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(54) Title: RECOMBINANT HUMAN ALPHA1-ANTITRYPSIN

(57) Abstract: The present invention relates recombinant human α 1-antitrypsin (rhAAT) comprising N-linked glycans, wherein at least 10% of said N-linked glycans are tetra-antennary glycans; and the degree of capping with sialic acid on said N-linked glycans (Z/A) is at least 50%. The invention further relates to rhAAT for use as a medicament, in particular for use in the prevention and/or treatment of a disease associated with AAT deficiency, and/or a disease involving neutrophil-mediated tissue damage.



RECOMBINANT HUMAN ALPHA1-ANTITRYPSIN

[0001] The invention relates to the field of pharmaceutical products, in particular to recombinant human α 1-antitrypsin, that can be used for *inter alia* the prevention and/or treatment of α 1-antitrypsin deficiency related emphysema.

BACKGROUND OF THE INVENTION

[0002] α 1-Antitrypsin (AAT) or α 1-protease inhibitor (α 1PI) is a natural inhibitor of proteases released by activated neutrophils. AAT is a glycoprotein consisting of 394 amino acids and having a molecular weight of 52 kD. Human AAT is expressed as a 418 amino acid precursor from which a 24 amino acid precursor is clipped to yield the 394 amino acid final product. AAT is synthesized primarily in the liver, but expression has also been demonstrated in neutrophils, monocytes and macrophages.

[0003] Low or zero plasma levels of AAT constitute a risk factor for the development of emphysema due to the unopposed and destructive action of neutrophil proteases in the lungs. For the prevention and/or treatment of AAT deficiency-related emphysema AAT augmentation therapy has been developed (Mulgrew *et al.*, 2007).

[0004] Currently, patients with AAT-deficiency-associated emphysema are treated with a high dose of plasma-derived (pd)AAT (60mg/kg/week; Prolastin[®] from Talecris, Aralast[®] from Baxter and Zemaira[®] from ZLB). Large quantities and frequent injections of AAT are required to relieve AAT-deficiency related emphysema. Major concern with the plasma-derived material is the safety of the preparations. As with all human-derived material, potential risk of pdAAT is contamination with prions or other adventitious agents. A second concern is the limited availability of the plasma material.

[0005] Human plasma derived AAT contains three N-linked glycans at Asn residues 46, 83 and 247. These glycans consist of mostly di- and tri-antennary structures. A high degree of sialylation of the glycans on recombinant proteins is of importance for optimal pharmacokinetics (PK).

[0006] Until now recombinant production of AAT has been hampered by low expression in most recombinant platforms (Karnaukhova *et al.*, 2006). Recombinant AAT

has been produced at high level in transgenic systems, but clinical development thereof has been stopped because of suboptimal pharmacokinetics (PK) and safety issues due to non-human contaminants (Spencer *et al.*, 2005).

[0007] There thus remains a need for recombinant human AAT (rhAAT) that is
5 active, can be obtained in sufficient quantities, and that has useful pharmacokinetic properties.

BRIEF SUMMARY OF THE INVENTION

10 [0008] According to the present invention, it has been shown that recombinant human α_1 -antitrypsin (rhAAT) having a suitable pharmacokinetic profile can be produced in large quantities in PER.C6 cells.

[0009] In the research that led to the invention, cell lines expressing human AAT and co-expressing an α -2,3-sialyltransferase (ST3) or an α -2,6-sialyltransferase (ST6)
15 were generated under serum-free conditions. The cell lines were capable of expressing high levels of rhAAT. Thus, yields of up to 22 picogram rhAAT per cell per day (pcd) were reached. Importantly, the rhAAT of the invention was shown to be active as a neutrophil elastase inhibitor. Analysis of the glycan profiles showed a good overall degree of capping with sialic acid. Interestingly, an increase in tetra-antennary glycans
20 was noted on the rhAAT of the invention, as compared to plasma-derived AAT (Prolastin[®]). Furthermore, rhAAT produced in cells co-expressing ST3 showed an increased mean residence time (MRT) in rats, as compared to plasma derived AAT. RhAAT produced in cells co-expressing ST6 showed a decreased mean residence time as compared to plasma-derived AAT.

25 [0010] In one aspect, the present invention therefore provides recombinant human α_1 -antitrypsin (rhAAT) comprising N-linked glycans, wherein:

(a) at least 10% of said N-linked glycans are tetra-antennary glycans;
and

(b) the degree of capping with sialic acid on said N-linked glycans
30 (Z/A) is at least 50%.

[0011] In another embodiment, at least 20% of said N-linked glycans are tetra-antennary glycans.

[0012] In a further embodiment, the degree of capping with sialic acid on said N-linked glycans that are tetra-antennary is at least 30%.

5 [0013] In a further embodiment at least 50% of the total sialylation on said N-linked glycans is α -2,3-sialylation. Preferably, at least 90% of the total sialylation on said N-linked glycans is α -2,3-sialylation.

[0014] In a further aspect, the invention provides preparations and pharmaceutical compositions comprising said rhAAT, and optionally one or more pharmaceutically
10 acceptable excipients.

[0015] In another aspect, the invention relates to the use of said rhAAT, preparations and/or pharmaceutical compositions, comprising said rhAAT, for the prevention and/or treatment of *inter alia* AAT-associated emphysema and/or inflammatory diseases with neutrophil-mediated tissue damage.

15 [0016] In a further embodiment, the invention provides a method for producing recombinant human α 1-antitrypsin, comprising the steps of providing a PER.C6 cell with a nucleic acid encoding human α 1-antitrypsin in such a way that said PER.C6 cell harbours said nucleic acid in an expressible form; and culturing said PER.C6 cell under conditions conducive to the production of said recombinant human AAT, wherein said
20 PER.C6 cell is modified to co-express α 2,3 sialyltransferase or α 2,6-sialyltransferase.

BRIEF DESCRIPTION OF THE FIGURES

[0017] FIG. 1. Frequency distribution of the specific productivities of the batch
25 cultures, both for cell lines overexpressing α 2,3 sialyltransferase (A: PER.C6-AAT-ST3) and cell overexpressing α 2,6-sialyltransferase (B: PER.C6-AAT-ST6).

[0018] FIG. 2. 4-12% BIS-Tris SDS-PAGE gel with cell culture harvests of PER.C6-rAAT (non-purified) samples, stained with colloidal blue.

[0019] FIG. 3. Schematic overview of the assay to determine the degree of
30 sialylation of the glycans on AAT by Z/A ratio.

[0020] FIG. 4. Specific productivities of the PER.C6 cell lines expressing AAT are not correlated with the degree of sialylation of the glycans of PER.C6-rAAT (Spearman's $r=-0.024$, $p=0.893$). ($\diamond$: PER.C6-AAT-ST3; $\circ$: PER.C6-AAT-ST6).

[0021] FIG. 5. Sialylation (Fig. 5A) and antennarity (Fig. 5B) of the glycans of PER.C6-rAAT from ECL negative samples in comparison with the glycans of PER.C6-rAAT from ECL positive FIG samples and Prolastin[®].

[0022] FIG. 6. Degree of sialylation on all glycans (Fig. 6A) and on the tetra-antennary glycans (Fig. 6B) of PER.C6-rAAT-ST3 in comparison with the glycans of PER.C6-rAAT-ST6 and Prolastin[®].

[0023] FIG. 7. Plasma concentration-time profiles for various doses of Prolastin[®] in buffer (PBS/T) or in PER.C6-CM following i.v. bolus administration to rats.

■: Prolastin[®] in PER.C6-CM, 50 $\mu\text{g/kg}$; ▲: Prolastin[®] in PER.C6-CM, 100 $\mu\text{g/kg}$;

●: Prolastin[®] in PER.C6-CM, 200 $\mu\text{g/kg}$; ○: Prolastin[®] in PBS/T, 2000 $\mu\text{g/kg}$.

[0024] FIG. 8. Plasma concentration-time profiles for PER.C6-rAAT-ST3, PER.C6-rAAT-ST6 and Prolastin[®]. ▲: PER.C6-rAAT-ST3, MRT ~18-23 hr; ■: PER.C6-rAAT-ST6, MRT ~4-4 hr, ●: Prolastin, MRT ~11-12 hr.

[0025] FIG. 9. Plasmid maps of the AAT expression vectors pAATopt-ST3 (A) and pAATopt-ST6 (B). CMV=Cytomegalovirus promoter, BGHp(A)= Bovine Growth Hormone polyadenylation sequence, fl ori= fl origin of replication, SV40=Simian Virus 40 promoter, Neo=Neomycin resistance marker, SV40p(A)=Simian Virus 40 polyadenylation sequence, AAT= α_1 -antitrypsin, ColE1=ColE1 origin of replication, Amp=ampicillin resistance marker, SIAT4C= gene coding for ST3=human sialyltransferase IV (L23767) (Fig. 9A), SIAT1= gene coding for ST6=human sialyltransferase I (NM_003032).

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DETAILED DESCRIPTION OF THE INVENTION

[0026] Alpha₁-antitrypsin (AAT) is a natural inhibitor of proteases released by activated neutrophils. AAT is secreted into the blood plasma but its primarily site of action is in the lung parenchyma. Human leukocyte elastase (HLE), (also known as

human neutrophil elastase (HNE) is a serine protease released from azurophilic granules of the neutrophils as part of the normal inflammatory response. Under normal homeostatic conditions AAT serves as an important regulator of proteolysis by human leukocyte elastase (HLE), thereby preventing damage of the lung alveolar matrix. Besides
5 HLE, AAT also inhibits two other proteases released into the lungs by neutrophils, namely cathepsin G (catG) and protease 3 (Pr3).

[0027] Plasma-derived AAT products have been developed and are currently being used for the treatment of patients suffering from AAT-deficiency (i.e. having low or zero plasma AAT levels) related emphysema, such as Prolastin®, (Talecris), Aralast®
10 (Baxter) and Zemaira® (ZLB). High amounts (approximately 60 mg/kg/ week) of this product are generally needed per patient. Besides emphysema, AAT is also a (potential) therapy for inflammatory diseases with neutrophil-mediated damage

[0028] Recombinant production of AAT in for example *E.coli* has been hampered by low expression in most recombinant platforms (Karnaukhova *et al.*, 2006).
15 Recombinant AAT has been produced at high level in transgenic systems, but clinical development thereof has been stopped because of suboptimal pharmacokinetics (PK) and safety issues due to non-human contaminants (Spencer *et al.*, 2005).

[0029] The present invention now provides a recombinant human $\alpha 1$ -antitrypsin (rhAAT) having suitable pharmacokinetic properties and that can be produced in large
20 quantities in for example PER.C6 cells. The recombinant AAT of the present invention has been shown to be functionally active, as determined by a chromogenic assay based on inhibition of human neutrophil elastase, and to have promising pharmacokinetic properties, as compared to plasma-derived AAT products, such as Prolastin®.

[0030] The present invention thus provides rhAAT comprising N-linked glycans,
25 wherein

- a) at least 10% of said N-linked glycans are tetra-antennary glycans; and
- b) the degree of capping with sialic acid on said N-linked glycans (Z/A) is at least 50%.

[0031] N-linked glycans are sugar chains that are covalently linked to asparagine
30 residues of a polypeptide (Varki *et al.* 1999). The process of N-glycosylation starts with the attachment of a dolichol oligosaccharide precursor to the asparagines precursor. This

precursor is subsequently modified into a high-mannose, hybrid, or complex-type oligosaccharide. In complex type N-linked sugars, both the α 3- and α 6-linked mannose residues are substituted by N-acetyl-glucosamine (GlcNAc) residues. Complex type N-glycans may contain two to five GlcNAc-bearing branches that are referred to as antennae. The ultimate structure of complex type N-linked sugars may vary extensively and depends on the protein to which they are attached, on the host cell and on the conditions under which the host cell is cultured. The GlcNAc-bearing branches may be modified with galactose (Gal) or N-acetyl-galactosamine (GalNAc) forming so-called LacNAc or LacdiNAc structures. Also, GlcNAc-bearing branches may contain multiple LacNAc structures forming so-called polylactosamine structures. Terminal galactoses may be modified with an α 2,3- or an α 2,6-linked sialic acid whereas terminal N-acetyl-galactosamines may only be modified with an α 2,6-linked sialic acid. The addition of sialic acids to terminal Gal or GalNAc is mediated by sialyltransferases. Probably more than 20 different sialyltransferases are encoded by the human genome (Harduin-Lepers et al., 2001). They differ in substrate specificity, tissue distribution and various biochemical parameters.

[0032] Native AAT has three glycosylation sites. Thus, AAT comprises three N-linked glycans in which branching can occur. These glycans may have 2,3 or 4 so-called “antennae” or branches. Surprisingly, it has been shown that at least 10%, preferably from about 10% to about 50%, of said N-linked glycans of the rhAAT of the invention are tetra-antennary glycans. In another embodiment at least 20 % of said N-linked glycans are tetra-antennary glycans, preferably from about 20 to 40% of said N-linked glycans are tetra-antennary glycans. According to the present invention, it has surprisingly been shown that the glycosylation profile, for example in terms of antennarity, as well as the pharmacokinetic properties, e.g. the mean residence time of the rhAAT of the invention differ from plasma-derived AAT, such as Prolastin®.

[0033] Since full sialylation of the glycans on recombinant proteins is of importance for optimal pharmacokinetic properties, the rhAAT of the present invention is expressed in PER.C6 cells in combination with a sialyltransferase to achieve highly sialylated isoforms of rhAAT. PER.C6 cells thus were co-transfected with either an α 2,3 sialyltransferase (PER.C6-ST3) or an α 2,6 sialyltransferase (PER.C6-ST6). In a

preferred embodiment of the invention at least 50% of the total sialylation on said N-linked glycans is α -2,3-sialylation. For example, at least 50, 70 or 80% of the total sialylation on said N-linked glycans is α -2,3-sialylation. In yet another embodiment at least 90% of the total sialylation on said N-linked glycans is α -2,3-sialylation. It has surprisingly been shown that in these embodiments the rhAAT of the present invention has different pharmacokinetic properties, as compared to plasma-derived AAT, such as Prolastin®, in particular a prolonged mean residence time. By sialylation is meant the amount of sialic residues present on the AAT carbohydrate structures: α -2,3-sialylation means sialylation at the 2,3 position, i.e. an α 2,3- linked sialic acid (as is well known in the art) and α -2,6-sialylation means sialylation at the 2,6 position, i.e. an or an α 2,6-linked sialic acid (also known in the art). With “at least 50% of the total sialylation on said N-linked glycans is α -2,3-sialylation”, it is thus meant that at least 50% of the total number of sialic acid residues present in the rhAAT is sialylated in the 2,3 position.

[0034] According to the invention it has been shown in PK studies in rat that the mean residence time (MRT) of both rhAAT produced by PER.C6-ST3 (rhAAT-ST3) and rhAAT produced by PER.C6-ST6 (rhAAT-ST6) differs significantly from that of plasma-derived AAT (Prolastin®). It has thus been shown that the MRT in rats of rhAAT-ST3 ranges from approximately 18-23 hours, whereas the MRT in rats of Prolastin® is approximately 11-12 hours. Using rhAAT having a prolonged MRT may for example reduce the dosage needed per patient. In contrast, the MRT in rats of rhAAT-ST6 is reduced as compared to that of Prolastin® (approximately 3-4 hours). The use of rhAAT-ST6 of the present invention may be advantageous due to the “more natural” 2,6-linkage of the sialic acid, which corresponds to the sialic acid linkage on plasma-derived AAT, such as Prolastin®. The use of rhAAT-ST6 may furthermore be advantageous in cases where a reduced MRT may be beneficial.

[0035] As stated above, the degree of sialylation of the glycans on recombinant proteins is of importance for optimal pharmacokinetic properties. In a preferred embodiment, the overall degree of capping with sialic acid on said N-linked glycans (as calculated by the Z/A ratio) of the rhAAT of the present invention therefore is at least 70%. More preferably, the degree of capping with sialic acid on said N-linked glycans

(Z/A) is at least 80%, even more preferably the degree of capping with sialic acid on said N-linked glycans (Z/A) is at least 90%.

[0036] In a further embodiment, the degree of capping with sialic acid on said N-linked glycans that are tetra-antennary is at least 20%, for example between 20% and 5 65%. A relatively low degree of sialylation on said tetra-antennary N-glycans may yield a rhAAT product having a reduced MRT, as compared to plasma derived AAT. In another embodiment, the degree of capping with sialic acid on said tetra-antennary N-linked glycans is at least 50%, for example between 50% and 97%, preferably at least 60%, more preferably between 70 and 95%. The higher level of sialylation in this embodiment 10 may contribute to the prolonged MRT, as compared to plasma derived AAT.

[0037] The invention furthermore provides preparations and pharmaceutical compositions, comprising said rhAAT and optionally one or more pharmaceutically acceptable excipients. By “pharmaceutically acceptable excipient” is meant any inert substance that is combined with an active molecule (such as a drug, agent, or protein) for 15 preparing a suitable or convenient dosage form. The “pharmaceutically acceptable excipient” is an excipient that is non-toxic to recipients at the dosages and concentrations employed and is compatible with other ingredients of the formulation comprising the active molecule.

[0038] The pharmaceutical compositions of the invention can be formulated into 20 various compositions for any route of administrations, well-known to the skilled person. The choice of the optimal route of administration of the pharmaceutical compositions will be influenced by several factors, including e.g. the physico-chemical properties of the active molecules within the compositions, the urgency of the clinical situation and the relationship of the plasma concentrations of the active molecules to the desired 25 therapeutic effect. The preparations and pharmaceutical compositions of the present invention are preferably formulated for intravenous administration or aerosol administration. Pharmaceutically suitable formulations of rhAAT can be prepared according to methods known to the person skilled in the art (see *Remington's Pharmaceutical Sciences*, 18th edition, A.R. Gennaro, Ed., Mack Publishing Company 30 (1990); *Pharmaceutical Formulation Development of Peptides and Proteins*, S. Frokjaer

and L. Hovgaard, Eds., Taylor & Francis (2000); and *Handbook of Pharmaceutical Excipients*, 3rd edition, A. Kibbe, Ed., Pharmaceutical Press (2000)).

[0039] Typically, pharmaceutical compositions must be sterile and stable under the conditions of manufacture and storage. The preparations and/or pharmaceutical compositions comprising the rhAAT of the invention can be in powder form for reconstitution in the appropriate pharmaceutically acceptable excipient before or at the time of delivery. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying (lyophilization) that yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

[0040] Alternatively, the rhAAT of the present invention can be in solution and the appropriate pharmaceutically acceptable excipient can be added and/or mixed before or at the time of delivery to provide a unit dosage injectable form.

[0041] The rhAAT, the preparations or pharmaceutical compositions, comprising said rhAAT, can be used as medicaments. The rhAAT, the preparations and pharmaceutical compositions can be suitably used in the prevention and/or treatment of diseases and/or disorders for which the administration of AAT has been proven beneficial. They can *inter alia* be used in the prevention and/or treatment, or combination thereof, of AAT-deficiency-associated emphysema. The rhAAT, preparations or pharmaceutical compositions of the present invention can further be used in the prevention and/or treatment of smoking-related emphysema, cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD). Since AAT has been described to have anti-inflammatory and antiapoptotic effects, the rhAAT, preparations or pharmaceutical compositions of the present invention may also be used as anti-inflammatory agents. (Lewis et al., 2008; Koulmanda et al., 2008)

[0042] In a further embodiment, the invention provides a method for producing recombinant human $\alpha 1$ -antitrypsin, comprising the steps of providing a PER.C6 cell with a nucleic acid encoding human $\alpha 1$ -antitrypsin in such a way that said PER.C6 cell harbors said nucleic acid in an expressible form; and culturing said PER.C6 cell under conditions conducive to the production of said recombinant human $\alpha 1$ -antitrypsin, wherein said cell further contains a nucleic acid sequence encoding a sialyltransferase,

preferably an alpha-2,6-sialyltransferase or an alpha-2,3-sialyltransferase, under control of a heterologous promoter. In an embodiment, the sialyltransferase is a human sialyltransferase. The rhAAT of the present invention may, however, also be produced in other mammalian cells, optionally co-expressing a sialyltransferase, such as for example
5 293 cells.

[0043] In an embodiment, the invention provides a method for producing recombinant human α 1-antitrypsin, comprising the steps of providing a PER.C6 cell with a nucleic acid encoding human α 1-antitrypsin in such a way that said PER.C6 cell harbors said nucleic acid in an expressible format, wherein said cell further contains a
10 nucleic acid sequence encoding a sialyltransferase, preferably an alpha-2,6-sialyltransferase or an alpha-2,3-sialyltransferase, under control of a heterologous promoter; culturing said PER.C6 cell in a serum-free medium and allowing expression of said human α 1-antitrypsin in said cell; harvesting the expressed human α 1-antitrypsin from said cell and/or from said culture medium; and optionally purifying said human α 1-
15 antitrypsin.

[0044] The use of PER.C6 cells as a production platform for proteins of interest has been described in WO 00/63403 the disclosure of which is incorporated herein by reference. As shown in WO 00/63403, PER.C6 can be suitably used for the production of recombinant proteins. In order to achieve large-scale (continuous) production of
20 recombinant proteins through cell culture, it is preferred to have cells capable of growing without the necessity of anchorage. The cells of the present invention have that capability. A PER.C6 cell according to this invention is a cell from an upstream or downstream passage or a descendent of an upstream or downstream passage of cells as deposited at the Center for Applied Microbiology and Research & European Collection
25 of Cell Cultures (ECACC) on 29 February 2006 under ECACC no. 96022940 (see, e.g., U.S. Patent 5,994,128). The use of PER.C6 cells for industrial processes has been extensively described, e.g. in Nichols et al, 2002, and more in particular for recombinant protein production, e.g. in Yallop et al, 2005a and 2005b.

[0045] The cells according to the invention, in particular PER.C6 cells, have the
30 additional advantage that they can be cultured in the absence of animal- or human-derived serum or animal- or human-derived serum components. Thus isolation is easier,

while the safety is enhanced due to the absence of additional human or animal proteins in the culture, and the system is very reliable (synthetic media are the best in reproducibility). Furthermore, the presence of the Early region 1A ("E1A") of adenovirus adds another level of advantages as compared to (human) cell lines that lack this particular gene. E1A as a transcriptional activator is known to enhance transcription from the enhancer/promoter of the CMV Immediate Early genes (Olive et al., 1990, Gorman et al., 1989). When the recombinant protein to be produced is under the control of the CMV enhancer/promoter, expression levels increase in the cells and not in cells that lack E1A.

[0046] In general, the production of a recombinant protein, such as rhAAT, in a host cell, such as a PER.C6 cell, comprises the introduction of nucleic acid in expressible format into the host cell, culturing the cells under conditions conducive to expression of the nucleic acid and allowing expression of the said nucleic acid in said cells. Alternatively, a protein that is naturally expressed in desired host cells, but not at sufficient levels, may be expressed at increased levels by introducing suitable regulation sequences such as a strong promoter in operable association with the desired gene (see e.g. WO 99/05268, where the endogenous EPO gene is over-expressed by introduction of a strong promoter upstream of the gene in human cells).

[0047] Nucleic acid encoding AAT in expressible format may be in the form of an expression cassette, and usually requires sequences capable of bringing about expression of the nucleic acid, such as enhancer(s), promoter, polyadenylation signal, and the like. Several promoters can be used for expression of recombinant nucleic acid, and these may comprise viral, mammalian, synthetic promoters, and the like. In certain embodiments, a promoter driving the expression of the nucleic acid of interest is the CMV immediate early promoter, for instance comprising nt. -735 to +95 from the CMV immediate early gene enhancer/promoter, as this promoter has been shown to give high expression levels in cells expressing E1A of an adenovirus (see e.g. WO 03/051927). The nucleic acid of interest may be a genomic DNA, a cDNA, synthetic DNA, a combination of these, etc.

[0048] Cell culture media are available from various vendors, and serum-free culture media are nowadays often used for cell culture, because they are more defined than media containing serum. The cells of the present invention grow well in serum-

containing media as well as in serum-free media. The cells of the invention in general grow adherently in serum-containing media, but are very proficient in growing in suspension to high cell densities (10×10^6 cells/ml and higher) in serum-free culture media, which means that they do not need a surface to adhere to, but remain relatively free from each other and from the walls of the culture vessel during most of the time. Processes for culturing the cells of the invention to high densities and/or for obtaining very high product yields from these cells have been described (WO 2004/099396).

[0049] The concept of genetic engineering to alter glycosylation of recombinant proteins produced in a cell has been amply established, and is for instance discussed in detail in e.g. US 2005/0164386. To this purpose, nucleic acid encoding the desired glycosylation enzyme in expressible format is or has been introduced into the cells according to the invention, and the desired glycosylation enzyme is expressed during the culturing of the cells according to the invention when the protein of interest is expressed. This results in an altered glycosylation pattern of the protein of interest as compared to the situation when no recombinant glycosylation enzyme is expressed in the cells. In the present invention, the glycosylation enzyme is a sialyltransferase, more preferred an α -2,3-sialyltransferase and/or an α -2,6-sialyltransferase. Preferably, the encoded sialyltransferase is a mammalian sialyltransferase, more preferably a human sialyltransferase. The nucleic acid encoding the sialyltransferase preferably is under control of a heterologous promoter, which should be active or have the possibility of being regulated in the cells of the invention. Preferably, the nucleic acid encoding the sialyltransferase is integrated into the genome of the cells, to ensure stable inheritance, and provide for stable expression of the sialyltransferase in subsequent generations of the cells.

[0050] The invention furthermore provides recombinant human AAT, obtainable by the methods according to the invention. According to the invention the rhAAT has a human glycosylation pattern different from the isolated natural human counterpart protein. The present invention in particular provided rhAAT comprising N-linked glycans, wherein at least 10% of said N-linked glycans are tetra-antennary glycans. In addition, the rhAAT obtainable according to the present invention has surprising pharmacokinetic properties.

[0051] Recombinant human AAT according to the invention includes full-length human AAT, but also may encompass biologically active polypeptide fragments, such as fragments of human AAT with one or more amino acid deletions at e.g. the N-terminus of the protein, as well as biologically active variants of human AAT, such as AAT with one or more amino acid substitutions (e.g. conservative substitutions), one or more deletions or additions of amino acids, which do not significantly change the functional activity of the protein. In an embodiment, the rhAAT of the present invention comprises an amino acid sequence provided herein as SEQ ID NO: 1. In some embodiments, amino acid sequences of AAT variants are at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% homologous to SEQ ID NO:1.

[0052] The present invention is further illustrated by the following non-limiting examples.

EXAMPLES

EXAMPLE 1

Cell line generation and specific productivities

[0053] Seven hundred PER.C6 cell lines expressing human AAT and co-expressing either an α 2,6-sialyltransferase (SIAT1; NM_003032) or an α 2,3-sialyltransferase (SIAT4C; L23767) (PER.C6-AAT-ST3/ PER.C6-AAT.ST6) were generated under serum-free conditions, as described in WO 2006/070011. Forty-seven serum-free PER.C6 cell lines producing either AAT-ST3 (27 cell lines) or AAT-ST6 (20 cell lines) were selected based on highest yield as determined by ELISA (AAT Elisa Kit, US Biologicals) from independent cell line generation programs using nucleofection as transfection tool. The expression plasmid contained the coding sequence for human AAT (SEQ ID NO:2) and the coding sequence for human ST3GalIV, both driven by a cytomegalovirus promoter that has been modified to achieve high levels of gene expression in PER.C6 cells (Yallop *et al*, 2005). The plasmid maps of the AAT expression vectors (pAATopt-ST3 and pAATopt-ST6) are shown in Figure 9.

[0054] The productivity of the cell lines varied between 8-22 picogram per cell per day (pcd) (PER.C6-AAT-ST3) and 7-19 pcd (PER.C6-AAT-ST6) (see Figure 1 and Tables 1 and 2). The cell lines were cryo-preserved in Proper-1 medium (Lonza) (6 vials, 3-5x10⁶ cells/ml), meeting all acceptance criteria (viability > 80%, good growth two passages post resuscitation). Batch cultures were generated (in Proper-1 medium), which were used for biochemical analysis (see Example 2).

EXAMPLE 2

Biochemical analysis

[0055] Integrity of PER.C6-rAAT

The supernatants of the 47 batch cultures were analyzed on SDS-PAGE stained with colloidal blue. Cell culture harvest of the PER.C6-rAAT (non-purified) samples showed AAT as the main protein band on SDS-PAGE. Furthermore, the gels showed intact AAT bands (shown in Figure 2) indicating the integrity of the material.

[0056] Activity of PER.C6-rAAT

Initially (in lieu of a chromogenic activity assay), an indication for activity was determined by analyzing the formation of a complex of PER.C6-rAAT with human neutrophil elastase. To this end, cell culture harvests of PER.C6-rAAT samples were incubated with 0, 0.1, 0.2 and 0.4 µg elastase for 30 min at 37°C and subsequently applied on a 4-12% BIS-Tris SDS-PAGE and stained with colloidal blue). All PER.C6-rAAT samples tested formed a complex with elastase (data not shown), indicating activity of the preparations. Further activity testing was done with a chromogenic assay based on inhibition of human neutrophil elastase using Prolastin[®] as reference (adapted from Bruin et al., 2005)). All PER.C6-rAAT samples tested, except for one (PER.C6-rAAT-ST3-234c), were at least as active as Prolastin[®] (>90%) (Tables 1 and 2).

[0057] Glycan analysis of PER.C6-rAAT

To screen for PER.C6-rAAT samples of which the glycans have a relative high degree of capping with sialic acid, needed for an optimal PK profile, a lectin assay was

applied, using *Erythrina cristagalli* lectin (ECL) for detection of exposed galactoses (see Figure 3 for a schematic overview of the used assay). From the 47 samples tested 15 showed a signal at OD405 with ECL > blank + 0.2, indicating a relatively high degree of exposure of terminal galactoses on the glycans of these AAT samples. For a few of these samples the low degree of capping of the glycans by sialic acid was confirmed by further glycan analysis using an HPLC-based method (Hermentin et al., 1996; Gervais et al., 2003). Therefore, the rhAAT samples with an ECL signal at OD405 > blank + 0.2 were not further subjected to extended glycan analysis and not tested in PK studies.

[0058] The extended glycan profiling method is schematically shown in Figure 3 and involves the release of the N-linked glycans from AAT with the enzyme N-glycosidase F (PNGaseF), and labelling the glycans with fluorescent 2-anthranilic acid (2-aa). Subsequently, the glycans are separated by anion exchange chromatography (AEX) into groups according to their negative charge, i.e. separation of glycans with different numbers of sialic acid. From this profile a Z-number, representing the degree of charge (i.e. sialylation) of the N-linked glycans, is determined.

The formula for Z:

$$Z = (A_{\text{Asialo}} \times 0) + (A_{\text{Monosialo}} \times 1) + (A_{\text{Disialo}} \times 2) + (A_{\text{Trisialo}} \times 3) + (A_{\text{Tetrasialo}} \times 4) \quad (A = \text{area, Hermentin et al., 1996}).$$

[0059] Furthermore, the desialylated glycans are separated by normal phase chromatography (NP) into groups according to their size, i.e. separation of glycans with different degree of branching (antennarity). From this profile an A-number, representing the degree of branching of the N-linked glycans, is determined.

The formula for A:

$$A = (A_{\text{Diantennary}} \times 2) + (A_{\text{Triantennary}} \times 3) + (A_{\text{Tetra-antennary}} \times 4).$$

[0060] Finally, by calculating the Z/A ratio, the degree (percentage) of capping by sialic acid of the N-linked glycans of a glycoprotein is obtained (Gervais et al., 2003).

[0061] The glycan profiles of PER.C6-rAAT are shown in Tables 1 and 2. The overall profiles of PER.C6-rAAT samples (Tables 1 and 2) show 12-54% di-antennae, 13-39% tri-antennae and 22-48% tetra-antennae and 0-55% di-sialo, 0-30% tri-sialo, 0-29% tetra-sialo. The degree of capping of the glycans by sialic acid of PER.C6-rAAT ranges from 0 to 92%, this degree of capping with sialic acid is not correlated with the specific productivities of the PER.C6-rAAT cell lines (Spearman's $r=-0.024$, $p=0.893$) (Tables 1 and 2 and Figure 4).

[0062] As expected the glycans the ECL positive rAAT contain a lower level of charge (sialylation) (Z-number range: 0-124) versus the ECL negative PER.C6-rAAT (Z-number range: 151-253) (difference is significant as tested by the Mann-Whitney test, $p<0.001$) (see Figure 5). The glycans of ECL-positive PER.C6-rAAT contain a higher level of antennarity (A-number range: 299-322) versus the ECL-negative PER.C6-rAAT (A-number range: 266-307) (difference is significant as tested by the Mann-Whitney test, $p<0.001$) (see Figure 5). Furthermore, there is a lower degree of sialylation on the tetra-antennary glycans of PER.C6-rAAT-ST6 (ECL negative), ranging from 6 to 62% versus PER.C6-rAAT-ST3 (ECL negative), ranging from 35 to 106% (difference is significant as tested by the Mann-Whitney test, $p<0.001$), while this is not the case for the overall degree of sialylation (Tables 1 and 2, Figure 6). The lower preference of ST6 for higher branched glycans (Joziasse *et al.*, 1987) may be the cause of the lower degree of sialylation on the tetra-antennary glycans of PER.C6-rAAT-ST6 versus PER.C6-rAAT-ST3.

[0063] The glycan profiles indicate a tendency for a higher degree of branching on the glycans of PER.C6-rAAT in comparison to plasma derived AAT (Prolastin[®]), as the profile for Prolastin[®] is: 80% di-antennae, 18% tri-antennae and 2% tetra-antennae (Tables 1 and 2). This phenomenon is also clear from comparison of the A-numbers (Figure 5 and Tables 1 and 2). The degree of capping of the glycans by sialic acid of Prolastin[®] is 96% (Tables 1 and 2).

EXAMPLE 3Pharmacokinetics of PER.C6-rAAT in a rat model

5 **[0064]** A rat model was established to determine the pharmacokinetics (PK) of PER.C6-rAAT. The model should be able to detect AAT present in unpurified PER.C6-conditioned medium (CM) at concentrations of about 40-100 µg/mL (at 2 mL/kg resulting in doses of about 80-200 µg/kg).

10 **[0065]** Four experiments were performed: the first two to set-up the model and the second, third and fourth experiments to select the PER.C6-rAAT cell lines producing rAAT with most optimal PK characteristics. In these studies, human pdAAT (Prolastin®) was used to establish the model and as reference. The experimental procedure was as follows: rats (male Wistar, age 8 weeks) were dosed i.v. in the tail vein with the AAT test samples (dose volume 2 mL/kg, dose range 50-200 µg/kg, n=3 rats per group). Blood was sampled from the tail tip pre-dose and at various time points following dose
15 administration. The blood was collected in K₂EDTA microvettes and plasma was prepared. The residual AAT concentration in the plasma samples was subsequently determined by ELISA (AAT Elisa Kit, Affinity Biologicals). The data obtained were modelled per rat with a 2-compartment model and PK parameters were subsequently determined.

20 **[0066]** As shown by the data from experiment 1 (see Figure 7) the model was found suitable to determine the PK profile in unpurified samples because the plasma concentration versus time curves of Prolastin® spiked in PER.C6-CM and buffer control (PBS, 0.01% w/v Tween-80 (PT) (doses 200 µg/kg) are similar. A similar result was found with Prolastin® doses of 100 µg/kg (data not shown). In Figure 7 it is also shown
25 that an increased response is observed with doses of Prolastin® spiked in CM from 50-200 µg/kg. From the data it was calculated that Prolastin® has a mean residence time (MRT) of 11-12 hr. Also the PER.C6-rAAT samples were detected, with PER.C6-rAAT-ST3-202c showing a slower clearance (MRT ~ 20.5 hr) as compared to Prolastin® and PER.C6-rAAT-ST6-530 showing a faster clearance (MRT ~3.6 hr) compared to
30 Prolastin® (Table 1 and 2). Based on these observations, it was therefore concluded that the model could be used for the unpurified PER.C6-rAAT samples, which were tested in

experiments 2-4 at a dose level of 100 µg/kg for all samples. In Figure 8 representative PK profiles for PER.C6-rAAT-ST3 and PER.C6-rAAT-ST6 in comparison to Prolastin[®] are shown.

5 [0067] The last column in Tables 1 and 2 shows the MRT values obtained in the PK experiments. Statistical testing on differences between samples and groups of samples with regard to the MRT was done by ANOVA on log transformed values. The MRTs of both the PER.C6-rAAT-ST3 and PER.C6-rAAT-ST6 preparations tested were significantly different from that of Prolastin[®]. The MRT for 8 out of 10 PER.C6-rAAT-ST3 preparations tested were significantly higher than that of Prolastin[®] and ranged from 10 18-23 hr. Between these 8 PER.C6-rAAT-ST3 preparations the differences in MRT were not significant.

[0068] The MRTs of PER.C6-rAAT-ST3-053b and -539 were significantly lower than that of Prolastin[®]. For PER.C6-rAAT-ST3-0539 this is due to the relatively low degree of sialylation (49%) as confirmed by the rescue of the PK profile of this 15 preparation in the first hour during blocking of the asialoglycoprotein receptor by co-injection with asialofetuin (data not shown). The glycan profile for PER.C6-rAAT-ST3-053b was inconclusive (no clear peak assignments possible), however, also in this case undersialylation is most likely the cause of the low MRT.

[0069] For all PER.C6-rAAT-ST6 preparations tested the MRT was significantly 20 lower than that of Prolastin[®] ranging from 3.4-3.9 hr. This may be explained by the relative low degree of sialylation of the tetra-antennary glycans on PER.C6-rAAT-ST6 (ranging from 6 to 62%) and/or by a potential different mechanism of clearance for α2,3 sialylated AAT versus α 2,6 sialylated AAT.

25 [0070] Conclusions

According to the present invention PER.C6 cell lines have been generated that produce human AAT with a high yield (up to 22 pcd) and with an acceptable quality, i.e. the preparations were active in an *in vitro* assay. Surprisingly and interestingly, promising PK profiles were demonstrated in a rat model. PER.C6 cells thus form a suitable platform 30 for the production of human recombinant AAT for use in *inter alia* AAT-deficiency related emphysema or for use in other inflammatory diseases with neutrophil-mediated

tissue damage. The rhAAT of the present invention may however also suitably be produced in other mammalian cell types co-expressing a 2,3- or 2,6-sialyltransferases.

Table 1. Specific productivities, activity and glycan profiles of PER.C6®-rAAT-ST3

PER.C6®-rAAT-ST3 cell line	Qp (pcd, n=1 or n=2)	Activity Ratio CrI/Ag (n=3)	Di-antennary (%)	Tri-antennary (%)	Tetra-antennary (%)	A-number	Asialo (%)	Mono-sialo (%)	Di-sialo (%)	Tri-sialo (%)	Tetra-sialo (%)	Z-number	Degree of Sia on tetra (%)	Degree of Sia ZIA (%)	MRT (hr)
ECL negative cell lines															
098a	22*	1.12	50.52	22.92	26.53	276	13.62	11.02	42.23	14.67	18.46	213	69.6	77.2	18.3
246c	20*	1.17	49.52	21.22	27.39	272	9.81	10.93	39.57	19.82	19.88	229	72.6	84.2	23.2
221c	19	1.03	52.24	24.46	21.97	266	7.98	12.94	39.4	22.08	17.6	228	80.1	85.7	18.3
202c	17*	1.06	48.89	23.31	26.23	272	5.64	9.8	40.41	19.43	24.71	248	94.2	91.2	20.5
019c	16	0.98	49.95	24.36	24.03	269	18.4	14.48	34.36	17.85	14.9	196	62.0	72.9	21.1
607	15	1.3	51.82	21.09	27.08	275	5.17	16.04	28.77	21.39	28.63	252	105.7	91.6	20.2
028c	15	1.03	42.17	27.83	29.44	286	20.71	11.52	33.3	19.35	15.11	197	51.3	68.9	21.7
539	14	1.17	23.71	39.18	35.53	307	31.52	25.63	15.21	15.23	12.41	151	34.9	49.2	5.1
013b	14*	1	50.97	23.24	23.93	267	7.85	14.04	37.47	22.04	18.59	229	77.7	85.8	
234c	11	0.68	39.98	26.62	33.42	294	18.23	20.58	33.72	16.59	20.89	201	62.5	68.4	
293c	10*	1.17	52.19	19.78	28.02	276	1.94	19.9	31.87	19.54	26.75	249	95.5	90.2	22.5
604	10	1.16	52.19	21.87	25.93	274	4.86	45.01	23.21	14.25	12.63	185	48.7	67.5	
602	10	1.1	47.23	20	31.85	282	2.48	7.93	47.01	19.33	23.25	253	73.0	89.7	
529	9*	1.2	51.25	22.72	24.88	270	6.41	14.42	38.21	20.39	20.57	234	82.7	86.7	
223c	9	1.03	47.34	25.45	25.92	275	10.05	13.38	35.6	21.83	19.13	227	73.8	82.5	
053b	9	0.94	51	51	51	51	51	51	51	51	51	51	51	51	5.7
ECL positive cell lines															
031c	21	0.99													
580	19*	1.04													
612	18*	1.05	28.85	37.51	33.63	305	84.01	10.19	4.25	1.15	0.41	24	1.2	7.9	
608	17*	1.15	30.29	37.37	31.47	299	>95					0	0.0	0.0	
045c	14*	1.14													
032c	14	1.08													
534	11	0.99	11.66	35.84	47.7	322	>95					0	0.0	0.0	
236c	10	0.98													
610	9	1.09													
354b	8	0.96	29.38	30.45	39.57	308	50.01	12.42	14.02	10.63	12.92	124	32.7	40.3	
040c		0.98	23.9	37.18	36.8	307	>95					0	0.0	0.0	
Plasma derived AAT (n=2 for glycan profiles, n=4 for PK)															
Prolestin®	na	1	80.27	17.92	1.85	222	0.05	8.06	71.3	19.23	1.37	214	74.3	96.2	11.2
[Inconclusive glycan profile AAT: α1-antitrypsin; On: specific productivity and bioassay per cell per day; Cr: chromogenic assay; As: antigen; ELISA: Sia: sialic acid; MRT: mean residence time; hr: hours; ECL: <i>Escherichia coli</i> ; Z-number: Z-number; PK: pharmacokinetics]															

SEQ ID NO: 1

Amino acid sequence of AAT

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Mpssvswgillllaglcclvpvslaedpqqdaaqktdtshhdqdhptfnkitpnlaefafs
lyrqlahqsnstniffspvsiatafamlslgtkadthdeileglnfnlteipeaqihegf
qellrtlnqpdsqqlttnnglflseglklvdkfledvkklyhseftvnfgdteeakkq
indyvekgtqgkivdlvkeldrdrtdvfalvnyiffkgkwerpfevkdteeedfhvdqvttv
10 kvpmmkrlgmfnihqckklsswvllmkylgnataiffpdegklqlhlenelthdiitkfl
enedrrsaslhlpklsitgtydlksvlgqlgitkvfsngadlsgvteeapklkskavhka
vltidekgteaagamfleaipmsippevkfnkpfvflmieqntksplfmgkvvnptqk*

SEQ ID NO: 2

15 Optimized CDS of AAT (based on NM_000295)

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atgccagcagcgtgagctggggcatcctgctgctggccggcctgtgctgcctggtgcc
gtgagcctggccgaggacccccagggcgacgcgcccagaaaaccgacaccagccaccac
gaccaggaccacccccaccttcaacaagatcacccccaacctggccgagttcgcccttcagc
ctgtaccggcagctggcccaccagagcaacagcaccaacatctttttcagccccgtgagc
atcgccaccgccttcgccatgctgtccctgggcaccaaggccgacacccacgacgagatc
ctggaaggcctgaacttcaacctgaccgagatccccgaggcccagatccacgagggcttc
caggaactgctgcggaacctgaaccagcccagacagccagctccagctcaccaccggcaac
ggcctgtttctgagcgagggcctgaaactgggtggacaagtttctcgaagatgtgaagaag
ctgtaccacagcgaggccttcaccgtgaacttcggcgacaccgaggaagccaagaagcag
atcaacgactacgtggagaagggcacccagggcaagatcgtggacctggtgaaagagctg
gaccgggacaccgtgttcgccctggtgaactacatcttcttcaagggcaagtgggagcgg
cctttcagaggtgaaggataccgaggaagaggacttccacgtggaccaggtgaccaccgtg
aaggtgcccattgatgaagcggctgggcatgttcaacatccagcactgcaagaagctgtcc
agctgggtcctgctgatgaagtacctgggcaacgccaccgccatctttttctgcccgcac
gagggcaagctgcagcacctggaaaacgagctgaccacgacatcatcaccaagtttctg
gaaaatgaggaccggcgagcgccagcctgcacctgcccagctgtccatcacccggcacc
tacgacctgaagagcgtgctggtggccagctgggcatcaccaaggtgttcagcaacggcgcc
gacctgagcggcgtgaccgaagaggccccctgaagctgtctaaggccgtgcacaaggcc
gtgctgaccatcgacgagaaggggaccgaagccgcccggagccatgtttctggaagccatc
cccatgagcatccccccgaggtgaagttcaacaagcccttcgtgttcctgatgatcgag
cagaacaccaagagacccccctgttcatgggcaaggtggtgaacccccacccaaaagtga

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CLAIMS

1. Recombinant human α 1-antitrypsin (rhAAT) comprising N-linked glycans, characterized in that:

- 5 (a) at least 10% of said N-linked glycans are tetra-antennary glycans; and
(b) the degree of capping with sialic acid on said N-linked glycans (Z/A) is at least 50%.

2. RhAAT according to claim 1, wherein at least 20% of said N-linked glycans are
10 tetra-antennary glycans.

3. RhAAT according to claim 1 or 2, wherein from about 10% to 50% of said N-linked glycans are tetra-antennary glycans.

15 4. RhAAT according to claim 1 or 2, wherein from about 20% to 40% of said N-linked glycans are tetra-antennary glycans.

5. RhAAT according to any one of the claims 1-4, wherein at least 50% of the total sialylation on said N-linked glycans is α -2,3-sialylation.
20

6. RhAAT according claim 5, wherein at least 90% of the total sialylation on said N-linked glycans is α -2,3-sialylation.

7. RhAAT according to any one of the preceding claims, wherein the degree of
25 capping with sialic acid on said N-linked glycans (as calculated by the Z/A ratio) is at least 70%, preferably at least 80%, more preferably at least 90%.

8. RhAAT according to any one of the preceding claims, wherein the degree of capping with sialic acid on said N-linked glycans that are tetra-antennary is at least 20%.
30

9. RhAAT according to any one of the preceding claims, wherein the degree of capping with sialic acid on said N-linked glycans that are tetra-antennary is at least 50%.

10. RhAAT according to claim 9, wherein the degree of capping with sialic acid on
5 said N-linked glycans that are tetra-antennary is from about 50% to about 97%.

11. RhAAT according to any one of the preceding claims, wherein the degree of capping with sialic acid on said N-linked glycans that are tetra-antennary is from about 70% to about 95%.

10

12. RhAAT according to any one of the preceding claims for use as a medicament.

13. RhAAT according to any one of the preceding claims for use in the prevention and/or treatment of a disease associated with AAT deficiency, and/or a disease involving
15 neutrophil-mediated tissue damage.

14. Method for producing recombinant human α 1-antitrypsin (rhAAT), comprising the steps of providing a PER.C6 cell with a nucleic acid encoding human α 1-antitrypsin in such a way that said PER.C6 cell harbors said nucleic acid in an expressible form; and
20 culturing said PER.C6 cell under conditions conducive to the production of said recombinant human α 1-antitrypsin, wherein said PER.C6 cell is modified to co-express a said sialyltransferase is α 2,3 sialyltransferase or α 2,6-sialyltransferase.

15. RhAAT obtainable by a method according to claim 14.

25

FIG. 1

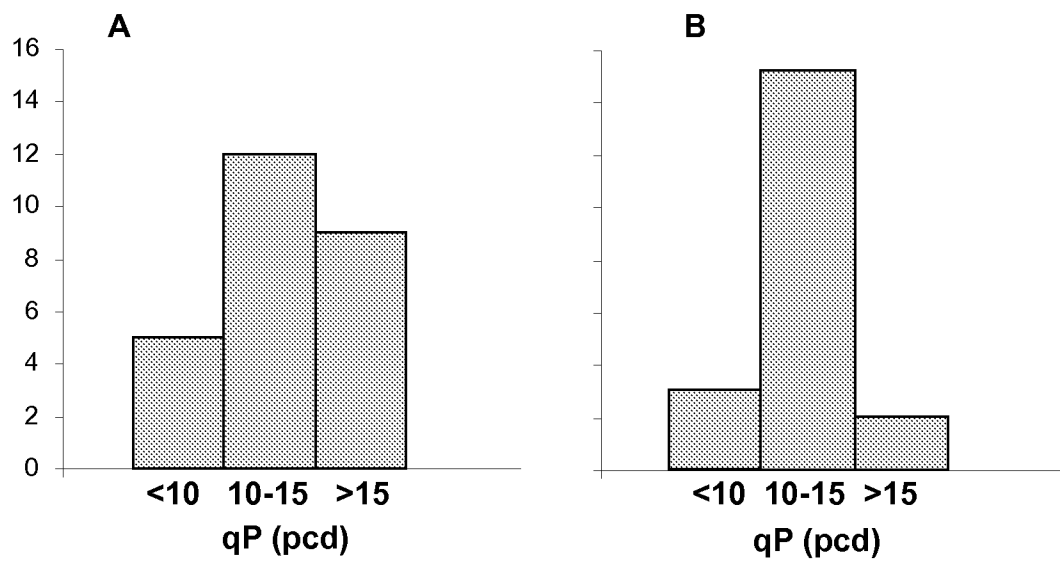


FIG. 2

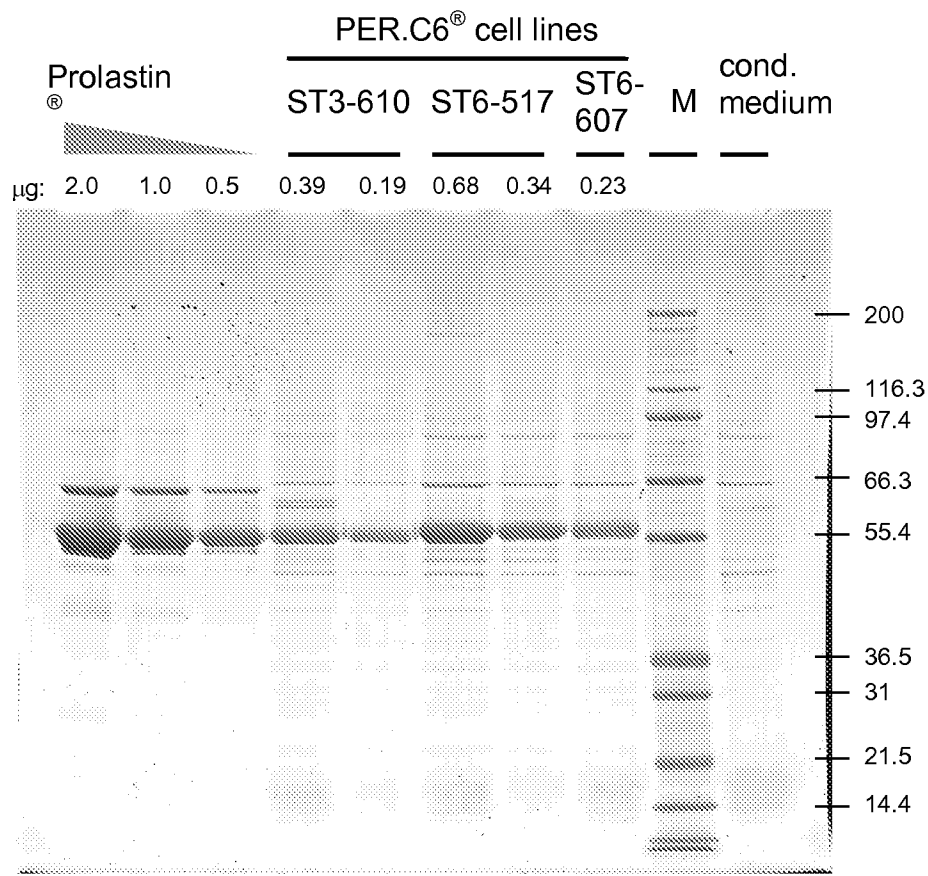


FIG. 3

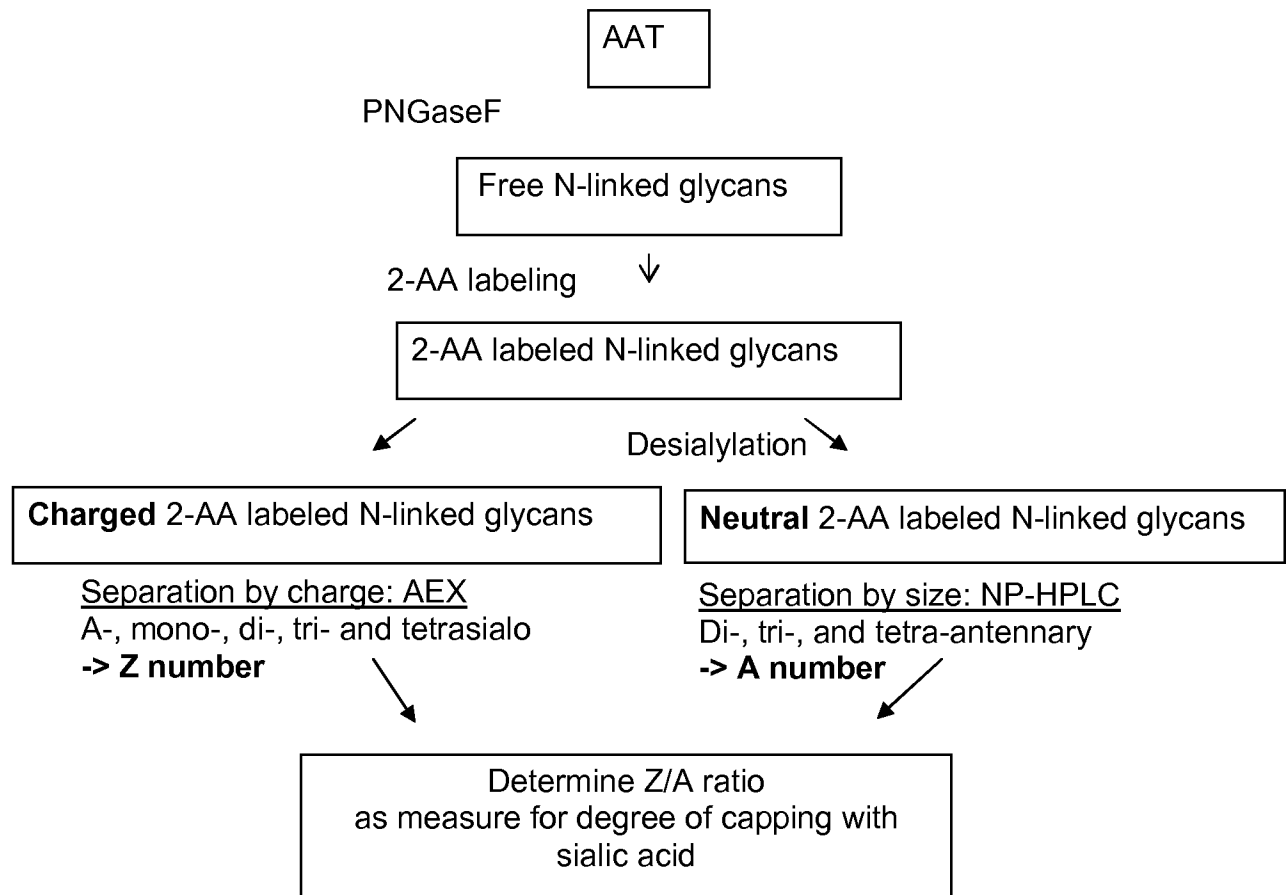


FIG. 4

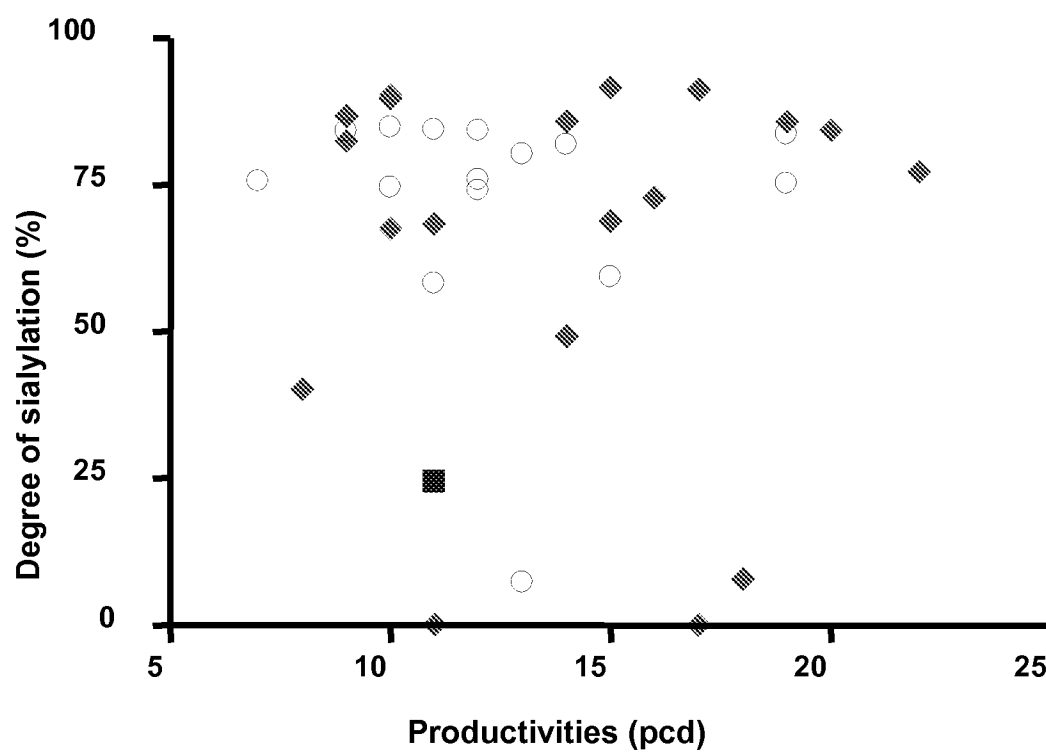


FIG. 5

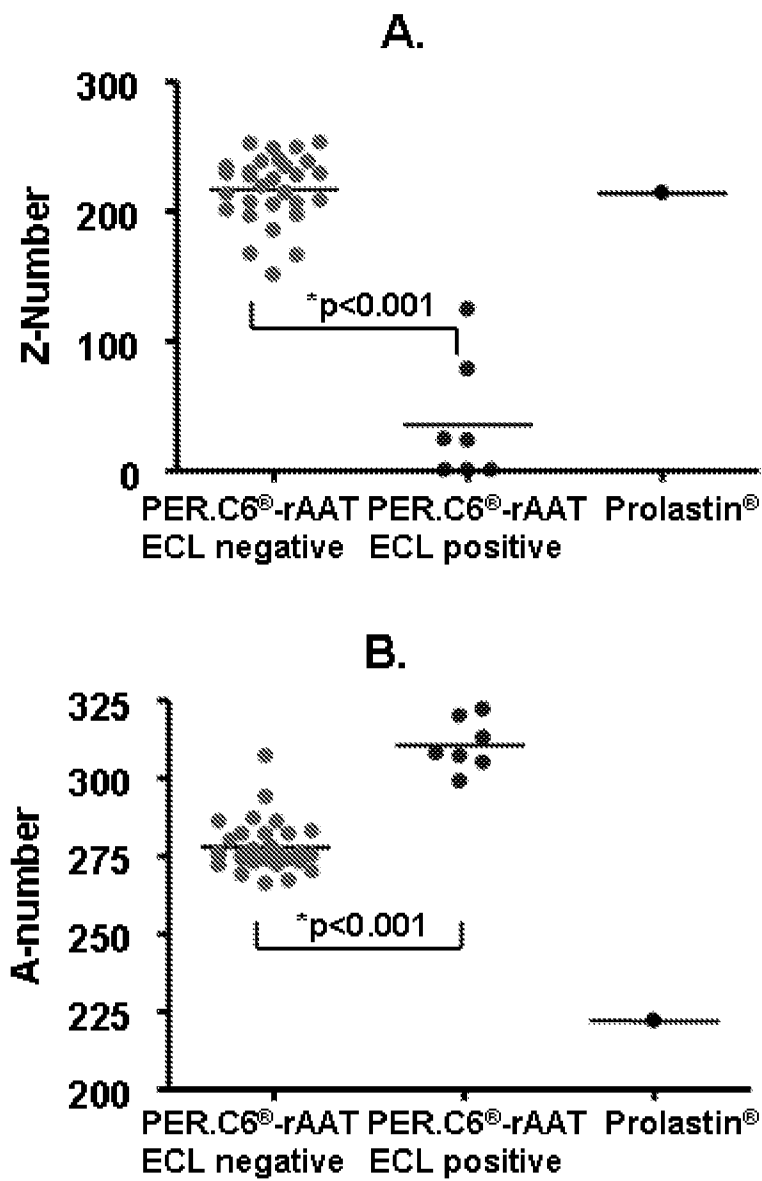


FIG. 6

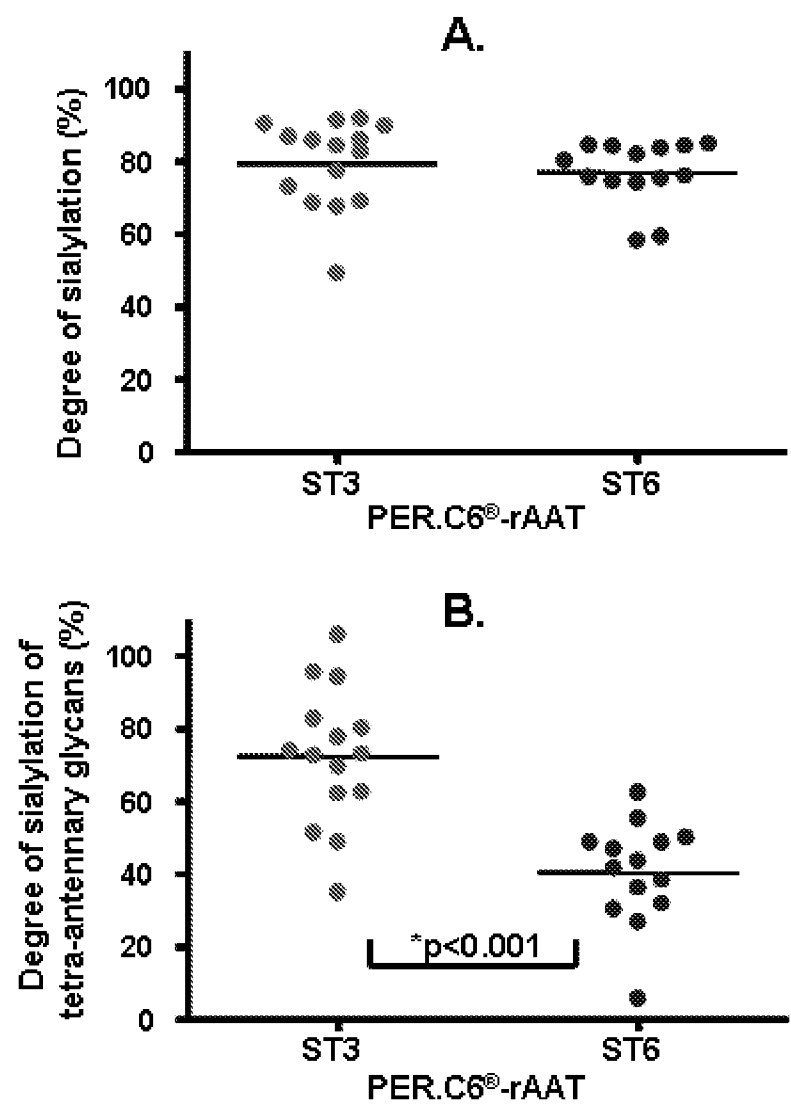


FIG. 7

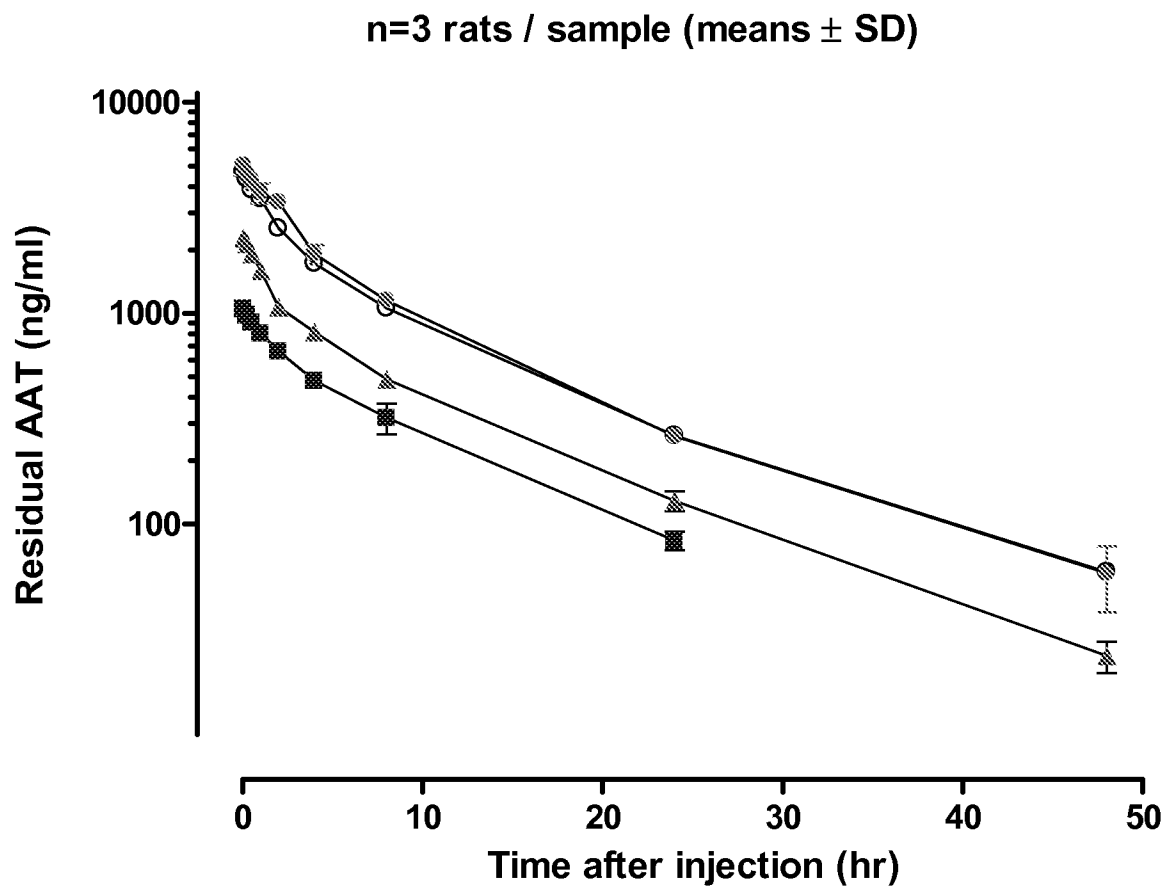


FIG. 8

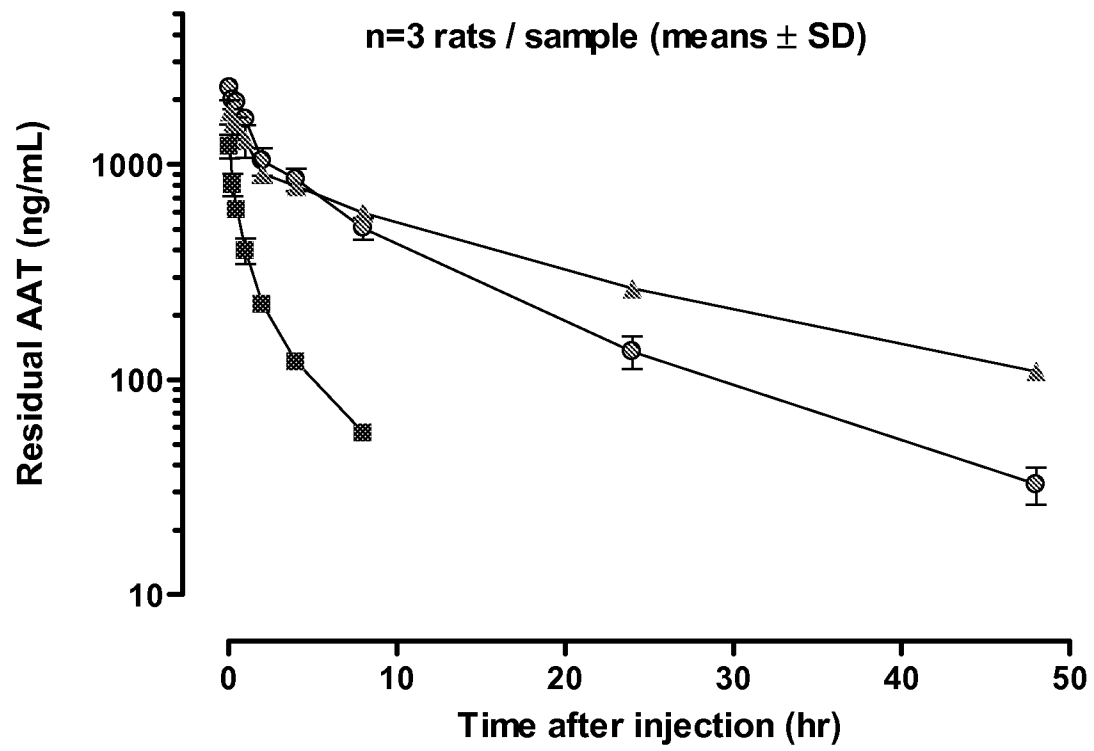
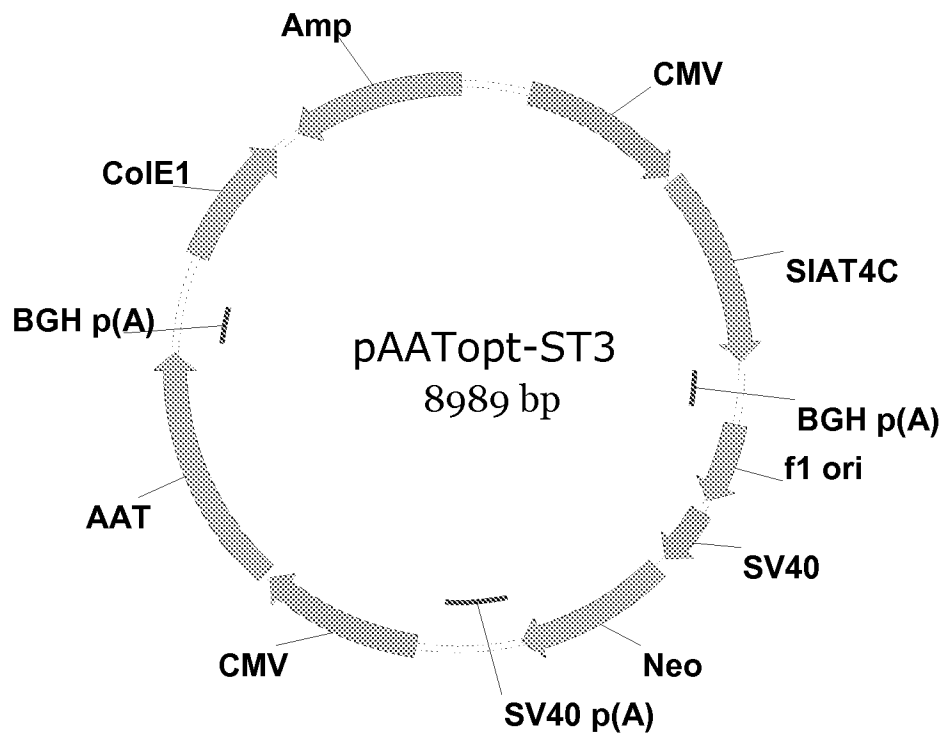
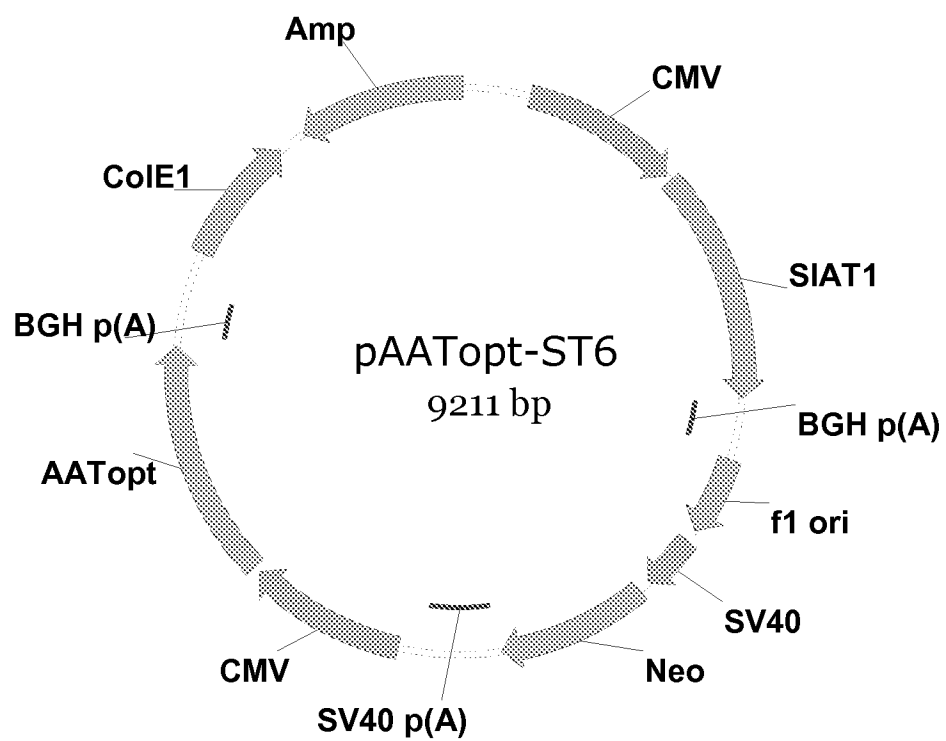


FIG. 9

A.



B.



INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2010/055177

A. CLASSIFICATION OF SUBJECT MATTER

INV. C07K14/81

ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHANG G-D ET AL: "Improvement of glycosylation in insect cells with mammalian glycosyltransferases" JOURNAL OF BIOTECHNOLOGY, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL, vol. 102, no. 1, 10 April 2003 (2003-04-10), pages 61-71, XP002387943 ISSN: 0168-1656 page 63, left-hand column, line 14	1-15
X	WO 2006/070011 A (CRUCCELL HOLLAND BV [NL]; OPSTELTEN DIRK JAN ELBERTUS [NL]) 6 July 2006 (2006-07-06) cited in the application paragraph [0052]	1-15
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☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:

A document defining the general state of the art which is not considered to be of particular relevance

E earlier document but published on or after the international filing date

L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

Date of the actual completion of the international search

6 July 2010

Date of mailing of the international search report

14/07/2010

Name and mailing address of the ISA/

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Fax: (+31-70) 340-3016

Authorized officer

Schmitz, Till

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2010/055177

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KOLARICH DANIEL ET AL: "Biochemical, molecular characterization, and glycoproteomic analyses of alpha(1)-proteinase inhibitor products used for replacement therapy" TRANSFUSION, AMERICAN ASSOCIATION OF BLOOD BANKS, BETHESDA, MD, US, vol. 46, no. 11, 1 November 2006 (2006-11-01), pages 1959-1977, XP002448797 ISSN: 0041-1132 page 1964, left-hand column, last paragraph - right-hand column, paragraph 1 -----	1-15
A	WO 2009/009086 A (SANGAMO BIOSCIENCES INC [US]; COLLINGWOOD TREVOR [US]; COST GREGORY J) 15 January 2009 (2009-01-15) paragraph [0121] paragraph [0123] -----	1-15
A	KOLARICH DANIEL ET AL: "Comprehensive glyco-proteomic analysis of human alpha(1)-antitrypsin and its charge isoforms" PROTEOMICS, WILEY - VCH VERLAG, WEINHEIM, DE, vol. 6, no. 11, 1 June 2006 (2006-06-01), pages 3369-3380, XP002502192 ISSN: 1615-9853 the whole document -----	1-15
A	GARVER R I JR ET AL: "Production of glycosylated physiologically "normal" human alpha 1-antitrypsin by mouse fibroblasts modified by insertion of a human alpha 1-antitrypsin cDNA using a retroviral vector." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA FEB 1987, vol. 84, no. 4, February 1987 (1987-02), pages 1050-1054, XP002540833 ISSN: 0027-8424 the whole document -----	1-15

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2010/055177

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

a. (means)

☒

on paper

☒

in electronic form

b. (time)

☐

in the international application as filed

☐

together with the international application in electronic form

☒

subsequently to this Authority for the purpose of search

2. ☒ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2010/055177

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 2006070011 A		06-07-2006	AU 2005321246 A1	06-07-2006
			CA 2592100 A1	06-07-2006
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			KR 20070110841 A	20-11-2007
			SG 158185 A1	29-01-2010
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			EP 2171052 A2	07-04-2010
			KR 20100052474 A	19-05-2010
			US 2009042250 A1	12-02-2009