review Article

Alpha-1 antitrypsin deficiency and recombinant protein sources with focus on plant sources: Updates, challenges and perspectives

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ABSTRACT

Alpha-1 antitrypsin deficiency (A1ATD) is an autosomal recessive disease characterized by low plasma levels of A1AT, a serine protease inhibitor representing the most abundant circulating antiprotease normally present at plasma levels of 1–2 g/L. The dominant clinical manifestations include predispositions to early onset emphysema due to protease/antiprotease imbalance in distal lung parenchyma and liver disease largely due to unsecreted polymerized accumulations of misfolded mutant A1AT within the endoplasmic reticulum of hepatocytes. Since 1987, the only FDA licensed specific therapy for the emphysema component has been infusions of A1AT purified from pooled human plasma at the 2020 cost of up to US \$200,000/year with the risk of intermittent shortages. In the past three decades various, potentially less expensive, recombinant forms of human A1AT have reached early stages of development, one of which is just reaching the stage of human clinical trials. The focus of this review is to update strategies for the treatment of the pulmonary component of A1ATD with some focus on perspectives for therapeutic production and regulatory approval of a recombinant product from plants. We review other competitive technologies for treating the lung disease manifestations of A1ATD, highlight strategies for the generation of data potentially helpful for securing FDA Investigational New Drug (IND) approval and present challenges in the selection of clinical trial strategies required for FDA licensing of a New Drug Approval (NDA) for this disease.

1. The disease: A1ATD

Alpha-1 antitrypsin deficiency (A1ATD) represents the most common respiratory hereditary disorders affecting Caucasians of European descent with an incidence of up to 1 of 1500 to 1 of 5000 people of this ancestry [1]. This includes up to 90,000 US individuals and an estimated 250,000 worldwide [2]. This highly undiagnosed orphan genetic abnormality is inherited in an autosomal co-dominant pattern and is phenotypically characterized by a predisposition for the development of emphysema, often presenting at an early age, and a lesser predisposition for liver disease [3]. The disease is biochemically characterized by

reduced serum levels of A1AT. Its onset and severity are known to be accelerated by smoking and by toxic environmental and occupational inhalant exposures [4,5]. The respiratory disease can also present as bronchitis, bronchiectasis and/or atypical asthma [4,5]. Individuals with liver and lung problems due to A1ATD are more likely to develop severe complications from COVID-19 infection.²

There is evidence that while individuals with severe A1ATD (PiZZ phenotype) who have never smoked have an increased risk of developing chronic obstructive pulmonary disease COPD/emphysema, they may not have a significant reduction in life expectancy compared to the general population [6]. A subset of A1ATD patients accumulate large amounts of intrahepatocyte aggregate/polymers of the abnormal

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² <https://www.alpha1.org/alphas-friends-family/resources/covid-19-updates>.

Abbreviations

A1AT	Alpha-1 antitrypsin
A1ATD	Alpha-1 antitrypsin deficiency
COPD	Chronic obstructive pulmonary disease
CHO	Chinese hamster ovary
CRP	C-reactive protein
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CS	Cigarette smoke
FDA	U.S. Food and Drug Administration
gRNA	guide RNA
HDL	High density lipoprotein
HDR	homology-directed repair

IND	Investigational New Drug
i.m.	Intramuscular
IL	Interleukin
iPSC	Induced pluripotent stem cell
LPS	Lipopolysaccharide
MMP	Matrix metalloproteinase
NDA	New Drug Application
PEG	Polyethylene glycol
rAAV	Recombinant adeno-associated virus
ROS	reactive oxygen species
RT	respiratory tract
SERPINA1	Serpin family A member 1 gene
TNF	Tumor necrosis factor

misfolded A1AT protein and develop liver cell injury, steatosis, cirrhosis and a significant incidence of hepatocellular carcinoma [7–11]. Similar to the case for A1ATD-related emphysema, this process is significantly potentiated by many other congenital and acquired liver diseases [9,12,13]. Both the respiratory and liver phenotypes of A1ATD are highly variable suggesting that other genetic and environmental factors play an interacting role in determining the incidence and degree of organ disease involvement [14,15]. The wide variation in the incidence and severity of the lung and liver disease makes it more challenging to recognize A1ATD. A smaller subset of A1ATD individuals can develop panniculitis, protease-3-associated vasculitis, autoimmune disease, bronchiectasis, bronchial asthma, or other occasionally reported conditions [16–19]. Importantly, it is well recognized that heterozygotes for milder forms of A1ATD not at de novo risk for lung or liver disease are at increased risk of both lung or liver disease given a “second hit” as can be delivered by inhaled toxins (such as cigarette smoke) or liver injurants.

The dominant emphysema component of A1ATD is primarily hypothesized to be caused by protease/antiprotease imbalances and results in chronic lung inflammation with progressive destruction of distal parenchymal gas exchanging constituents with widespread loss of alveolar septa and the supporting elastic matrix [20]. Neutrophilic proteases are believed to represent the major, but not only, causative proinflammatory protease mediators [21,22]. The recognized major lung tissue protective function of A1AT is to inhibit neutrophil elastase, protease 3, cathepsin G and alpha-defensins, the major neutrophilic proteases. However, numerous studies have demonstrated A1AT to have a wide array of immunomodulatory functions unrelated to its antiprotease properties [23–28]. It is thus likely that the multifaceted A1AT plays a protective role modulating molecular and cellular determinants of emphysema that go beyond its primary antiprotease functions [29]. It is likewise apparent that clinical manifestations of A1ATD may be impacted by genetic factors including those related to immune homeostasis genes [14,15,30]. It can thus be concluded that patients lacking A1AT are likely to have an enhanced neutrophilic inflammatory response to pulmonary insults, including infection, in addition to their deficiency of antiprotease activity [2,15].

2. The protein: A1AT

A1AT, the largest and archetypal member of the serpine family of proteases, is a 52 kDa glycoprotein, encoded by the SERPINA1 (serpin family A member 1) gene on chromosome 14. It is secreted mainly by hepatocytes (over 90%) although lesser amounts can be produced by pulmonary epithelial and alveolar cells, neutrophils, eosinophils, monocytes, and tissue macrophages [20,31,32]. The protein consists of a single polypeptide chain of 394 amino acid residues. It is N-glycosylated at positions Asn-46, Asn-83 and Asn-247 (numbering from the N-terminus of the mature, processed protein), with oligosaccharides

comprising about 12–16% of the glycoprotein mass [33]. It is organized around a central globular region and has an exposed and mobile reactive center loop site for establishing a covalent ester bond with target serine proteases, thus being a suicide substrate inhibitor [4,34–36].

The specific structural conformation, electrostatic surface charges and molecular dynamics of the 20–24 residue reactive center loop serves as a bait sequence for its target proteases. This reactive loop is thus critical for the particular inhibitory specificity and kinetics of the reacting serine protease [8,37–39]. Like other serpins, some mutated forms of A1AT accept the loop site of another A1AT molecule to form chains of unbranched polymers, which accumulate as inclusion bodies within the hepatocyte endoplasmic reticulum, inactivating the polymerized protein and resulting in both decreased A1AT secretion and liver pathobiology including ER stress, cirrhosis, and cancer [8,40].

A1AT is an acute phase protein being produced in increased amounts during infection or inflammation under interleukin (IL) 6 driven mechanisms [4,41]. It is uncertain as to the fraction of A1AT that is increased by extrahepatic sources as compared with hepatic sources under inflammation conditions. A1AT has considerable microheterogeneity due to branching differences in attached carbohydrates, particularly at Asn-83, presumably resulting from variations in specific cellular and species glycosylating enzyme profiles. The functional variabilities of these glycan isoforms have not been fully characterized, but have been shown to impact significant activities beyond serine protease inhibition (see later discussions). It has also been documented that non-enzymatic glycosylation of A1AT can occur in human plasma [42]. The plasma half-life of secreted A1AT is 4.5 days [43].

A1AT contains nine methionine groups some of which are exposed at the molecule's surface and susceptible to oxidizing conditions (e.g., such as at sites of inflammation or cigarette smoke exposure) and which potentially themselves contribute to the antioxidant properties of A1AT [44–46]. More importantly, oxidations of either methionine 351 or methionine 358 in the reactive center loop result in significant loss of inhibitory activity against neutrophil elastase (and has consequently led to intense study) [46,47]. Like albumin, A1AT also has a single reactive cysteine thiol group at position 232 that has been shown to participate in mixed disulfide formation with many plasma components harboring reactive free thiols and affecting its' intracellular retention and pharmacokinetic activity [34,44,48].

To date, nearly 300 protein sequence changing variants in the SERPINA1 A1AT gene have been described [2]. Over 120 of these allelic variants have known, or are predicted to produce, clinically significant deleterious effects, e.g. producing a quantitative or qualitative deficiency of secreted A1AT protease inhibitor [49–51]. PiM is the wild type normal allele and the associated homozygote genotype/phenotype PiMM is accompanied by plasma levels of 1–2 g/L. Levels less than 1–1.1 g/L suggest the presence of a genotype with an enhanced potential risk of clinical lung disease consequence [52].

The most prevalent deficiency variants associated with reduced serum A1AT levels are the PiZ and PiS alleles. They both have altered native stability as a dominant susceptibility to polymerize, PiZ much more so than PiS [53]. Recently, it has been shown that intrahepatic heteropolymerization can occur between PiZ and PiM alleles [40,53]. Severe forms of A1ATD are most commonly associated with homozygosity of the PiZ allele (PiZZ), representing approximately 95% of cases of severe A1ATD. It is caused by a homozygous inheritance of a single base mutation (NC_000014.9.c.1096G > A) of the SERPINA1 gene resulting in Glu342Lys [2,54]. The PiZ allele is present in up to 1 of 25 North European Caucasians of whom approximately 1 of 2000 are homozygote. Carriers of this PiZZ phenotype also make up 95% if the approximately 5000 U.S. severely deficient patients receiving weekly infusions of 3–5 g of plasma A1AT replacement therapy and having the greatest risk for A1ATD-related lung disease [35]. Homozygous PiZZ individuals have only 10–15% of serum A1AT levels compared to individuals carrying the normal PiMM allele [55].

A1AT serum levels of over 11 μ M (0.57 g/L) are theoretically estimated to protect the normal lung from proteolytic destructions in non-smokers [20,56,57]. For decades the dose of the 60 mg/kg/week has been established as the dose that would maintain patients with severe A1ATD above this theoretical protective plasma threshold of 11 μ M and alveolar fluid threshold of 1.2 μ M and optimally increase levels of neutrophilic antiproteases in lung parenchymal tissue [43,58,59]. More recently considerable controversy has arisen surrounding the finding that higher replacement doses of A1AT may be more beneficial in selected patients [57,60,61]. Of related interest, it has been shown that exogenously administered A1AT (replacement therapy) downregulates SERPINA1 gene expression [62].

It should be remembered that A1AT is an acute-phase protein and can be transiently elevated as much as 2–3 fold by inflammatory processes, potentially masking disease risk. Thus it has been recommended that C-reactive protein (CRP) levels be taken into account (preferably synchronously), especially when interpreting A1AT levels in the range of the theoretical “protection” threshold [52,63]. The increase in A1AT seen in inflammatory conditions triggered by IL-6 is generally thought of as an anti-inflammatory response (as is the IL-10’s response). The relative blunting of the increase in A1AT as compared to IL-6 in COVID-19 serious illness has given rise to a clinical trial of A1AT therapy in this infection (Royal College of Surgeons, Ireland; ClinicalTrials.gov Identifier: NCT04547140) [64–66].

The PiM allele shows a stronger inflammation-induced response than the PiZ allele [63]. There is also an issue of functional capacity of the various A1AT genotypes which could impact interpretations of A1AT levels, spectrum of activities, and reaction kinetics. Although the PiZ allele appears to express branched chain glycans similar to the PiM allele, the presence of increased total fucosylation in the PiZ A1AT, including Le^x type fucosylation, may contribute to subtle differences in some immune modulating properties between the two alleles [67,68]. Importantly, it should be recognized that polymorphisms in many genes have been shown to influence (COPD)/emphysema development in A1AT patients [15].

Individuals with the second most common mutant allele, also caused by a single nucleotide polymorphism producing the PiS allele (Glu264Val), have a serum level of 45–60% of normal levels [64,69]. Of the most common A1ATD genotypes, only the PiSZ genotype produces A1AT concentrations that straddle the theoretical “protection” threshold of 11 μ M, with 10–20% of these subjects expressing serum levels below this threshold [69]. However, PiSZ, like PiMZ individuals, who are never or very minimal smokers, have a practically insignificant incidence of emphysema due solely to their reduced plasma A1AT levels but may have a small greater annual decline in lung function not related to smoking (but a feature of established emphysema) [20,69,70].

A1ATD patients with missense, nonsense, or splicing mutations, collectively known as null mutations, have no or very minimal circulating A1AT and have worse lung function than PiZZ individuals [71,

72]. This strongly suggests that concentrations of A1AT play a role as a risk factor for emphysema (although smoking clearly continues to play a strong central role irrespective of the specific A1AT concentration). It has recently been shown that a patient homogeneous for the null allele had some circulating authentic A1AT protein of a truncated size of limited antiprotease and anti-inflammatory function [73]. Some of the rarer variant alleles produce measurable but dysfunctional serum proteins, the F variant, which binds neutrophil elastase weakly, being the foremost example. A1AT protein levels and protein phenotyping by such techniques as allele-specific sequence genotyping using customized PCR panels have the potential to detect the common M, S, Z, F, and I alleles. Table 1 summarizes the specific allelic mutation, effect, and population frequency of selected mutations.

Rarer and novel alleles require SERPINA1 gene sequencing when A1ATD variants cannot be identified by allele-specific sequence genotyping [75,76]. Substantial expertise is required in the diagnosis of the deficient alleles in more rare variants. Many inherited A1AT mutations may not automatically result in A1ATD or disease development. Other mutations may not alter the reactive center loop anti-elastase activity but may alter non-anti-elastase anti-inflammatory activities [77]. Recent authoritative reviews further detail A1AT phenotyping, genotyping, and gene sequencing [52,76].

The PiZZ phenotype confers a proinflammatory phenotype in cells in which it is produced, e.g. intracellular misfolding, aggregation, and polymerization of mutant ZZ has been shown to induce ER stress and thus promote a potentially enhanced degree of secondary inflammatory responses [30,34,35]. An impressive portfolio of mechanical insights into the folding, structure, function and dysfunction of A1AT has accumulated and informed the overall field of misfolded protein pathobiology [34,36,78]. Oxidative factors, including exposure to cigarette smoke and inflammatory oxidants/redox milieu, may influence PiZ A1AT polymerization [15].

As of 2020, two new small molecule strategies have been initiated in early therapeutic stage clinical trials to modify mutant PiZZ A1AT protein accumulations in the liver, one to shut off its synthesis with a siRNA [36] and the other to inhibit its misfolding and accumulation in the hepatocyte and facilitate secretion of the abnormal protein (Vertex Laboratories, VX-814). Z Factor Ltd has also introduced ZF874, another effective small molecule targeting PiZ folding and secretion (<http://zfactor.co.uk>).

For the past four decades the basic therapeutic strategy to protect individuals with severe levels of A1ATD (e.g., A1AT levels below 11 μ M with evidence of emphysema) from the development and progression of emphysema has been to aggressively discourage smoking and to supplement their reduced levels of plasma A1AT to levels above the theoretical protective threshold of 11 μ M [20,43,59]. A recent review

Table 1
Selected alleles in SERPINA1 leading to Alpha-1 Antitrypsin Deficiency selected from Ref. [1,74].

Allele	Mutation	Effect of Mutation	Estimated allele frequency in population
M	N/A	N/A	85%–95% worldwide
S	Glu264Val	~50% of normal serum levels due to protein misfolding, heteropolymers	3.5% North American, 3.7% European
Z	Glu342Lys	Polymerization and low serum levels (10–15%)	1.2% North American, 2% Northern Europe
F	Arg223Cys	Reduced functional ability to inhibit neutrophil elastase (but not PR3)	0.4% Europeans
I	Arg39Cys	Protein misfolding with heteropolymers and decreased serum levels (~68%)	Rare
Null	Numerous	Zero functional secreted protein	Rare

summarizes 14 published studies of varying rigor regarding attempts to verify clinical efficacy of A1AT replacement therapy [15].

The present review is designed to familiarize readership of the status of emerging therapeutic opportunities finally hitting the clinical trial horizon in A1ATD with the aim of describing the potential for plant produced A1AT therapeutics. Our focus is to update strategies for developing recombinant plant sources of A1AT and to address strategies whereby they could possibly gain regulatory approval.

3. Intersections of oxidative and protease pathobiology in A1AT/A1ATD

There are numerous instances where the effects of oxidant-antioxidant and protease-antiprotease balance intersections can contribute to the incidence, development and severity of various inflammatory diseases including A1ATD related COPD/emphysema. In A1ATD this interaction is best exhibited by the findings that cigarette smoke toxicants including reactive oxidants, aldehydes and components of particulates are capable of overcoming respiratory tract (RT) antioxidant defenses, inactivating the major anti-protease defenses by oxidizing the methionines in the reactive loop of A1AT that irreversibly binds serine proteases such as leukocyte elastase, and initiating neutrophilic dominant inflammatory processes at RT surfaces and thereby amplifying oxidant inactivation of A1AT [46,79–83]. This oxidation deactivation of the A1AT antiprotease activity has stimulated the engineering of recombinant and gene therapy A1AT molecules resistant to oxidants [58,84,85] and has provided a rationale for therapeutic co-administration of an antioxidant with A1AT [86]. The inflammatory processes initiated at the RT surfaces, dominated by neutrophils and macrophages, feeds forward the unbalanced coordinated oxidant-protease tissue injuries [32,87–90]. Finally, it should be noted that oxidative inactivation of A1AT not only is capable of potentiating serine protease-induced lung injury but also, as this protease activates matrix metalloproteinases (MMPs), calls further lung tissue injury mechanisms into play.

A potpourri of other oxidative biological considerations are related to A1AT and A1ATD pathobiology. A1AT itself, like albumin, is often considered a reactive oxygen species (ROS) scavenger via its multiple exposed reactive methionines and its reactive thiol group [81]. Of additional note, oxidized A1AT can activate monocytes [91], stimulate the release of chemokines from lung epithelial cells [92], and in the case of circulating PiZ alleles can polymerize the A1AT which in turn can then serve as an additional neutrophil chemotactic factor capable of further stoking oxidative and protease airway surface injury [32,79]. Of a related interest, determinations of oxidative A1AT levels have been proposed as a potential biomarker for the severity of COPD/emphysema and A1ATD [93,94].

Several other functions of A1AT are reported to have significant cross-over effects with oxidative stress processes. A1AT can form complexes with free heme, such as that derived from hemoglobin, thus reducing or abolishing free heme ROS production and further neutrophil activation. This activity also holds for the oxidized form of A1AT devoid of anti-elastase activity [32,95]. Oxidants are involved in pathways leading to apoptosis [96] a process known to be inhibited by A1AT [97]. Other studies have shown A1AT to suppress neutrophilic NADPH oxidase activity and generation of ROS although it is uncertain as to the degree that neutrophil extracellular trap activity, a process requiring both elastase and ROS, is affected by A1AT [32].

Another potential interrelated A1ATD relationship with redox medicine relates to events secondary to accumulations of toxic polymerized A1AT mutants (largely but not exclusively PiZ alleles) within the ER which have escaped proteasome and autophagic proteostatic processes, thus initiating ER overload, ER stress and unfolded protein responses. For example, of the seven selenoproteins identified in the ER, some have been reported to contribute protective redox homeostatic functions including the limiting of the increased ROS production related

to ER stress responses [98–101]. It seems possible that ER-related ROS production could contribute to the oxidative stress noted to be present in liver tissues of murine models of A1ATD [102]. Of note, the exaggerated inflammatory responses associated with lesser amounts of aberrantly folded A1AT polymers known to be present in A1ATD phagocytic cells and lung epithelial cells may also relate to considerations of ER stress related activities yet to be fully characterized [92,103].

As is the case for many of the other 35 serpine protease inhibitors, it is problematic as to whether A1AT interactions with oxidative stress (redox active) pathways can be ascribed to its canonical antiprotease activities or it's anti-inflammatory non-antiprotease pathways many of which are still to be fully characterized. For example, mechanisms accounting for A1AT suppressing such clinical entities as eclampsia [104] or ischemia-reperfusion injury [105] or such important pro-inflammatory processes as inflammasome activation [106] are yet to be fully detailed. It can be speculated that both oxidative and protease pathways are capable of impacting multiple signaling pathways influencing such inflammatory disease modifying pathways as those related to nuclear factor erythroid 2-related factor 2, hypoxia-inducible factor 2 alpha, and nuclear factor kappa-light-chain-enhancer of activated B cells.

It can be concluded that both oxidant/antioxidant and protease/antiprotease balances and their interactive metabolic processes undoubtedly play strong relevant roles in numerous lung pathobiologies characterized by inflammatory processes. This can be well illustrated by the ameliorating effects of antioxidants on protease-induced tissue injury and that of antiproteases on oxidant-induced tissue injury in select experimental models [89,104]. In the case of inhaled organic dust pollutants, both proteases and oxidants are simultaneously initiating lung cell injuries [107]. It is important to recognize the impacts of protease/antiprotease activities in relationship to the impact of oxidative/antioxidant activities, and vice versa, in studies designed to unravel their respective and their interactive contributions to pathobiology in various lung inflammatory scenarios including COPD/emphysema and A1ATD. It is likely that much remains to be characterized regarding the details of this interdisciplinary field of investigation in the years ahead.

4. Small molecular elastase inhibitors

Knowledge that the lack of A1AT predisposes individuals to a premature and severe form of emphysema, taken alongside experimental animal studies of various elastase-related forms of lung injury including inductions of emphysema [108,109], has spurred many investigators and pharmaceutical companies to develop synthetic or natural inhibitors of neutrophil elastase (Santhera Pharmaceuticals, POL6014; ClinicalTrials.gov Identifier: NCT03748199) [110–121].

Numerous studies have investigated the potential therapeutic role of neutrophil elastase inhibitors (both endogenous and synthetic) in models of neutrophilic inflammatory lung damage with contradictory results as presented in critical literature reviews (e.g., Ref. [122]). The past phase 1 and phase 2 clinical trials investigating neutrophilic elastase inhibitors do not show clear positive results in inflammatory neutrophilic lung diseases in spite of showing positive results in numerous animal studies, including recent studies performed on an engineered A1ATD mouse [123,122].

There is also the issue of elastase-related intracellular killing of microbes subsequent to phagocytosis, elastase-related degradations of extracellular matrix during chemotactic movements through tissue matrices, and resolutions of extracellular debris to be taken into account [124]. In spite of some demonstrations of experimental efficacy in acute lung injury (e.g. Refs. [125,126]), after several decades of preclinical, and in some cases small clinical studies, no FDA approved molecules have as yet emerged [122]. That said, a recent Phase 2 Mereo BioPharma oral neutrophil elastase inhibitor (Astraeus) trial in A1ATD patients is underway in 2020 (ClinicalTrials.gov Identifier: NCT03636347), the primary endpoint being decreases in desmosine/isodesmosine

(biomarkers for neutrophil elastase activity) plasma levels. Another neutrophil elastase inhibitor, POL6014, is even more advanced in clinical trials, being presented in aerosol form as a treatment for cystic fibrosis patients [127].

Finally, in another provocative recent study, inhibition of the dipeptidyl peptidase that activates neutrophilic elastase significantly decreased the incidence of exacerbations of patients with bronchiectasis confirmed by computed tomography and a history of frequent exacerbations. Although elastase is known to play a role in bacterial killing, there was no evidence of decreased antibacterial defenses in this short six-month trial [128].

It remains unclear as to what fraction of lung protease damage seen in A1ATD could be ameliorated by inhibition of neutrophil elastase alone. It is unknown as to what fraction of the protective effects of A1AT can be ascribed to the inhibition of neutrophilic elastase vs the inhibition of a wider spectrum of serine proteases and to the additional contributions of its non-elastase inhibiting anti-inflammatory properties. For example, neutrophilic protease 3 tracheal instillations are capable of inducing emphysema in rodents [129]. It is also likely that kinetic considerations will play an important role in determinations of efficacies of the plethora of small molecule elastase inhibitors being proposed for therapeutic use (as is the case for all enzymatic protein inhibitors) [22, 122, 130]. It cannot be safely concluded that small molecule inhibitors of elastase can be regarded as comparable and equivalent to A1AT in the protection of the lung proteolytic processes leading to COPD/emphysema pathobiology in A1ATD.

5. Small molecule Z protein modifiers

For almost three decades considerable efforts have been made to screen for small molecules that would bind to regions of the A1AT mutant Z protein and inhibit the peptide from forming polymers, thus treating the liver phenotype of A1ATD [7, 36, 131–133]. Of the over 1 million compounds screened, some were even shown to be able to not only decrease polymerization but to also increase secretion of the mutant Z protein [36, 133–135]. Although understandings of the pathophysiology of mutant A1AT polymerization has allowed the development of small molecule strategies to block polymerization with the aim of curing liver A1ATD disease, these molecules have yet to enter clinical trials. Other approaches have identified small molecules to enhance degradations of intracellular accumulations of polymerized mutant A1AT by stimulating intracellular proteasomic and autophagy-enhancing processes [136].

Based on their strategies used to develop seminal corrector and potentiator molecules used to modify defects in misassembly, misprocessing and mis-trafficking of CFTR and thus revolutionizing the treatment of CF [137], Vertex Laboratories has implemented a revolutionary new treatment for A1ATD. They have gained IND approval for early clinical trials of their VX-814 compound (ClinicalTrials.gov Identifier: NCT04167345). This compound is designed to prevent intracellular A1AT Z protein polymerization and enhance its secretion to achieve plasma levels hypothesized to protect the lung from proteolytic breakdown (e.g., increase Z levels to above 11 μ M, Z protein having over 80% of the anti-elastase activity of the normal M protein A1AT). Another drug development company, a Cambridge University spin-out, Z Factor Ltd, with the aid of a proprietary crystal structure, has patented a compound that allows the Z protein to fold correctly (ZF874).

The non-antiprotease functions of the mutant Z protein have not been characterized to the extent as they have for the normal M protein A1AT. Interestingly, there is the suggestion that some of the non-anti-elastase functions of the Z protein may be different than those of the M protein [138]. However, the Z A1AT has been shown to have anti-apoptotic effects comparable to the M protein [139].

Although to date in 2020 there are no formal peer reviewed publications on the Vertex or Z Factor Ltd compounds, both companies have revealed in publicly available online data that administration of VX-814

or ZF874 to the transgenic Z mouse mobilizes and induces efficient secretion of the hepatocyte retained Z protein into the plasma. This revolutionary small molecule strategy thus has the potential to provide for transformative treatment of both the A1ATD related liver and lung diseases, and in fact the compounds are both in preliminary clinical trials in late 2020. Unfortunately as of November 2020 the VX-814 clinical trial has been put on hold for possible complications. However, one can safely predict that other new oral small molecule products facilitating secretion of misfolded Z protein will be uncovered. It remains to be rigorously demonstrated that the enhanced secreted intra-hepatic Z protein remains insignificantly polymerized and does not exhibit pro-inflammatory properties in the oxidant environment of lung tissues and which could potentially exacerbate emphysema development [30, 140].

6. Gene and cell therapy

Since 1987, the only specific FDA licensed therapy for A1ATD has consisted of weekly intravenous infusions of pooled human plasma A1AT. This A1AT replacement therapy is dependent on supply availability and has been costly despite the licensing of several competitive human plasma protein products [141]. Gene therapy approaches would allow for sustained *in vivo* production of A1AT over a long period of time, potentially offering a lasting protective circulating level for A1ATD patients and obviating the need for frequent A1AT infusions. A large variety of gene and cell therapy approaches have been explored over the past three decades. Only a few representative studies are reviewed here. More detailed and comprehensive aspects of gene and cell therapies of A1ATD are reviewed in numerous recent publications [20, 142–145].

Many of the early experimental animal studies in the 1990s, using a variety of different viral and non-viral vector systems, transfection strategies, promoters, and routes of A1AT gene delivery, were successful with high efficacy and long-term expression. However, most of these studies yielded only very low levels of circulating PiM A1AT with vector-associated cytotoxicity/immunotoxicity and risks of insertional mutagenesis [146, 145]. Using recombinant adeno-associated virus (rAAV) vectors, which do not integrate into the genome have lesser immunogenicity, and more have efficient promoter factors, Song and colleagues were able to obtain stable therapeutic ranges (up to 80 mg/dL) of plasma human A1AT expression after intramuscular (i.m.) skeletal muscle transduction with varying degrees of elicited immune responses. This study, along with other similar studies, validated the concept and has laid the groundwork for *in vivo* human trials [147]. This same group also used their more efficient rAAV vector system for liver-directed A1AT gene delivery via the portal vein, again resulting in high plasma levels of A1AT (e.g. 60 mg/dL) in the murine model without evidence of liver toxicity [148].

In an early human biochemical efficacy and safety study, a normal A1AT gene in a plasmid-cationic liposome complex was successfully transfected into nostril cells of five A1ATD patients. This study resulted in normal A1AT protein being found in the fluid of the transfected nostril at up to a third of normal nostril lavage levels, peaking at 15 days, thus demonstrating an important proof of concept [149].

In another early proof of concept human phase 1 trial a single i.m. dose of rAAV vector expressing normal PiM A1AT transfected to several A1ATD subjects was shown to result in a sustained microtherapeutic plasma level of the normal transgene A1AT protein (0.1% of the therapeutic plasma level) for at least a year. This study further showed that, despite the demonstrated effector T-cell responses, normal A1AT was able to be transfected and secreted from the myofibers for long periods of time [150, 151]. Using a similar i.m. administration strategy with an improved rAAV vector, these investigators were able to demonstrate a linear dose-response effect in plasma although the subsequent transfected PiM A1AT plasma levels only reached 5% of levels required to reduce the risk of COPD/emphysema (e.g. 11 μ M). Although the

maximal dose required over 100 i.m. injections, the dose was received without clinical symptoms of ongoing myositis (although biopsies suggested some subclinical inflammation) [152,153].

It has long been appreciated that therapeutic expressions of a normal A1AT transgene that would be sufficient to achieve therapeutic plasma A1AT concentrations would be challenging. Thus, the concept of transfection of lung tissue cells has actively been pursued. In this regard, gene therapy strategies utilizing lung epithelial, macrophage, and endothelial cells have been considered [145,154,155]. Wilson and colleagues intratracheally instilled a lentiviral system able to deliver human PiM A1AT that not only successfully delivered human A1AT to 70% of the alveolar macrophages of infused mice but also ameliorated a mouse model of emphysema [155].

A route using novel rAAV gene transfer vectors directly injected into the easily accessible pleural surface cavity has received considerable sustained attention [20,144,156]. This A1AT gene transfer unit is targeted for pleural mesothelial cells and by absorption into pleural lymphatic drainage systems for distribution to lung parenchymal cells to liver cells. The combined preclinical supportive efficacy and toxicology data in two species has advanced this intrapleural injection strategy to a current IND approved phase 1/2 clinical trial (NCT02168686) [20]. An improved second-generation gene therapy prototype has already evolved in the laboratory for this general therapeutic approach [58].

Gene therapy and gene editing technologies using such tools as ever evolving rAAV vectors, CRISPR/Cas-9 systems [157], and gRNA/HDR donor template systems are increasingly being advanced as a therapeutic solution for use in a wide variety of genetic diseases with over 300 gene product candidates in various stages of pipeline development [20, 158]. A handful of these are approved for therapy. These include those for hemophilia [159], hemoglobinopathies [160,161], and Leber congenital amaurosis, a form of congenital blindness [162]. Others, such as those for cystic fibrosis, are poised to enter clinical trials (or are already in preliminary clinical trials) [163]. These multiple early successes have incentivized recent experimental and clinical efforts to advance gene therapy and editing technologies for many monogenetic diseases including A1ATD.

Advances in genetically engineered animal models of A1ATD have strongly contributed to the advance of the discipline of A1ATD gene-based therapeutics. Transgenic PiZ and A1AT KO mouse models have been critical aids to the development of the effectiveness of mutant A1AT genomic and base editing strategies. This is elegantly demonstrated by a study utilizing a combination of CRISPR/Cas-9 gene editing tools and rAAV gene delivery vectors to correct the disease phenotype in the hepatocytes of the A1ATD humanized transgenic PiZ mouse [164]. Strategies used in this study not only reduced the circulating PiZ protein >99% without induction of liver toxicity while reducing the intracellular accumulation of the abnormal PiZ protein 1 week after rAAV-CRISPR delivery but also changed the circulating PiZ protein to the wildtype M allele, albeit the PiZ protein downregulation in plasma was more impressive than the PiM protein augmentation. This suggests the feasible possibility of a two-step gene-based hepatocyte A1ATD strategy, first to turn off synthesis of the abnormal PiZ allele and second to transfect in the PiM allele gene, presumably to a healthier hepatocyte population.

A new base editing technology [165] has recently been applied to A1ATD gene therapy strategies. Based editors have two principal constituents fused together to form a single protein including a CRISPR protein bound to a guide RNA that leverages the established DNA-targeting ability of CRISPR modified such as causing a single-strand break rather than a double-strand break and a base editing enzyme such as a deaminase which carries out the desired chemical modification of the target DNA base. This exciting technology can be designed to repair the PiZ allele SERPINA1 G > A transition mutation, thus enabling correction of the pathogenic PiZ mutation in a one step process [166]. Studies utilizing the transgenic PiZ mouse have thus not only opened the door for the A1ATD liver disease research community,

but have also advanced a strategy for treating A1AT lung disease [145, 164,167–169].

Another genetic approach that has been used for several of the blood coagulation diseases has recently used modified mRNA encoding human A1AT formulated in lipid nanoparticles transfected into cultured hepatocytes and *in vivo* into PiZ transgenic mice to generate functioning PiM human protein [170,171]. Although this strategy would seem to be a viable potential treatment for A1ATD, the technique would require repeated mRNA administrations, but could be useful administered as a depot system for patients requiring only transient doses of A1AT.

Like the case for gene-targeted technologies, several cell-based approaches have also been successful in validating “proof of concept” therapies for A1ATD. An early study transfected a human PiM gene into murine alveolar macrophages then intratracheally instilled the macrophages into the lung. The investigators demonstrated human PiM protein to be present in the alveolar fluids of the instilled recipient mice for over 30 weeks [172].

The cell-based approach is best exemplified by seminal studies using host induced pluripotent stem cells (iPSCs) to achieve biallelic correction of point mutations in the A1AT SERPINA1 gene responsible for A1ATD [173]. Other examples of cell-based experimental animal transplantation of the normal PiM SERPINA1 gene have included passage of lentivirally transduced hematopoietic murine stem cells into irradiated mouse recipients with sustained systemic expressions of PiM antitrypsin for over 30 weeks [174]. There is no doubt that cellular transplants capable of A1AT secretion can be successfully engrafted into A1ATD patient tissues. However, there remain major challenges in these cells delivering the quantities of A1AT protein required for lung parenchymal tissues. Finally, there is the issue that the transplanted cells be successfully engrafted to proliferate and compete with native stem cell populations. The emergences of more refined and powerful *in vivo* gene transfer and gene and base editing technologies have likely set back enthusiasms for advancing cell-based approaches for lifetime therapeutics of most monogenetic diseases including A1ATD.

In summary, after three decades of progress in A1ATD gene therapy approaches using a large spectrum of biomolecular techniques, strategies for effective and safe A1AT genetic engineering are increasingly undergoing laboratory-to-clinic translation after a complex multi-decade journey [20,145,175–178]. This is further evidenced by the more than 17 gene and cellular therapy drugs already approved by the FDA and the hundreds of clinical trials at various stages of being applied to therapies of rare monogenetic diseases [143,159,162]. However, in spite of impressive advances, they are probably still another decade away from being capable of delivering efficient amount of effective, safe and licensed gene-related therapy to the A1ATD lung disease community (although existing technologies are likely to soon be available to treat the A1TD liver disease community). We can expect that further advances in gene and base editing tools being developed in pre-clinical models for many monogenetic diseases including A1AT will eventually reach the efficacy and safety levels that will inevitably take the spotlight as the key future therapy in the treatment of A1ATD.

In a notable departure from this conjecture, two lysosomal storage disease disorders caused by enzyme deficiencies which coincidentally are also the targets of recombinant plant protein replacement therapies, Gaucher's disease (the target of the first recombinant plant product licensed by the FDA) and Fabry's disease (which also has a recombinant plant produced product), now both have gene therapies to express their functional missing enzyme in 2020 clinical trials (AVR-RD-0, NCT 04145037 phase 1/2 and AVR-RD-01, NCT 03454893 phase 2, respectively).

7. Recombinant A1AT approaches

Recombinant protein-based therapeutic products continue to be among the fastest growing areas of drug development for years to come. This is no exception for treatment of A1ATD, which has been the target

of plasma-derived protein therapeutics for over 30 years. Protein-based therapeutics represent an opportunity to decrease the prohibitive costs of human plasma A1AT (up to U.S. \$200,000 per patient per year in 2020) while developing platforms to potentially introduce improved A1AT-based products [84]. Supply chain limitations of human plasma-harvested A1AT are a potential concern to the safety of the A1ATD population. Concerns are present in both supply and demand, considering a shrinking supply of plasma (e.g. stocks diverted for other applications, lowered volume of plasma donations) and a growing demand should the optimal dose or population in need increase, or if indications for A1AT expand.

There is a growing need to bring a more personalized approach to replacement therapy to patients with A1ATD. It seems likely that a subset of patients with continuing accelerated loss of lung function or parenchymal tissue CT density would benefit from more than standard conventional doses of A1AT [61,179,180]. Additional supplies of A1AT will also be needed if more severe A1ATD patients requiring therapy are identified, and if patients with lesser degrees of A1ATD (e.g. MZ or SZ heterozygotes) with rapidly accelerated trajectories of lung function or parenchymal tissue loss are shown to be helped by replacement therapy.

At the current time A1AT is not available even for A1ATD patients in many countries because of the economic barrier. Cost issues thus represent a strong further impetus for development of recombinant A1AT products [181]. Development of an additional supply of A1AT would allow for A1AT treatments of an expanded number of non-emphysema related indications [16,17,27,104,130,182–206].

The feasibility of using plasmid constructs and/or retroviral vectors and recombinant DNA technology to produce functionally active A1AT with anti-elastase behavior similar to or better than that of human plasma A1AT has been aggressively investigated for 3–4 decades. A1AT has been recombinantly produced successfully from both prokaryotic and eukaryotic expression systems. Expression in *Escherichia coli* has been widely studied [207,208]. Although the unglycosylated *E. coli* form lacks the 3 N-linked glycans on human A1AT, glycosylation does not appear to affect its molecular function as a protease inhibitor [207,209]. Limitations include its lack of demonstrated ability to function in non-elastase immune modulating activities and its short circulation half time. The latter issue has been addressed with specific conjugation of the unglycosylated A1AT free thiol with polyethylene glycol (PEG) while preserving its anti-elastase activity [210,211]. This product has been further developed using triple-mutant engineering to produce an A1AT with enhanced thermal stability and oxidant resistance while remaining unglycosylated [85]. Of note, engineered glycan modifications have been shown to influence the stability and flexibility of A1AT as well as to considerably alter the circulatory half-lives of other recombinant forms of human A1AT [109,212].

Expression systems utilizing yeast [213], fungus [214], insect cells, mice, goats, rabbits, and sheep have all had some successes but have been limited by such issues as non-human glycan related immunogenicity, reduced stability and biological activity, and production feasibility issues [215]. Recombinant human A1AT has likewise been produced successfully in numerous human cell types by transfecting A1AT cDNA including into human embryonic PER.C-6, and liver HepG2 cell lines [216,217]. When co-transfected with a single recombinant human sialyl transferase gene, this product exhibited indistinguishable pharmacokinetic properties and anti-elastase functions as compared to human plasma A1AT [217].

Immortalized Chinese Hamster Ovary (CHO) cells have provided for an attractive commercial alternative to human cell lines for more than half a century. They have generated over half of the recombinant protein biopharmaceuticals approved over the past decade, albeit with glycosylation patterns showing significant different profile patterns than human profiles [215]; with differences between different CHO lines and other mammalian cell lines [218] and also dependent on bioreactor operating conditions [219]. At the current time CHO systems are central to the manufacture of recombinant proteins.

Like most of the non-human recombinant A1AT preparations, CHO cell produced A1AT products have shown anti-elastase activities similar to those of human plasma. However, only a paucity of studies have attempted to characterize the degree of their similarity to the many reported non-elastase anti-inflammatory immune modulating properties of human plasma A1AT. It is likely that many of these latter functions of A1AT will be influenced by specific glycan profiles [220], as will be discussed later. The availability of the CHO genome sequence has enabled the development of genome-scale models to examine the metabolic signatures upon various metabolic bioprocessing conditions. Investigators are now applying genomic insights and sophisticated technologies for glycoengineering to led to breakthrough innovations in the field of CHO cell technological innovations, accelerating their optimizations for human protein production.

Among the technological innovations that have accelerated the development of downstream protein products has been the development of Fc-fusion chimeric proteins, a process that has resulted in considerable improvement in circulating protein half-life while enhancing downstream protein purification procedures [221]. These chimeric proteins are increasingly being recognized as safe and useful therapeutic products. Further engineering of glycosylation profiles of either the Fc or the fused protein component have the potential for broadening the kinetic repertoire of therapeutic products [222].

A1AT-Fc, a CHO cell-derived recombinant chimeric form of A1AT fused with the Fc domain of human IgG1 to the C-terminus of A1AT, has been demonstrated by several investigators and companies to have a greatly prolonged plasma half-life compared to human plasma A1AT and has been shown to possess both antiprotease and non-antiprotease anti-inflammatory effects [223]. One A1AT-Fc CHO cell formulation has shown a significantly greater anti-inflammatory potency over plasma derived A1AT above and beyond its increase in plasma pharmacokinetic differences [224]. Recombinant A1AT-Fc formulations are likely to translate into a more convenient dosing schedule (e.g. perhaps every 4 weeks for routine maintenance IV therapy instead of every week for human plasma A1AT). Most of these formulations have generally been shown to have anti-elastase activities comparable to that of human plasma A1AT. Several of these preparations have shown non-antiprotease related anti-inflammatory potencies beyond their easier to demonstrate antiprotease activities [24,223–226]. A single subcutaneous low dose of one A1AT-Fc formulation has been shown to be highly effective in reducing an experimental model of acute inflammatory gouty arthritis, illustrating one of the non-A1ATD possible utilizations of recombinant A1AT [223]. As is the case for a new class of hemophilia A factor VIII replacement product [227], which is an Fc-fusion protein augmented with additional polypeptide fusion partners, one can expect other novel fusion molecules to be fused to the A1AT-Fc product to further favorably modify recombinant A1AT.

A recent CHO cell-derived A1AT-Fc preparation has initiated a Phase 1 clinical trial (INBRX-101, [ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study?term=INBRX-101&rank=1) Identifier: NCT03815396). Detailed reports of the specific glycan profiles of the three A1AT sites and the one Fc site are not reported but are likely to have been modified so as to further increase the preparation's plasma stability and pharmacokinetic profile. The Inhibrx pipeline A1AT-Fc product also has mutations in the two methionines in the A1AT reactive loop so as to render the product less sensitive to oxidant inactivation of the A1AT portion of the fused protein.

Although the Inhibrx product and several developing CHO cell recombinant A1AT-Fc preparations look to be promising and bio-better from the standpoint of oxidant resistant enzymatic functions and antiprotease pharmacokinetics, there are likely to be residual issues related to upscale manufacturing challenges, patent disputes, market uncertainties, and possible effects secondary to differences between the engineered A1AT-Fc CHO cell compound and the human plasma A1AT glycosylation profiles. These later differences have the potential to affect transport and non-protease anti-inflammatory immune modulating functions of A1AT. Similar to the case of the previously mentioned

Vertex Z protein small molecule corrector preparation designed to increase the liver secretion of the misfolded Z-A1AT variant (responsible for 95% of severely deficient A1ATD patients), the A1AT-Fc recombinant product raises regulatory issues in that it may be challenging to make the case for bioequivalence to the existing human plasma PiMM A1AT products. This would particularly be true of other potent anti-inflammatory formulations of A1AT-Fc that have anti-inflammatory effects but no inhibitory effects on elastase activity [225,226].

More specific excellent reviews of newer therapeutic strategies to treat the lung and liver aspects of A1ATD, including updated public A1ATD clinical trial databases [228] and details of specific strategies for engineering A1AT (including glycoengineering), have recently been published [39].

8. Recombinant plant A1AT

As compared to other recombinant protein production platforms, plant-based production, also known as molecular pharming, can provide an array of advantages including improved product safety, lower cost, and reduced production cycle time in both developed and developing countries [229,230]. In 2012, the FDA approved a glucocerebrosidase enzyme produced in carrot cell suspension culture, Elelyso® (Taliglucerase Alfa, Protalix BioTherapeutics, Karmiel, Israel), for treatment of Gaucher's disease, representing the first plant-made recombinant therapeutic protein commercially available for human patients. While Elelyso® was able to demonstrate clear advantage over the established mammalian cell-derived product as a less expensive (with 25% lower price per dose with maintained profit margins) and bio-better (due to the naturally occurring high-mannose glycan structure of vacuole-targeted plant cell-expressed protein), it was the reported viral contamination and subsequent world-wide shortage of the mammalian cell-derived

production that expedited the regulatory approval process for the first commercial molecular pharming product [231].

During the Ebola crisis in 2014–2016, the company Kentucky Bio-processing leveraged the fast production cycle time of molecular pharming to rapidly respond to the outbreak with the production of ZMapp, a mixture of three monoclonal antibodies produced in *Nicotiana benthamiana* using transient agroinfiltration. ZMapp showed promise as a post-exposure Ebola therapy in animal studies [232] and in humans [233] but ultimately did not reach commercial approval as the outbreak died down and a lack of Ebola-positive subjects halted clinical trials.

In the current COVID-19 pandemic, molecular pharming is being used to rapidly respond to the outbreak. With the enormous scale of global impact, this plant-based production response is addressing a variety of needs ranging from SARS-CoV-2 research reagents to vaccines and therapeutics, reaching beyond the scope of antibody therapy developed for Ebola. An array of molecular pharming companies, including Medicago, iBIO, Kentucky Bioprocessing, Novici Biotech, Plant Biotechnology, PlatForm, Leaf Expression Systems, Cape Bio Pharms, Mosaic Immunoengineering, and Baiya Phytopharm, alongside with academic groups, are working on plant-based production of SARS-CoV-2 vaccines, diagnostic reagents, and receptor decoy proteins [234–237].

The capacity to use plants to produce complex glycoproteins with controlled and/or modified glycosylation patterns through subcellular localization [238], process modifications [239], and host engineering [240] is further discussed in section 9 of this review, followed by elaboration of techno-economic advantages in section 10.

A1AT production specifically has been attempted in transgenic rice cell suspension culture (*Oryza sativa*) [241–244], transgenic tobacco cell suspension culture (*Nicotiana tabacum*) [245,246], transgenic tomato plants (*Solanum lycopersium*) [247,248], transgenic tobacco leaf

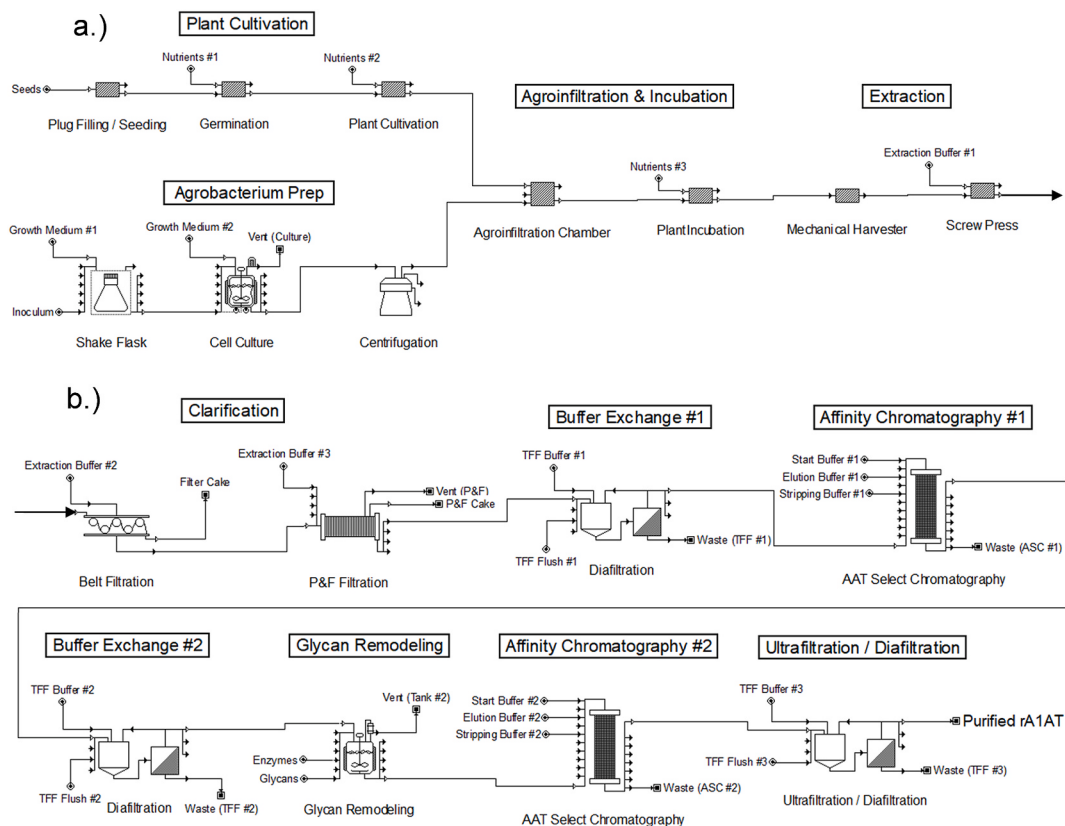


Fig. 1. A schematic of a hypothetical commercial-scale plant-made pharmaceutical manufacturing facility designed to produce and purify recombinant A1AT, broken down into the a) upstream, and b) downstream process flows. The process flow is based on the authors' vision of scaling up previously published bioprocessing results that were performed at an academic level. An optional glycan remodeling step is included for consideration. P&F, plate and frame.

chloroplasts (*N. tabacum*) [249], and transgenic rice seed (*O. sativa*) [250]. Active A1AT has also been produced transiently in the leaves of tobacco (*Nicotiana benthamiana*) plants [84,251–254]; (Padgett, unpublished).

Fig. 1 depicts a simplified process diagram for the transient expression strategy. In this scheme, greenhouse-grown plants are transformed using *Agrobacterium tumefaciens*-mediated gene transfer, a process called agroinfiltration or agroinfection [255,256], and begin expressing recombinant protein within 3–7 days post-exposure. Agroinfiltrated tissues can then be harvested by means of homogenization and protein extraction [257] for further downstream processing typically involving clarification and ultrafiltration of the tissue extract and homogenate followed by chromatography or other methods of protein purification. For A1AT in particular, the development of an immunoaffinity chromatography resin by Cytiva Inc. has greatly reduced the difficulty of protein purification while increasing the single-step yield and purity [84]. While this lab-scale purification scheme posits a simpler approach to purify A1AT, further refinement and definition for production at commercial scale is needed and underway.

Although transient expression in plants has the advantage to produce high quantity of active A1AT, isolating the fully intact A1AT from minor degradation products is still a challenge. Castilho and team have reported that secreted recombinant A1AT was truncated at both the N- and C-termini [251]. It is likely that the recombinant A1AT is being cleaved by endogenous plant proteases in the apoplastic compartment and that the reaction may be inhibited by serine protease inhibitors (unpublished data). However, it is still unclear if recombinantly produced plant-derived A1AT is actually cleaved by these proteases, and in which cell compartments the degradation is primarily occurring. On the other hand, it has been shown that plant-derived A1AT, whether produced in transgenic plant cell culture [241], transgenic plant seeds [250], or transiently in plant leaves [84], are all as active as human plasma-derived A1AT in anti-elastase inhibitory activity and band shift assays. Furthermore, the recent study from Silberstein and team has preliminarily reported bio-better characteristics, showing that transiently produced plant-made recombinant A1AT with a point substitution in the C-terminal reactive loop not only shows comparable antiprotease activity to human A1AT but that it also retains full activity under oxidative conditions that deactivate its native counterpart.

In summary, plant-based production of recombinant A1AT is now showing significant promise. Considerable research efforts in academic and industrial laboratories are being directed toward finally achieving reliable and economical production of intact and fully active plant-derived A1AT. Novel strategies to enable this have recently been developed and demonstrated (Novici Biotech LLC, unpublished results); [258]. Collaborative efforts among the authors to expand upon those encouraging early studies are currently underway.

9. Further considerations of recombinant A1AT glycan heterogeneity

Asparagine N-linked glycosylation represents a major co- and post-translational modification of secretory proteins including A1AT. Although glycan profiles do not appear to greatly affect A1AT anti-elastase activities *in vitro*, the three N-glycosylation structures are known to play a significant role in A1AT plasma pharmacokinetics, stability and interactions with other proteins [259,260]. There is considerable evidence that the A1AT glycan components have been strategically placed such that their profiles contribute to the apparent increasingly more versatile molecular properties of the protein [261].

For example, the augmented A1AT production as an acute phase reactant in response to IL-6 hepatocyte stimulation is accompanied by subtle variations in A1AT glycan chains characterized by more heavy sialylated forms having an increased affinity for IL-8 [41,67] and impacting innate cell responses [261]. Differences in A1AT glycan spectrums associated with high density lipoprotein (HDL) compared

with plasma A1AT glycan profiles have been described [262]. The detailed functions of the linear, branched and polymeric oligosaccharide structures of both human plasma and most recombinant forms of A1AT are yet to be fully characterized.

A phase I approved commercially developed CHO cell recombinant A1AT-Fc compound has undergone considerable molecular engineering of its A1AT and Fc glycan components that are yet to be published (NCT04167345 Inhibrx, 2019). It is readily apparent that glycan engineering represents an important component of multifunctional recombinant proteins such as A1AT [263].

The effects of the profiles of plant A1AT glycosylation on the stability, physical properties, pharmacokinetics and non-elastase inhibiting properties of plant produced A1AT are yet to be fully characterized. In a similar vein, discrepancies in N- glycosylation pathways and efficiencies between mammalian cells (e.g. CHO cells) and plant cells are yet to be fully detailed. The initial glycosylation pathway in the ER including trimming of the Man 9 (GlcNAc2Man9) precursor oligosaccharides and modification to form GnGn (GlcNAc2Man3GlcNAc2) structures are the same in plants and mammals. However plants can add a α 1,3 core fucose rather than the α 1,6 core fucose found in mammals, a β 1,2 bisecting xylose, and β 1,3 galactose and α 1,4 fucose on terminal GlcNAc's, a structure known as a Lewis a epitope [264].

Although immunogenicity of plant-specific glycans on glycoproteins has not been seen to date in FDA approved plant-made biologics on the market (e.g. Elelyso®) [265] there are existing established strategies to minimize, modify or eliminate plant-specific glycans if it proves to be a concern. For example, elimination of the N-linked glycosylation sites altogether, adding an ER retention signal, or using plant host lines in which enzymes responsible for the addition of plant-specific xylose and fucose sugars are knocked down [240] or knocked out will prevent plant-specific xylose and fucose linkages in the product. Adding small molecule inhibitors of enzymes in the glycosylation pathway such as kifunensine to the infiltration medium is another simple bioprocessing approach [239,266]. *In planta* deglycosylation followed by *in vitro* reglycosylation after primary purification is another approach [267]. Plant glycoengineering is rapidly developing [222,251,268–277]; and with the advent of CRISPR/Cas9 technologies, it is likely that glyco-engineered plant hosts will be available in the near future. It can be expected that prospects for engineering of plant produced N-glycosylation modifications represent a promising source for incorporation into plant A1AT production platforms, thus producing an increased quality product more closely biosimilar to human plasma A1AT.

10. Techno-economic outlook for recombinant plant-based production

Plant-based production of recombinant human A1AT has been carried out in research laboratories for several decades [278]. A limited set of studies to date suggest that there may be significant cost savings in manufacturing recombinant A1AT with whole plant-based systems as compared with the more traditional biomanufacturing platforms that use mammalian cells, largely CHO cells grown in bioreactors at similar production scales [279,280]. The commercial feasibility reported in these studies is in part supported by the existing breadth of commercial-scale molecular pharming production facilities – including those of iBio CDMO in Bryan, TX, Medicago in Quebec, Canada, and Kentucky Bioprocessing in Owensboro, KY. Holtz and team have very nicely described iBio CDMO's (formerly Caliber Biotherapeutics) vision and implementation of commercial-scale manufacturing of plant-made pharmaceuticals in a recent review [281]. There is also evidence to suggest that plant cell culture bioreactor-based production is an economically viable strategy for production of human therapeutics [282].

Table 2 shows the purchase prices for commercially available A1AT products alongside recent literature that reports simulated cost of goods sold for plant-based pharmaceutical production.

Table 2

Purchase prices for human plasma-derived A1AT on Apr 30, 2019 (Retrieved from [GoodRx.com](https://www.goodrx.com)) and simulated cost of goods sold for commercial-scale plant-based pharmaceutical production.

Purchase Price of Human Plasma-Derived A1AT		
Human A1AT Formulation	Quantity	Price (\$)
Glassia	50 ml (1 g)	552.90
Aralast NP	50 ml (1 g)	519.90
Zemaira	4 g	2054
Prolastin-C	No data available	No data available
Cost of Goods Sold for Plant-Based Pharmaceutical Production		
Source	Product	Cost (\$/g)
[283]	Antiviral protein	105.80
[280]	Monoclonal antibody	90–121
[284]	Butyrylcholinesterase	1180
[282] (Plant cell culture)	Butyrylcholinesterase	676–1610

Based on the most recent analyses from Ref. [280,283]; it is reasonable to project that plant recombinant A1AT could be manufactured for a comparable cost of ~\$100/g at a production capacity of 300 kg product/year. Applying an estimated gross margin of 66.86%, an average gross margin obtained from data on 503 biotechnology drug firms (retrieved from New York University's Stern School of Business; http://pages.stern.nyu.edu/~adamodar/New_Home_Page/datafile/margin.html), this translates into a purchase price of ~\$300/g. This calculation estimates ~40% purchase price reduction over human plasma-derived A1AT. However, it is critical to understand that the techno-economic models can be highly sensitive to parameters such as expression level, production capacity, and downstream recovery and that the techno-economics for plant recombinant A1AT may vary from published models depending on the exact processing. For example, in Refs. [280] the cost increases from the base case of ~\$100/g at an expression level of 1 g product/kg leaf fresh weight to ~\$300/g at an expression level of 0.25 g product/kg leaf fresh weight. It is also important to note that monoclonal antibody production

benefits as a platform molecule with a breadth of commercially available equipment specifically designed to process monoclonal antibodies (e.g. protein A chromatography). Recent developments of A1AT-specific immunoaffinity chromatography resin, as previously described, have helped to close the gap in processing efficiency between A1AT and platform molecules like monoclonal antibodies.

However, as of 2020, there has been little movement in plant-based production of recombinant A1AT from the stage of academic laboratory to larger-scale pilot or commercial biomanufacturing facilities. There is a nascent yet established regulatory pathway for commercial production of human therapeutic proteins that corroborates plants as a viable production vehicle for a wide variety of human therapeutic proteins; the FDA has already approved a handful of plant derived therapeutic proteins for human use and additional products are in various stages of review [283,285].

Potential obstacles to commercialization of plant-based recombinant A1AT include further optimization of bioactive A1AT accumulation levels, convincing demonstrations that plant-made recombinant A1AT is reasonably biosimilar or biobetter as compared to present commercial preparations, and a direct techno-economic analysis of plant-based A1AT production to evaluate profitability against other emerging A1AT products reaching the market. One barrier to comparing different recombinant production platforms is the lack of publicly available and robust techno-economic analyses for CHO and microbial production [99,286]. As a precursory comparison, Fig. 2 gives a recent snapshot of comparative yields of CHO cell and plant leaf extraction levels of functional A1AT.

Despite this difficulty in obtaining direct comparisons between platforms, a strategy to minimize risk of recombinant plant A1AT techno-economics would be to model manufacturing flows that include processes to negate the impact of plant-specific contributions. As previously mentioned, the impact of plant A1AT glycoforms to product critical quality attributes are not yet fully characterized. Inclusion of chemical processing steps of glycan remodeling to obtain fully humanized glycoforms would represent a “worst case” scenario, from the

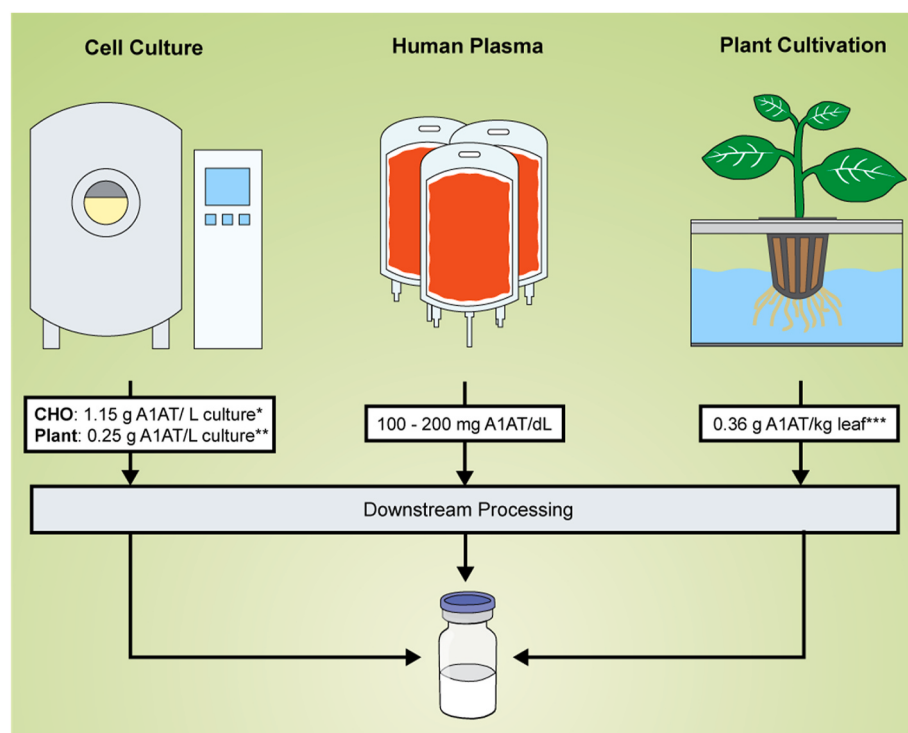


Fig. 2. An illustration of established and promising A1AT protein therapeutic production platforms compared by specific productivity. * [287]; values are based on total A1AT, no information on functionality; ** [242]; values are based on functional A1AT; *** [84]; values are based on functional A1AT (780 mg/kg leaf total A1AT).

techno-economic perspective. The proposed manufacturing flow shown in Fig. 1 includes an example of how that glycan remodeling step may be included for transient production of A1AT in *N. benthamiana*.

11. Preclinical modeling for therapeutic development of recombinant A1AT

In the U.S., successful drug development depends on a tedious navigation through the early academic or commercial product development phase. The latter preclinical stages of this innovative product is focused on refinements of optimal product production, purification, physical characterization and relevant molecular, cellular and animal biological activities that will allow for successful recruitment of commercial interest including financing and development of a basic packet in support of issuance of an FDA investigational new drug (IND) application, an important required step prior to initiation of preliminary staged clinical trials. This requires selection of strategies for determining pharmacokinetics, bioavailability to sites of action, and considerations of an experimental animal model for testing *in vivo* efficacy and toxicity. It is important that resources not be wasted on collection of excessive or irrelevant data or animal use in order to conserve development resources.

The FDA offers no rigid guidelines for this other than assurances that the “totality of data” submitted to them will be taken into account. Thus, there is some room for creativity and discovery in preclinical data collections. Considerations in this section are limited to those relevant to recombinant A1AT products with active *in vitro* anti-elastase activities not too dissimilar to that of native human plasma A1AT.

Challenges facing investigators include considerations around the data which would determine the degree of bioequivalence of the new recombinant product to the existing commercial replacement therapies, not only in pharmacokinetic and antiprotease activities but also in select non-protease anti-inflammatory activities. The full extent of the myriad of overlapping immune modulating functions unrelated to A1AT's dominant serine protease inhibiting functions are yet to be fully clinically clarified.

This is confounded by the fact that elastase itself initiates so many downstream pro-inflammatory responses that are ameliorated by A1AT via its irreversible inhibition of elastase. This is well exemplified by the fact that elastase may be the trigger for more complex inflammatory and proteolytic cascades which contribute to lung tissue destruction and thus be inhibited by both A1AT and inhibitors of these complex downstream pathways. These pathways include those related to MMPs, IL-8, IL-1 beta and tumor necrosis factor (TNF), all of which are themselves amenable to a host of specific inhibitors in addition to anti-elastase and which could confound attempts to disentangle A1AT antiprotease functions from those not related to its antiprotease activities [288–290]. Leukocyte elastase is not the only important serine protease causing destruction of lung parenchyma in A1ATD [22,289,291–293]. It is likely that suppression of serine protease activity is insufficient to prevent the full accelerated emphysema seen in A1ATD patients [294].

Parameters of relevance to recombinant A1AT preclinical modelling could logically include: (i) rigorous determinations of recombinant A1AT *in vivo* stability and pharmacokinetic assessments; (ii) determination of susceptibilities to oxidative inactivation, particularly those due to inflammatory and environmental oxidants; (iii) characterization of the site-specific glycans on the recombinant product; (iv) characterization of protein-protein interactions many of which may be dependent on glycan profiles; (v) determination of the full spectrum of antiprotease activities; (vi) characterizations of anti-apoptotic activities, a major biologic process related to emphysema and inhibited by A1AT; (vii) survey of the multifaceted effects on neutrophils including chemotactic activities; (viii) measurements of the degree of penetrance of new A1AT products into lung tissues and alveolar spaces; (ix) effects on relevant cellular model systems; (x) effects on appropriate animal models; (xi) assessment of toxicity issues. As the FDA may consider bioequivalence as

an important criterion, it would be important to conduct all biological assays in a comparative manner with an existing commercially approved human plasma antitrypsin (e.g., Prolastin, Prolastin C, Zemaira, Glassia, Aralast). It would also be helpful to provide practical economic assessments (not presently an FDA consideration).

Many of the recombinant products have mutations of one or both of the reactive loop methionines to provide for increased resistance to oxidative deactivation. As the lung antiproteases including A1AT often are functioning in an oxidative inflammatory milieu or directly exposed to oxidative inhaled oxidants (e.g. cigarette/environmental smoke, ozone), it is important that oxidative susceptibilities be determined. The spectrum of recombinant A1AT glycans are important to determine as they can be expected to influence non-protease inhibiting functions of A1AT including overall molecular stability, pharmacokinetics, and interactions with other molecular species including receptors for mediators of inflammatory and anti-inflammatory processes [295]. It is important to identify potential recombinant A1AT binding partners and binding kinetics using a variety of state-of-the-art techniques such as biolayer interferometry [295,296]. Of the many inflammatory mediators to be considered, interactions with IL-8, lipid mediator leukotriene B₄, A disintegrin and metalloprotease 17, and TNF are likely to be important [16,73,297–299]. To date, the precise nature and biological significance of the interactions between A1AT and other molecules have received suboptimal attention and this is especially true for recombinant forms of A1AT. Interactions of new forms of A1AT with lipoproteins and free fatty acids would also be useful to document [300,301].

It is important that the full spectrum of antiprotease activities should also be documented [22]. As apoptosis plays such a major role in emphysema processes and is reported to be directly inhibited by human plasma A1AT, comparative roles of various forms of A1AT on this important cellular function are worth documenting [97,302]. There is even a recent report of A1AT inhibiting another important inflammation-related process, inflammasome activation [106].

As numerous apparent non-protease functions of A1AT relate to reported interactions with neutrophil functions, including chemotaxis and inflammatory mediators, it would seem worthwhile to document comparative effects of human plasma and recombinant forms of A1AT on a selected spectrum of these parameters [28,32,303–306].

As it is still unclear as to the precise mechanisms that enable A1AT transport into lung tissues and alveolar surfaces, it is possible that facilitated transport processes are involved, including those related to lipid metabolism [298,301,307–310]. It is thus important that new recombinant forms of A1AT undergo studies of lung uptake and transport to alveolar surfaces and be compared to uptake rates of plasma-derived A1AT [309–311]. Comparative measurements of A1AT in exhaled breath condensate should help in this regard [312,313].

Since abundant evidence exists that TNF is involved in cigarette smoke (CS) inductions of COPD and that plasma A1AT ameliorates actions of TNF, comparisons of plasma and recombinant A1AT effects on TNF lung cell effects would seem relevant to perform [297,314–317].

Finally, in support of the regulatory theme of bioequivalence, it would be useful to use such techniques at RNAseq to interrogate similarities and differences in gene expression between native human plasma A1AT and recombinant forms of A1AT in experimental models where A1AT can be expected to modify LPS and/or TNF effects on such cellular systems as lung epithelial and endothelial cells.

The cornerstone of potential therapeutic preclinical studies relies on animal models of human disease. Such animal models provide insights into potential mechanistic targets and help validate treatment strategies to be translated safely to clinical trials. Animal models for human pulmonary diseases as a precursor to human clinical trials have been extensively discussed in the literature [126,318–324]. Inductions of COPD-like lesions in lungs of animal models using noxious inhalants have included such agents as proteases, lipopolysaccharide (LPS), TNF, and air pollutants. Not surprisingly, many studies have utilized mice exposed to CS as the “gold standard” animal model of emphysema [324].

Studies of CS exposure in mouse models have been invaluable in studying mechanisms of emphysema development and have made significant contributions to understanding CS related human lung biology. These models have included transgenic and knockout mice that either develop “emphysema-like” changes spontaneously or become more resistant or susceptible to CS exposure [321,325]. Although, lung injury-inflammatory processes in mice and humans show significant differences [326], there are also significant correlations between the biological processes of CS injury in mice and humans [323].

A wide range of therapeutic interventions have been shown to ameliorate rodent lung responses to CS [327–329]. These ameliorating interventions have included N-acetylcysteine [330], SOD [331], thio-redoxin [332], roflumilast [333], clarithromycin [334], MMP inhibitor [335], statins [327], TNF receptor deficient mice [317], hydrogen-rich water [29], exercise [336], and as taxanthin, a xanthophyll carotenoid [337]. It is worthy to note that many of the reported interventions have been touted as being anti-inflammatory agents or antioxidants. It should also be noted that there are many transgenic and gene-targeted mouse models of emphysema, some of which have been examined for susceptibility to CS [317,338,339].

In elegant studies Dhami et al. showed that human A1AT supplementation to mice almost completely ameliorated a short-term acute CS exposure as documented by decreases in inflammatory biomarkers and desmosine measurements [340]. Churg et al. also showed that A1AT administration also inhibited short term and long-term inflammatory effects of CS exposure while reducing CS-induced effects on increasing airspace size. This group also demonstrated that oxidized A1AT with inactive protease activity also ameliorated CS-induced inflammatory activity [314]. CS exposures with inhaled recombinant A1AT showed that inhaled A1AT ameliorated emphysema even though it was demonstrated that inactivating mouse antibodies against A1AT were generated [341]. Finally, Takeda et al. have also used a recombinant A1AT preparation that did not have anti-elastase activity to demonstrate the efficacy of A1AT to inhibit CS-induced mouse emphysema [224]. This latter finding would seem to provide convincing evidence supporting significant non-elastase inhibiting anti-inflammatory activities of native human plasma and recombinant forms of A1AT in CS inductions of inflammation and emphysema in mouse models.

Although CS delivery to mice is among the most widely used method for modeling COPD/emphysema and for the ameliorating effects of A1AT [342], its limitations merit mentioning. It cannot recapitulate features of progressive advanced emphysema as observed in human past smokers. The CS induced emphysema changes in mice are mild and do not progress after cessation of CS exposure [328]. Thus they do not model the quantitative trajectories of COPD disease progression, a hallmark of past human smokers with COPD. This seriously limits the translational value of the “gold standard” CS mouse model of COPD as recombinant forms of A1AT and other biosimilar products are not being designed to administer to active smokers.

It should additionally be recognized that the rodent CS exposures themselves require near fumigating levels of inhaled particulate and reactive gaseous CS constituents to deliver these most toxic constituents past the very effective filtering capabilities of the rodent upper respiratory tract (nose). Thus, unphysiologically high levels of less reactive and less soluble gases (e.g., including CO) are delivered to lung parenchyma, resulting in toxic levels of HbCO (20–30% range). The deliveries of toxic CS constituents to rodent lung tissues are quantitatively unlike the mixture of CS toxins delivered to distal lung parenchyma of smoking humans. Finally, the mice are relatively CO intoxicated during and after their CS exposures if they are being used to generate emphysema.

An ideal model for relevant head-to-head comparisons of effectiveness of human plasma A1AT vs. recombinant A1AT, including plant produced recombinant preparations, would be to use a knockout model of severe A1AT deficiency that spontaneously develops emphysema. Such a model has been engineered [343] and has been shown to be adaptable for the testing of both elastase inhibitors and short term A1AT

replacement therapy investigations [123]. This mouse closely recapitulates the clinical characteristics of human A1ATD and is accelerated by CS exposure. These mice received one dose of an optimized AAV vector encoding human A1AT protein, ameliorating both its development of spontaneous emphysematous changes and its CS-induced emphysema [344]. However, challenges may arise in using this knockout mouse model for the testing of repeated doses of recombinant or plasma-derived human A1AT products until the model has been desensitized to human A1AT due to rapid production of antibodies inactivating the antiprotease function of the infused recombinant human A1AT.

Intratracheal neutrophilic elastase injections have often replaced CS as a technically simpler and more efficient method to induce experimental emphysema and to evaluate amelioration by potentially therapeutic agents in COPD/emphysema models [126,321,345–349]. Another common acute lung injury experimental model that has been used is LPS [350], another form of acute lung injury shown to be ameliorated by human plasma A1AT [347]. This model has also demonstrated that both human plasma-derived A1AT, as well as a recombinant form of A1AT without anti-elastase activity, ameliorated LPS induced neutrophilic-dominated lung inflammatory responses in both wild type and elastase deficient mouse strains [24].

These models are relevant to human COPD/emphysema as unlike the mild disease induced by CS in mouse models, human COPD/emphysema in past smokers is characterized by persistent respiratory tract inflammation, disease progression, and acute episodes of exacerbation [351, 352], not unlike the neutrophilic inflammatory changes seen in A1ATD [15]. It is useful to consider such tracheal instillations of elastase, LPS or TNF in post CS-induced rodent models of emphysema in order to more rigorously model evaluations of human therapeutics for inflammation-related COPD/emphysema progressions and exacerbations [297,317,321,350,353,354]; or in simplified elastase models followed by LPS models [355].

It is recognized that non-human primates have been developed as a model for COPD/emphysema [356]. Although they would be useful for short term modeling recombinant A1AT pharmacokinetics and lung uptake, their use in longer term studies would seem to present both moral and economic limitations. Finally, a recognized challenge is to select which spectrum of studies will be most “clinically relevant” to satisfy basic regulatory hurdles for an IND, to guide clinical trial strategies, and to attract commercial funding.

From the developmental perspective, the pre-clinical data package would include standard tests for A1AT protein yield and enzymatic activity, stability, manufacture reproducibility and aforementioned biological testing with both acceptable state-of-the-art testing methodologies and prudent use of most relevant strategies concerning comparative quantitative determinations of bioequivalences to the approved commercial human plasma A1AT products. The success of this stage of development is not only critical to gain FDA approval for an IND status but is also critical to gain funding sources for the next stage of development.

12. Regulatory issues and clinical trials

As is the case for FDA IND approvals, the regulatory landscape governing FDA licensing of New Drug Approval (NDA) of A1ATD products, including recombinant A1AT formulations, is not clearly defined. The original commercial human purified plasma A1AT product was primarily approved by the FDA in 1987 based on its biochemical efficacy based on similarity to plasma A1AT [59]. Presumably, recombinant forms would, at least in part, require both basic science and *in vivo* data demonstrating a relevant degree of bioequivalence with the existing commercially available purified human plasma products.

Over the next several decades, although impressive increased understandings of the molecular basis of the disease occurred and the safety and antiprotease efficacies of other commercial human plasma

[357] and recombinant [358] A1AT preparations were characterized, it continues to be challenging to document efficacy in clinical trials and to obtain approval of A1ATD therapeutic products, other than for other purified plasma derived biosimilar human A1AT products. A summary of select preclinical and clinical trial activities in the A1ATD landscape are shown in Table 3.

The challenges and expenses of carrying out conventional clinical trials documenting clinical efficacies in A1ATD have been well recognized [35,72,359–362]. Until recently, in spite of the fact that numerous potential therapies, including those related to recombinant A1AT products, have been proposed in the last three decades, regulatory successes have been essentially nil [358,359,363]. Conventional FDA therapeutic approvals have been based on randomized, rigorously controlled clinical trials confirming clinical efficacy based on outcomes related to objective disease modifying measurable parameters. Studies of the last 5 years have opened up the landscape of newer appreciated endpoints recognized by investigators seeking regulatory approvals of novel A1ATD therapies including recombinant A1AT preparations [60, 151,364].

The RAPID program using CT chest densitometry represents the most convincing randomly controlled trial demonstrating the clinical efficacy of A1AT replacement therapy [365,366]. This seminal 2–4 year clinical study solidified earlier concepts that lung density measurements represent not only the most non-invasive direct clinical measure of emphysema and its progression but also reflects the most sensitive anatomical or functional parameter to demonstrate a significant change documenting the efficacy of A1AT replacement therapy over a 2 year period as compared to other symptomatic and functional objective parameters [365,367,368]. Of important relevance, lung density measurements have even been correlated with survival in A1ATD patients [4]. This compelling newer evidence of A1AT replacement therapy would seem to solidify lung density measurements as a clinically valid primary endpoint in which to evaluate new A1AT therapeutic products.

The RAPID studies additionally confirmed the value of elastin degradation products as a viable clinical trial endpoint [369–371]. In an elegant experimental study the intrapulmonary delivery of a volatile reporter of neutrophilic elastase activity released and expelled in breath has been shown to reflect lung elastase activity following induced mouse lung inflammation, thus reflecting a rapid breath readout for changes in lung elastase activity following the administration of small molecule elastase inhibitors or A1AT [123]. The hope is that eventual credibility of biomarkers to reflect accelerated inflammation and protease-induced emphysema progression beyond that expected from normal aging could potentially bypass requirements for 2 year long clinical trials with lung

density measurements to meet regulatory licensing approvals. Such validation of biomarkers to replace years long clinical validating trials would presumably significantly reduce development costs, and accelerate regulatory approvals.

The availability of effective approved therapies for A1ATD adds complexities to the design of other stand alone novel agents including recombinant A1ATs because most trials require a wash-in phase where existing effective therapies have to be discontinued for a period of time before initiation of a “biosimilar” therapy. The published report that even relatively short-term withdrawal of an approved replacement therapy (e.g. 2–3 months) may have health consequences confounds strategies for new clinical trials and excludes performing a placebo trial on ethical grounds [65]. It is likely that clinical trials of recombinant A1AT replacement products would be designed utilizing an active control (licensed human plasma commercial product) and using a non-inferiority or superiority clinical trial.

Other parameters that are well recognized to reflect progression of COPD/emphysema have included standard pulmonary functional tests of forced expiratory volume in 1 s and diffusion capacity measurements. While such parameters are necessary to measure in lung-related clinical trials, issues of test variability and sensitivity necessitate several years to generate significant effects on these pulmonary functional parameters as shown by the SELECT and RAPID trials which failed to show significant effects of replacement therapy over a 1–2 year span [191,365,372]. Another issue using pulmonary function test endpoints is that regulatory authorities often challenge the clinical meaningfulness of small but statistically significant changes (especially in non-bronchodilator settings).

As discussed in two recent FDA-related workshops held with representatives of NIH and the A1AT scientific patient and industry communities on 09-16-2019 and 03-23-2020, many surrogate outcome biomarkers have been interrogated as possible endpoints reflecting either mechanisms or markers of accelerated progression of COPD/emphysema beyond that of aging itself and which could be used by industry and the FDA as regulatory validations of drug efficacy. Not surprisingly, since COPD/emphysema is well recognized as an inflammatory disease [351], many biomarkers of inflammation and neutrophil activation in plasma or alveolar fluid compartments have been proposed [35,61,367,373–377]. These biomarkers have included fibrinogen, CRP, sRAGE, SP-D, CC-16, endostatin, circulating polymers, complement, and other imprints of inflammatory processes in plasma and alveolar wash fluids [15,35,61,376,378]. It would seem likely that future biomarker profiles would include composite multivariate dataset endpoints of inflammatory parameters. It is also likely that developing proteomic, metabolomic, and genetic profiling studies will be able to discriminate between different COPD/emphysema risk and progression profiles.

Although many of these biomarkers have been useful to predict disease progression and even shown to be associated with lung density, they have yet to be developed to a degree of serving as a surrogate for density loss progression on CT [35]. They could however, along with serial lung density and pulmonary function measurements, offer the possibility of identifying A1ATD populations most likely to progress more rapidly [290,379]. It is known that not all A1ATD subjects have the same risk of developing COPD/emphysema. Identifying those at greatest risk would be useful in clinical design strategies that could be implemented for all phases of new trials for lung disease therapeutics, thus allowing for increased flexibility, decreased sample size, and shorter time-frames to demonstrate positive outcomes.

Other endpoints recently used for assessing efficacy of COPD/emphysema such as quality of life improvement, questionnaires, and exacerbation rates, are useful secondary endpoints, and have the advantage of assessing disease impact from the perception of the patient (a significant FDA consideration). Respiratory questionnaire use as an appropriate endpoint for clinical trials in COPD/emphysema has been challenging [380]. Exacerbation endpoints have been successfully

Table 3

Novel Therapeutic Approaches to Alpha-1 Antitrypsin Deficiency and Active Studies (November 2020). [Clinicaltrials.gov](https://clinicaltrials.gov) identifiers and references for active studies: 1, NCT03815396 2 [258], 3, NCT04204252 4, NCT03636347 5 [128], 6, NCT03945292 7, NCT04167345, NCT04474197, NCT04443192 8, NCT02168686 9 [157], 10 [170,171] 11, [172].

Target	Approach	Active Studies
Lung	CHO cell recombinant A1AT synthesis	INBRX-101 ¹
Lung	Plant-based recombinant A1AT synthesis	Pre-clinical ²
Lung	Inhaled A1AT delivery	InnovAAte ³
Lung	Small molecule elastase inhibitors	Astraeus ⁴
Lung	Inhibition of the dipeptidyl peptidase	Pre-clinical ⁵
Liver	Missense RNA	SEQOIA study ⁶
Liver	Small molecule Z protein modification	VERTEX 814/864, Z-Factor ZF8747 ⁷
Liver	rAAV gene transfer vectors	ADVANCE study ⁸
Liver	CRISPR/Cas-9	Pre-clinical ⁹
Liver	AAT mRNA	Pre-clinical ¹⁰
Liver	Cell based gene therapy	Pre-clinical ¹¹

utilized in some COPD/emphysema [381–383] and bronchiectasis [75] trials but can be confounded by such issues as definitions of subjective endpoints, variabilities of inciting causes, and heterogenous confounding illnesses.

Although the only approved A1ATD therapies are certified by the IV route, alternative routes have been considered and even undergone clinical trials, particularly via aerosol delivery [184,384,385]. Other delivery routes have included subcutaneous and even transepidermal injections [386]. Clinical trial endpoint strategies will have to take into consideration details related to route of administration issues. It is worth noting that after 30 years of experimental trials, aerosol forms of replacement A1AT have undergone successful early clinical trials but are yet to be fully approved by the FDA as of late 2020 [184,384,385,387,388].

As is the case for new therapeutics for pulmonary fibrosis [389], the availability of a well-recognized effective approved therapy for A1ATD adds to the complexities in the design of clinical studies to test recombinant A1AT and other novel therapies for treatment. Both the A1ATD therapeutic development community and the regulatory network, as emphasized in the recent workshops, are likely to have to consider novel goals and requirements to develop the most efficient strategy designs to move A1ATD recombinants and other newer therapies forward to the clinic.

Currently, only a single plant-made biopharmaceutical product is available for therapeutic use (Elelyso® for Gaucher's disease [390,391]). Guidelines for approval and for large-scale biomanufacturing of plant-made products would likely follow similar requirements that govern current Good Manufacturing Practices (cGMP). A significant difficulty in this regard is that there is no clear direct path to approval for a plant-made biopharmaceutical product, although models similar to those used by ZMapp during its emergency clinical trial in 2014–2015 may serve as a guideline [285]. The success of NDA applications will be facilitated by close interactions with the FDA at all stages of trial design and management.

13. Conclusions and perspectives

Over two decades of research have increased our understanding of the many technical aspects of recombinant protein synthesis in plants and have provided a strong basis for continuing their therapeutic development. In the case of A1AT, more basic scientific studies are needed to provide for a solid evidence-based platform for scientific advancements to a larger intermediate production format stage.

Aggressive incorporation of state-of-the-art strategies will be needed to in order to approximate the presently greater yields of functional A1AT produced by CHO cell technologies. Technological advances such as further optimization of recombinant plant-based protein production and purification processes are needed so as to obtain larger quantities of appropriately glycosylated and active A1AT protein. In this regard, it will be important to further optimize antiprotease and antioxidant cocktails for use during transient production and leaf A1AT extractions. Reproducible batch-oriented recombinant plant-derived A1AT production strategies will need to be optimized. In addition to upstream refinements in plant production strategies, it will be important to achieve a better understanding of the roles of post-translational modifications, including the influence of glycans on the antiprotease functions of A1AT as well as its non-enzymatic functions that appear to be unrelated to its well characterized anti-elastase activities. Finally, overall economic cost analysis will be important if plant production of A1AT is to compete favorably with the emerging advances in CHO cell-based production of human A1AT [392].

Further challenges include the need to complete an array of pre-clinical studies showing bioequivalence and/or superiority of plant recombinant A1AT product compared to the existing FDA approved plasma A1AT preparations from both physical and biological perspectives. Although preparations of oxidant-resistant plant-derived

recombinant A1AT have been shown to be as effective at inhibiting elastase activity as the unmodified form [84], there remain such challenges as characterization of *in vivo* pharmacokinetics, including penetration into pulmonary tissues, immunological considerations and assessment of the numerous non-elastase inhibiting functions of plant produced A1AT. Notwithstanding these challenges, a number of specific pharmacotherapeutic successes of plant-derived recombinant human proteins [285] including the relative tolerance of humans to proteins displaying plant glycans [265] now provide encouraging support for a continuing and aggressive pursuit of further basic scientific developments focused on the plant-based production of A1AT, as well as many other promising protein candidates for use as human therapeutics.

Author contributions

All the authors discussed and contributed to the writing of the paper.

Declaration of competing interest

All authors are working to develop plant produced human alpha-antitrypsin for therapeutic application. DS is employed by BioMarin Pharmaceuticals Inc, a company focused on developing therapeutics for orphan diseases. KM is a cofounder and Chief Scientific Advisor for Insergen, Inc., a plant biotechnology company focused on biologics for orphan diseases, including A1AT. HP is a cofounder, CEO and CSO of Novici Biotech LLC, a company focused on protein engineering, plant host improvement, and plant-based manufacturing.

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