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WO-A1-2014/177576
WO-A2-2004/085478
WO-A2-2008/080623
WO-A2-2010/003766
HOLLER N ET AL: "Development of improved soluble inhibitors of FasL and CD40L based on oligomerized receptors", JOURNAL OF IMMUNOLOGICAL METHODS, ELSEVIER SCIENCE PUBLISHERS B.V.,AMSTERDAM, NL, vol. 237, no. 1-2, 1 April 2000 (2000-04-01), pages 159-173, XP004192503, ISSN: 0022-1759, DOI: 10.1016/S0022-1759(99)00239-2
LIU ET AL: "Murine coronavirus-induced oligodendrocyte apoptosis is mediated through the activation of the Fas signaling pathway", VIROLOGY, ELSEVIER, AMSTERDAM, NL, vol. 360, no. 2, 28 March 2007 (2007-03-28), pages 364-375, XP005938080, ISSN: 0042-6822, DOI: 10.1016/J.VIROL.2006.10.044
R&d Systems: "Recombinant Rat Fas/TNFRSF6/CD95 Fc Chimera", , 10 December 2015 (2015-12-10), XP055247173, Retrieved from the Internet: URL:https://resources.rndsystems.com/pdfs/ datasheets/2159-fa.pdf [retrieved on 2016-02-03]

DESCRIPTION

[0001] The present invention relates to a pharmaceutical composition comprising a mixture of isoforms of the fusion protein APG101, formulations providing such composition in a stable form as well as a method for producing such a composition.

[0002] Fusion proteins comprising the extracellular domain of the death receptor CD95 (Apo-1; Fas) fused to an immunoglobulin Fc domain are described in WO2004085478. However, it turned out difficult to provide such fusion proteins in sufficient amounts with a sufficient stability.

[0003] According to a first aspect the present invention relates to a pharmaceutical composition comprising a mixture of APG101 isoforms wherein unmodified APG101 is represented by SEQ ID NO:1 said mixture comprising APG101 variants N-terminally truncated and whose first amino acid is amino acid 17, 21 and 26 with respect to SEQ ID NO:1 and less than 1 mol% unmodified APG101 according to SEQ ID NO:1 and distributing within a pI range of 4.0 - 8.5, and wherein said mixture of APG101 isoforms is obtainable by a method comprising the steps

1. (a) producing a mixture of APG101 isoforms by a fed-batch production process providing a cell harvest using a peptone-containing basal medium, and
2. (b) isolating the mixture of APG101 isoforms from the cell harvest,

wherein step (a) comprises a series of cultivation steps of a given master cell batch until relevant harvest parameters are reached, followed by cell sedimentation and filtration of fusion protein containing supernatant, wherein the relevant harvest parameters are an APG101 titer within a range of 0.5 g/l - 5 g/l and a cell density of about 1×10^6 - 1×10^8 cells/ml and
step (b) comprises capture chromatography, virus inactivation, a series of anion and/or cation chromatography, virus filtration and adjustment to a desired final protein concentration. APG101 comprises an extracellular CD95 domain (APO-1; Fas) and a second domain being an Fc domain. Accordingly, the extracellular CD95 domain as used herein may be also called "first domain", while the Fc domain may be called "second domain".

[0004] The first domain protein is a human protein, i.e. a human extracellular CD95 domain.

[0005] As defined by SEQ ID NO: 1 APG101 can be a fusion protein comprising a human extracellular CD95 domain (amino acids 26-172) and a human IgG1 Fc domain (amino acids 172-400), further optionally comprising an N-terminal signal sequence (e.g. amino acids 1-25 of SEQ ID NO: 1). The presence of the signal peptide indicates the immature form of APG101. During maturation, the signal peptide is cleaved off. According to an especially preferred embodiment the signal sequence is cleaved off. APG101 with the signal sequence is also comprised by the term "unmodified APG101".

[0006] The term "APG101" describes a fusion protein of position 26-400 of SEQ ID NO: 1, with

and/or without a signal peptide.

[0007] The pharmaceutical composition according to the invention comprises a mixture of APG101 isoforms. The term "isoform" as used herein designates different forms of the same protein, i.e. different forms of APG101 as defined in the claims. Isoforms can differ, for example, by protein length, by amino acid deletion, and/or post-translational modification when compared to the corresponding unmodified protein, i.e. the protein which is translated and expressed from a given coding sequence without any modification. Different isoforms can be distinguished, for example, by electrophoresis, such as SDS-electrophoresis, and/or isoelectric focussing which is preferred according to the present invention.

[0008] Isoforms differing in protein length can be, for example, N- terminally and/or C-terminally extended and/or shortened when compared with the corresponding unmodified protein. A mixture of APG101 isoforms according to the invention comprises less than 1 mol-% APG101 in unmodified form as well as N- terminally shortened variants whose first amino acid is amino acid 17, 21 and 26, respectively, of SEQ ID NO:1.

[0009] Such N-terminally shortened fusion proteins may in terms of the present invention also be named -17, -21, and -26 N-terminally shortened variants of unmodified APG101. The numbering refers to the APG101 protein including signal sequence according to SEQ ID NO: 1, wherein the number refers to the first amino acid in the N-terminally truncated APG101 variant.

[0010] This means a shortened fusion protein having SEQ ID NO:1 N-terminally truncated by 16 amino acids corresponds to a APG101 variant designated -17 and results in a protein having amino acids 17-400 of SEQ ID NO:1, N-terminally truncated by 20 amino acids corresponds to -21 (amino acids 21 - 400 of SEQ ID NO:1) and N-terminally truncated by 25 amino acids corresponds to -26 (amino acids 26 - 400 of SEQ ID NO:1).

[0011] An example for a C-terminal shortening of APG101 isoforms is C-terminal Lys-clipping.

[0012] According to the present invention the mixture of APG101 isoforms of the pharmaceutical composition according to the present invention comprises less than1 mol-% unmodified APG101. Most preferred is an embodiment comprising a mixture of APG101 isoforms that does not comprise any unmodified APG101.

[0013] As outlined above, isoforms can also differ by amino acid deletion and/or addition of amino acids. Such a deletion may comprise one or more amino acids.

[0014] Isoforms according to the invention can also differ with regard to post-translational modification. Post-translational modification according to the present invention may involve, without being limited thereto, the addition of hydrophobic groups, in particular for membrane localisation such as myristoylation, palmitoylation, isoprenylation or glypiation, the addition of cofactors for enhanced enzymatic activity such as lipoyation, the addition of smaller chemical groups such as acylation, formylation, alkylation, methylation, amidation at the C-terminus,

amino acid addition, γ-carboxylation, glycosylation, hydroxylation, oxidation, glycilation, biotinylation and/or pegylation.

[0015] According to the present invention the addition of sialic acids, Fc-based glycosylation, in particular Fc-based N-terminal glycosylation, and/or pyro-Glu-modification are preferred embodiments of post-translational modification.

[0016] According to a preferred embodiment the fusion proteins comprised by the pharmaceutical composition of the invention comprise high amounts of sialic acids. According to the present invention the content of sialic acid is preferably from about 4.0 to 7.0 mol NeuAc/mol APG101, more preferably from 4.5 to 6.0 mol NeuAc/mol APG101 and most preferably about 5.0 mol NeuAc/mol APG101. As used herein, sialic acids refer N- or O-substituted derivatives of neuraminic acid. A preferred sialic acid is N-acetylneuraminic acid (NeuAc). The amino group generally bears either an acetyl or glycolyl group but other modifications have been described. The hydroxyl substituents may vary considerably. Preferred hydroxyl substituents are acetyl, lactyl, methyl, sulfate and/or phosphate groups. The addition of sialic acid results generally in more anionic proteins. The resulting negative charge gives this modification the ability to change a protein's surface charge and binding ability. High amounts of sialic acid lead to better serum stability and thus, improved pharmacokinetics and lower immunogenicity. The high degree of sialylation of APG101 isoforms of the present invention could be explained by the high amount of diantennary structure. It has to be regarded as highly surprising that the APG101 isoforms in the pharmaceutical composition of the invention obtained by the inventive method show such a high grade of sialic acid addition.

[0017] According to the present invention, glycosylation designates a reaction in which a carbohydrate is attached to a functional group of a fusion protein, functional fragment thereof as defined herein. In particular, it relates to the addition of a carbohydrate to APG101 or an isoform thereof. The carbohydrate may be added, for example, by N-linkage or O-linkage. N-linked carbohydrates are attached to a nitrogen of asparagine or arginine site chains. O-linked carbohydrates are attached to the hydroxy oxygen of serine, threonine, tyrosine, hydroxylysine or hydroxyproline side chains. According to the present invention, N-linkage, in particular Fc-based N-terminal glycosylation is preferred. Particularly preferred N-linked glycosylation sites are located at positions N118, N136 and/or N250 of APG101 (SEQ ID NO: 1).

[0018] Fucosylation according to the present invention relates to the adding of fucose sugar units to a molecule. With regard to the present invention such an addition of a fucose sugar unit to APG101, represents an especially preferred type of glycosylation. A high portion of fucosylated forms leads to a reduced antibody-dependent cellular cytotoxicity (ADCC). Thus, the mixture of fusion protein isoforms is characterised by reduced ADCC, which is beneficial for pharmaceutical and diagnostic applications.

[0019] Of course, beside the first and second domain as defined herein, the APG101 isoforms according to the invention may comprise further domains such as further targeting domains, e.g. single chain antibodies or fragments thereof and/or signal domains.

[0020] The pharmaceutical composition according to the present invention may comprise N-terminally blocked fusion proteins, which provide a higher stability with regard to N-terminal degradation by proteases, as well as fusion proteins having a free N-terminus, which provides a higher stability with regard to N-terminal degradation by proteases.

[0021] Modifications blocking the N-terminus of protein are known to a person skilled in the art. However, a preferred post-translational modification according to the present invention blocking the N-terminus is the pyro-Glu-modification. Pyro-Glu is also termed pyrrolidone carboxylic acid. Pyro-Glu-modification according to the present invention relates to the modification of an N-terminal glutamine by cyclisation of the glutamine via condensation of the α-amino group with a side chain carboxyl group. Modified proteins show an increased half-life. Such a modification can also occur at a glutamate residue. Particularly preferred is a pyro-Glu-modification, i.e. a pyrrolidone carboxylic acid, with regard to the N-terminally shortened fusion protein -26.

[0022] In a preferred embodiment of the present application the pharmaceutical composition according to the present invention comprises 80-99 mol-% N-terminally blocked fusion proteins and/or 1-20 mol-% fusion proteins having a free N-terminus.

[0023] According to a further preferred embodiment the pharmaceutical composition comprises 0.0 to 5.0 mol-%, more preferably 0.0 to 3.0 mol-% and even more preferably 0.0 to 1.0 mol-%, of fusion protein high molecular weight forms such as aggregates. In a preferred embodiment the pharmaceutical composition according to the present invention does not comprise any aggregates of fusion protein isoforms, in particular no dimers or aggregates of APG101. Dimers or aggregates are generally undesired because they have a negative effect on solubility.

[0024] The functional form of APG101 comprises two fusion proteins, as described herein, coupled by disulfide bridges at the hinge region at positions 179 or/and 182 with reference SEQ ID NO:1 of the two molecules (see Figure 7). The disulfide bridge may also be formed at position 173 with reference to SEQ ID NO:1 of the two molecules, resulting in an improved stability. If the disulfide bridge at position 173 with reference to SEQ ID NO:1 is not required, the Cys residue at this position can be replaced by another amino acid, or can be deleted.

[0025] The pharmaceutical composition according to the present invention is provided by the method according to the present invention described herein.

[0026] According to the invention, the mixture of APG101 isoforms distributes within a pI range of about 4.0 to about 8.5. In a further embodiment the pI range of the mixture of APG101 isoforms comprised by the pharmaceutical composition according to the invention is about 4.5 to about 7.8, more preferably about 5.0 to about 7.5.

[0027] The isoelectric point (pI) is defined by the pH-value at which a particular molecule or

surface carries no electrical charge. Depending on the pH range of the surrounding medium the amino acids of a protein may carry different positive or negative charges. The sum of all charges of a protein is zero at a specific pH range, its isoelectric point, i.e. the pI value. If a protein molecule in an electric field reaches a point of the medium having this pH value, its electrophorectic mobility diminishes and it remains at this site. A person skilled in the art is familiar with methods for determining the pI value of a given protein, such as isoelectric focussing. The technique is capable of extremely high resolution. Proteins differing by a single charge can be separated and/or fractionated.

[0028] The pharmaceutical composition according to the present invention described herein may be used for pharmaceutical, diagnostic and/or research applications. It may be applied in human medicine as well as veterinary medicine.

[0029] Another aspect of the present invention relates to a formulation comprising a pharmaceutical composition according to the invention.

[0030] According to a preferred embodiment the formulation comprises

1. (a) phosphate, more preferably about 1 mM to about 100 mM phosphate buffer, more preferably about 5 mM phosphate to about 85 mM phosphate, more preferably about 20 mM to about 80 mM phosphate, more preferably about 30 mM to about 70 mM phosphate, even more preferably about 40 mM to about 60 mM phosphate, most preferred about 50 mM phosphate,
2. (b) a viscosity enhancing agent, preferably about 0.1-10 weight-% viscosity enhancing agent, more preferably 1 to 8 weight-% viscosity enhancing agent, more preferably about 3 weight-% to about 7 weight-% viscosity enhancing agent, even more preferred about 6 weight-% to about 7 weight-% viscosity enhancing agent, and most preferred about 5 weight-% viscosity enhancing agent, and
3. (c) has a pH value in the range of 4-8.

[0031] In terms of the present invention, the term "phosphate" is comprises any suitable phosphate buffer known to the person skilled in the art. According to an especially preferred embodiment the phosphate buffer is Na-phosphate.

[0032] Viscosity enhancing or increasing agents are well-known to a person skilled in the art and comprise alginic acid, carboxymethyl cellulose, dextrin, gelatin, guar gum, hydroxyethyl cellulose, magnesium aluminum silicate, polyvinyl alcohol, polyethylene oxide, silicon dioxide, starch, xanthan gum, etc. Further excipients which of course might be present comprise Saccharose, sorbitol and/or glycine. However, a viscosity enhancing agent which is especially preferred according to the present invention is sorbitol. According to an especially preferred embodiment, the viscosity enhancing agent sorbitol is present with about 5 weight-%.

[0033] The pH value of the formulation according to the present invention is within the range

of about 4 to 8. According to a preferred embodiment, it is within the range of 5 to 8, more preferably 6 to 8 and even more preferably 6.5 to 8. According to an especially preferred embodiment, the pH is about 6.5, about 7.0 or about 7.5.

[0034] According to an especially preferred embodiment, the formulation according to the present invention comprises about 30 mM Na-phosphate or about 50 mM Na-phosphate, about 5 % sorbitol and shows a pH value of about 6.5 (cf. buffers 5 and 6 of Figure 10).

[0035] Surprisingly, the pharmaceutical composition of the invention provided in that type of formulation is very stable and does not tend to form aggregates. For example, the formulations of the present invention are further characterized by reduced fragmentation of APG101. Moreover, it was possible to provide high protein concentrations, e.g. about 20 mg/ml in stable form.

[0036] The pharmaceutical composition and/or formulation according to the invention can be administered to a subject in need thereof, particularly a human patient, in a sufficient dose for the treatment of the specific conditions by suitable means. For example, the pharmaceutical composition and/or formulation according to the invention may be formulated together with pharmaceutically acceptable carriers, diluents and/or adjuvants. Therapeutic efficiency and toxicity may be determined according to standard protocols. The pharmaceutical composition may be administered systemically, e.g. intraperitoneally, intramuscularly, or intravenously or locally such as intranasally, subcutaneously or intrathecally. The dose of the pharmaceutical composition and/or formulation administered will, of course, be dependent on the subject to be treated and on the condition of the subject such as the subject's weight, the subject's age and the type and severity of the disease or injury to be treated, the manner of administration and the judgement of the prescribing physician. For example, a daily dose of 0.001 to 100 mg/kg is suitable.

[0037] Another aspect of the present invention relates to a pharmaceutical composition or formulation comprising the pharmaceutical composition or formulation according to the invention, which contains at least one further active agent. Which further active agent is used depends on the indication to be treated. For example, cytotoxic agents such as doxorubicin, cisplatin or carboplatin, cytokines or other anti-neoplastic agents may be used in the treatment of cancer.

[0038] The formulation and/or pharmaceutical composition according to the invention may further comprise pharmaceutically acceptable carriers, diluents, and/or adjuvants. The term "carrier" when used herein includes carriers, excipients and/or stabilisers that are non-toxic to the cell or mammal being exposed thereto at the dosages and concentrations employed. Often the physiologically acceptable carriers an aqueous pH buffered solutions or liposomes. Examples of physiologically acceptable carriers include buffers such as phosphate, citrate and other organic acids (however, with regard to the formulation of the present invention, a phosphate buffer is preferred); anti-oxidants including ascorbic acid, low molecular weight (less than about 10 residues) polypeptides; proteins such as serum albumin, gelatine or

immunoglobulins; hydrophilic polymers such as polyvinyl pyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose or dextrins, gelating agents such as EDTA, sugar, alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as TWEEN, polyethylene or polyethylene glycol.

[0039] According to a preferred embodiment the pharmaceutical composition and/or formulation according to the invention can be used to inhibit the CD95 signalling pathway, in particular the extrinsic apoptotic pathway triggered by CD95L, i.e. the CD95 receptor ligand. In particular, the pharmaceutical composition can be used in the prophylaxis and/or treatment of disorders selected from autoimmune disorders, AIDS, heart disorders, e.g. myocardial infarction, graft-versus-host disorders, transplant rejection, brain damage, e.g. stroke, spinal chord injuries, sepsis, hepatitis, disorders associated with inflammation, ischemic reperfusion injury and renal disorders. Of course, the pharmaceutical composition and/or formulation described herein may be used for the treatment of cancers, preferably solid cancers, e.g., brain cancers, e.g., glioblastomas. Alternatively, the cancer to be treated may be a cancer of lymphoid or myeloid origin.

[0040] Another aspect of the present invention relates to a method for producing a composition according to the invention comprising the steps

1. (a) production of a composition according to the present invention by a feed batch production process, providing a cell harvest using a peptone-containing medium and
2. (b) isolation of the composition of the present invention from the cell harvest,

wherein step (a) comprises a series of cultivation steps of a given master cell batch until relevant harvest parameters are reached, followed by cell sedimentation and filtration of fusion protein containing supernatant, wherein the relevant harvest parameters are an APG101 titer within a range of 0.5 g/l - 5 g/l and a cell density of about 1×10^6 - 1×10^8 cells/ml and
step (b) comprises capture chromatography, virus inactivation, a series of anion and/or cation chromatography, virus filtration and adjustment to a desired final protein concentration.

[0041] One advantage of the method of the present invention compared to the methods known from the prior art is its high yield.

[0042] Step (a), i.e. the "method for producing a composition according to the present invention by a feed-batch production process providing a cell harvest" will also be designated as "upstream process (USP)" in the following. The method according to step (a) of the present invention is also referred to as "inventive USP". Figure 1 shows a comparison of the upstream process according to the prior art and a preferred embodiment of the upstream process of the present invention.

[0043] Step (b), i.e. "isolation of the composition of the present invention from the cell harvest" will also be designated as "downstream process (DSP)" in the following.

[0044] Step (a) comprises a series of cultivation steps of a given master cell batch until relevant harvest parameters are reached followed by sedimentation and filtration of fusion protein, containing supernatant. In a preferred embodiment of the present invention the process steps of the upstream process may be summarised as a series comprising the following steps.

[0045] Thawing,
subcultivation,
50 l bioreactor,
200 l bioreactor,
1000 l bioreactor,
sedimentation,
depth filtration and
0.2 µm filtration.

[0046] Of course, carrying out the cultivation steps from subcultivation to the 1000 l bioreactor is only one way of carrying out the invention. For example, the cultivation steps may be carried out in bioreactors with varying sizes as well. Of course, during the series of subcultivation steps, the person skilled in the art can determine suitable parameters like temperature, growth time, media, etc. The crucial factor is to achieve relevant harvest parameters, which may be a titer, the cell density, with the titer being within a range of 0.5 g/l to 5 g/l, more preferably 1 g/l to 3 g/l, more preferably 1.5 g/l to 5 g/l and most preferably being 1.8 g/l to 2 g/l. Examples for values of cell density are about 1×10^6 to 1×10^8 cells/ml, preferably about 1×10^7 cells/ml. In an especially preferred embodiment of the present invention the titer is about 1.8 g/l to about 2 g/l and the cell density is about 1×10^7 cells/ml.

[0047] The method according to the present invention is carried out in a pepton-containing basal medium and a chemically defined medium.

[0048] Step (b) comprises capture chromatography, virus inactivation, a series of anion and/or cation chromatography, virus filtration and adjustment to a desired final protein concentration.

[0049] The downstream process of purifying a composition comprising APG101 isoforms obtained by step (a) according to step (b) defined above comprises chromatography steps, a virus inactivation step, an ultrafiltration step, a diafiltration step and a virus filtration step. According to a preferred embodiment, this downstream process comprises three different chromatographic steps. The first chromatography step is carried out with a resin to capture the target protein and/or to remove process-related impurities (e.g., HCPs, DNA) and or to reduce the volume of the product-containing fraction. A corresponding resin can be selected by the person skilled in the art. An example of a resin is Mab Select SuRE, which is also a preferred embodiment according to the invention.

[0050] After this first chromatography step a virus inactivation step follows. Preferably, this

virus inactivation step is performed under acidic conditions (e.g., pH 3.5 ± 0.2) followed by a conditioning of the inactivation pool or at a less acidic pH value such as pH 5.0. The buffer matrix for virus inactivation and subsequent pH 5.0 adjustment may be solely based on 20 nM Na-citrate buffer.

[0051] After this virus inactivation step chromatography, an ion exchange step is carried out in order to reduce process-related impurities such as DNA. According to the present invention an anion exchange chromatography (AIEX) step is preferred, particular in a flow-through mode. The target protein passes the AIEX column, whereas DNA binds to the resin. Preferably, the AIEX flow-through pool is subsequently processed without any conditioning using a further column-based step. This optional further step contributes to the overall reduction of virus contamination and residual HCP, DNA and bleached protein-A ligand. According to a preferred embodiment a mix-mode resin capto-MMC operated column in bind/eluate mode is used.

[0052] The eluate is passed through a virus filter (VF) and applied to an ultra-diafiltration step (UF/DF) subsequently. According to the invention a specific volumetric load of ≤ 100 l/m^2 can be obtained on the virus filtration step. Preferably, a membrane with about a 30 kD cut-off is used. Of course, single purification steps described above can be replaced by steps known to the person skilled in the art achieving the same or a comparable effect.

[0053] Finally, the UF/DF retentate is formulated and the concentration of the APG101 composition according to the present invention is adjusted to the desired protein concentration such as 20 ± 2 mg/ml.

[0054] Figure 2 illustrates the flow scheme of a preferred embodiment of a downstream process according to the present invention. As can be taken from Figure 3 the inventive downstream process is characterised by a number of advantages over downstream processes known from the prior art.

[0055] For example, after virus inactivation no holding step at a neutral pH value is required. With regard to the virus filtration step, a volumetric load ≤ 100 l/m^2 is possible compared to 37 g/m^2 known in prior processes. Further, using the formulation buffer of the present invention high protein concentrations such as 20 mg/ml can be reached compared to 10 mg/ml in PBS.

[0056] The method for producing a composition according to the present invention, which is described herein, results in APG101 isoforms in pI range of 4.0 to 8.5. A composition provided this way only contains very small amounts of unwanted higher molecular weight forms such as dimers or aggregates. APG101 isoforms provided this way are characterised by high amounts of sialic acid content as well as Fc-based N-terminal glycosylation comprising high amounts of fucosylated forms.

Figures

[0057]

Figure 1:
Comparison of the inventive upstream process with a non-inventive upstream process

Figure 2:
Flow scheme showing a preferred embodiment of the downstream process of the invention

Figure 3:
Comparison of the inventive downstream process with a non-inventive downstream process

Figure 4a:
IEF of AEX fraction of an APG101 mixture obtained by the inventive method.

Figure 4b:
IEF of AEX fraction of an APG101 mixture obtained by the non-inventive method.

Figure 5:
Schematic overview of the potency assay.

Figure 6:
In vitro biological activity (EC_{50}) of APG101 isoform mixtures obtained by the inventive method compared with APG101 obtained by non-inventive methods.

Figure 7:
Functional APG101 molecule.

Figure 8:
Thermofluorescence assay results of an APG101 composition buffer comparison.

Figure 9:
Thermofluorescence assay results of an APG101 composition excipient comparison.

Figure 10:
Analytical results of forced degradation stability study: Excipients (+) shows good and (-) shows poor performance of the buffer regarding APG101 stability.

Figure 11:
Thermofluorescence Assay Assessing the Thermal Stability of Two Batches APG101 from Apogenix in Direct Comparison to Commerically Available Fas/Fc.

Example 1

Method for producing a composition according to the invention

[0058] The method for providing a composition according to the present invention comprises an upstream process and a downstream process as defined above.

1. Upstream process

1.1 Batch definition

[0059] The composition comprising APG101 isoforms is produced in a fed-batch cultivation. Two vials of the master cell bank MCB1AGA are thawed. The viability of the third subcultivation has to be > 90%, the viable cell count has to be >1.5x10^6 cells/mL. If both vials fulfill these specifications, the culture with the higher viability is used for the fourth subcultivation and inoculation of the seed reactor. The culture with lower viability will be discarded after third subcultivation. The cell culture is expanded in shake flasks up to ≥ 4 L total volume before inoculating the first seed bioreactor. As first seed bioreactor a 50 L Xcellerex disposable bioreactor (XDR) is used. The cell culture is cultivated for three days before being transferred into the second seed reactor 200 L XDR. After another 3 days of cultivation, the 1000L production reactor is inoculated. Harvesting procedure is started at day 13 or earlier, if the viability drops below 61%.

1.2 Cell line

[0060] The utilized Master Cell Bank (MCB) is designated "MCB1AGA".

1.3 Thawing and subcultivations

[0061] Two cryo vials of the MCB are resuscitated consecutively. The following thawing procedure is applied for each vial: The cryo vials are thawed in a beaker with WFI at 36.8 °C (setpoint) until a small ice crystal remains. Cells are then transferred into approx. 10 mL of cooled (at 5 ± 3 °C) growth media (media no. 3001772, purchased from PAA), supplemented with 6 mM L-glutamine (final concentration) and 50 nM MTX (final concentration). To remove residual DMSO, a washing step in cooled (5 ± 3 °C) medium is performed via centrifugation. The cell pellet is resuspended in 50 mL of prewarmed (36.8±1 °C) medium after the centrifugation step. Cell concentration and viability are measured with the Cedex cell counter. This Out-of-Freeze culture is finally incubated in a shaker incubator with a working volume of 50 ml using 250 ml shake flasks.

[0062] The first and second subcultures are stability splits performed with a working volume of 120 ml (first subculture) and 150 mL (second subcultures) using 500 ml shake flasks. The third and fourth subcultures are the first expansion phase and performed in 2000 mL shake flasks with a working volume of 800 mL. For these inital four passages, the prewarmed (at 36.8 ± 1 °C) growth media (media no. 3001772, purchased from PAA), supplemented with 6 mM L-

glutamine and 50 nM MTX (final concentrations), is used as cultivation medium.

[0063] Measurement of cell concentration and viability is performed prior to each cultivation step using a Cedex cell counter. The next subculture is prepared depending on the cell growth.

[0064] At subculture no. 5, the shake flasks are pooled in a 5L glass bottle. This pool is sampled for cell concentration and viability. Depending on the actual VCC the required cell culture volume is then transferred into a 50 L seed bioreactor.

1.4 Seed Bioreactor (50L)

[0065] The 50 L seed bioreactor is equipped with a bottom-mounted magnetic drive agitator system and 1 mm sparger discs. Prior to inoculation the 50 L bioreactor is filled with approx. 20 L of growth media (media no. 3001772, purchased from PAA) supplemented with 6 mM L-glutamine (final concentration). These parameters apply to the medium pre-conditioning and to the seed train cultivation process.

[0066] When the process parameters are stable within their acceptable ranges the inoculum transfer is started. After inoculation the reactor is filled up with medium to a final working volume of 25 L. During cell mass expansion in the 50 L bioreactor no feed addition is applied to the process. The pH is controlled with CO2 via sparger. The oxygen level is controlled by submerse aeration with oxygen on demand. An overlay gas flow of air is applied to the headspace. Submerse aeration with pressurized air with a flow rate of 0.1 L/min, which can be adapted for adjusting pCO2, is performed. The expected cultivation time in the seed bioreactor is 3 days before inoculation of a 200 L seed bioreactor.

1.5 Seed bioreactor (200 L)

[0067] The 200 L seed bioreactor is equipped with a bottom-mounted magnetic drive agitator system and 1 mm sparger discs. Prior to inoculation the 200 L bioreactor is filled with approx. 100 L of growth media (media no. 3001772, purchased from PAA) supplemented with 6 mM L-glutamine (final concentration). These parameters apply to the medium pre-conditioning and to the seed train cultivation process.

[0068] When the process parameters are stable within their acceptable ranges the inoculum transfer is started. After inoculation, medium is added to a final working volume of 120L. During cell mass expansion in the 200 L bioreactor no feed addition is applied to the process. The pH is corrected with CO_2 gas. The oxygen level is controlled by submerse aeration with oxygen on demand. An overlay gas flow of air is applied to the headspace. Submerse aeration with pressurized air with a flow rate of 0.4 L/min, which can be adapted for adjusting pCO_2, is performed. The expected cultivation time in the seed bioreactor is 3 days before cells are

transferred into a 1000 L production bioreactor.

1.6 Fed-Batch Production Process

[0069] The production process of a composition comprising APG101 isoforms is a fed-batch cultivation. A 1000 L production bioreactor is equipped with a bottom-mounted magnetic drive agitator system and 1 mm sparger discs. Prior to inoculation the 1000 L bioreactor is filled with approx. 580 L growth medium (media no. 3001829, purchased from Becton Dickison) and supplemented with 6 mM L-glutamine (final concentration, calculated on the final starting volume of 720L). When the process parameters are within their acceptable ranges the inoculum transfer from the seed bioreactor to the production bioreactor is started. The target cell concentration after inoculation in the production bioreactor is $0.3*10^6$ viable cells/mL in a total volume of 720L. The required volume of the seed bioreactor cell culture is transferred to the production reactor, which is then filled up with growth medium (media no. 3001829, purchased from Becton Dickison) until the starting volume of 720L is reached. The cell culture is fed with Feedmedium A (PM30728) starting at day 3, and Glucose Feedmedium B (PM30729).

[0070] Daily feeding is started with Feed B separately, Feed A and Glucose can be fed simultaneously.

- Feedmedium B:

  Bolus feed starts at day 3 after sampling.

  Feeding rate day 3-6: 5.184 g/L/d (calculated on start volume of 720L)

  Feeding rate day 7-12: 2.592 g/L/d (calculated on start volume of 720L)

- Feedmedium A:

  Bolus feed starts at day 3 after sampling.

  Feeding rate day 3-5: 43.2 g/L/d (calculated on start volume of 720L)

  Feeding rate day 6-12: 21.6 g/L/d (calculated on start volume of 720L)

- D-Glucose Feed: Glucose is added when the actual D-glucose concentration is < 5 g/L starting at day 7. The concentration of D-glucose is adjusted to 5 g/L by adding the required amounts of D-Glucose feed.

[0071] The oxygen level is controlled by application of a oxygen controller cascade with 3 priorities: Priority 1 consists of a flow of process air on demand until an gas flow of 10 L/min is reached. Then the agitation (priority 2) is increased continuously until a stirring speed of 100

rpm is reached. The third priority consists of submerse sparging 02 on demand.

[0072] An air overlay flow is applied to the headspace.

[0073] The pH is controlled with CO_2. If necessary 1 M Na_2CO_3 is prepared to be added if necessary. Formation of foam is observed regularly and antifoam is added if necessary.

[0074] The harvesting procedure is started at cultivation day 13, or earlier if the viability drops below < 61% (Cedex). First step of the procedure is the sedimentation of cells, where the cell broth is cooled down to 10 ± 5 °C. When the temperature is below 20 °C, stirrer, aeration and pH control are switched off. After a minimum of 12 h of sedimentation the clarification step is started. Supernatant is clarified by a two step depth filtration and 0.2 µm filtration.

1.7 Sedimentation

[0075] The harvesting procedure is started by a sedimentation step. Culture broth is cooled down to finally 10 ± 5°C. When the temperature is < 20°C, a gitation, pO2 and pH-control are inactivated. After min. 12 h and max. 22 h hours of sedimentation, depth filtration is started.

1.8 Filtration

[0076] The depth filtration is performed with the Stax™ Disposable Depth Filter Systems from Pall, loading 7x PDK5 and 2x PDD1 depth filters. The clarification is followed by a 0.2 µm filtration. The depth filters are flushed with approx. 900 L PBS at a flux rate of ≤ 100 L/m2/h. The residual liquid is blown out of the system with air. Filtration process is run with a pump flow rate of ≤ 3,5 L/min and a maximum pressure of 1.0 bar. To increase the product recovery, the filters are rinsed afterwards with approx. 60L PBS pH 7,25 and blown out with pressurized air at a maximum pressure at 0.8 bar. Filtrated harvest is transferred directly through the wall duct into the GD suite, collected in a 1000L Mixtainer and stored at room temperature.

2. Downstream Process

[0077] For illustration purposes a description of the individual purification steps during the downstream process will be given in the following.

2.1 Protein A capture (C10)

[0078] The filtration material from above was transferred depth filtrated (0.2 µm) and tempered to 5 ± 3 °C. Prior to processing the harvest was split in four equal aliquots and stored

at room temperature over night to achieve final process temperature of 21 ± 3 °C. The processing of harvest was carried out without any further conditioning on in four cycles.

[0079] The elution was induced via a low pH step. The UV280 profiles of the four cycles were highly congruent and show the expected shape including the typical single peak. within the elution step.

[0080] The yields of the Protein A runs varied between 94 to 98 %. Hence, the product recovery of the Protein A runs was in the expected range. Furthermore, all recoveries were comparable with each other and confirmed the data acquired during process transfer and adaptation.

2.2 Virus inactivation (V10)

[0081] Immediately after collecting the Protein A eluate a fixed volume addition (specification: pH 3.5 ± 0.2) was executed with 20 mM citric acid within 5 min to inactivate enveloped viruses. The obtained virus inactivation solutions were incubated separately for 75 ± 15 min at room temperature (21 ± 3 °C). Finally, the pH of the inactivation solutions was adjusted to pH 5.0 ± 0.2 via addition of a fixed volume of 20 mM Na_3-Citrate to stop virus inactivation. The entire conditioning schemes were as follows:

Subsequently, the conditioned virus inactivation solutions were filtered via a 0.22 µm filter (Sartobran P) to separate potentially formed precipitates and to inhibit microbial growth in the process solution. Each conditioned virus inactivation batch was stored at 21 ± 3 °C and finally pooled prior to processing via the AIEX (C20) step.

2.3 AIEX (C20) -Column

[0082] Subsequently to the virus inactivation, pH adjustment and filtration the conditioned virus inactivation pool was processed over a Capto Q column in FT mode in two cycles. The method comprises the cleaning in place step 1 (buffer 0.5 M NaOH), an equilibration step (20 mM Na-citrate, pH 5), a load step of conditioned virus inactivation solution, a washing step (20 mM Na-citrate, pH 5.0), a regeneration step (20 mM Na-citrate, 1 M Na-Cl, pH 5.5), a cleaning in place step 2 (0.5 M NaOH) and a storage step (0.01 M NaOH).

[0083] The UV280 profiles of the two cycles are congruent and show the expected increase of the UV280 absorption profile during application of the conditioned Protein A eluate.

[0084] Each obtained flow through fraction was finally 0.22 µm filtered (Sartobran P) in order to address bioburden reduction. Afterwards, the separate fractions were pooled and stored at

21 ± 3 °C until further processing via the MMC step (C30). A comparison of AIEX fractions of a mixture of APG101 isoforms obtained by the inventive method and of APG101 obtained by a non-inventive method by IEF is shown in Figures 4a and 4b.

[0085] The yields of the AIEX runs were around 100 %.

2.4 MMC (C30) - Column

[0086] After the C20 step the AIEX product pool was processed over a Capto MMC column in three cycles. The method comprised a cleaning in place step (buffer 0.5 M NaOH), an equilibration step (20 mM Na-citrate, pH 5.0), a load step using the AIEX product/wash, a wash step (20 mM Na-citrate, pH 5.0), an elution step (50 mM Na-phosphate, 105 mM NaCl, pH 7.4), a regeneration step (3 M NaCl, pH 11), a cleaing in place step (0.5 M NaOH), a conditioning step (50 mM Na-phosphate, 105 mM NaCl, pH 7.4) and a storage step (20 mM Na-phosphate, 20% ethanol, pH 7.5).

[0087] The elution was induced via an increase of the pH. The UV280 profiles of the three cycles are highly congruent and show the expected shape including the typical single peak within the elution step.

[0088] Subsequently, the eluate fractions were each filtered over a 0.22 µm filter (Sartobran P) and stored at 21 ± 3 °C. Prior to further processing via the virus filtration step the particular Capto MMC eluate fractions were pooled. The yields of the MMC runs range around 100 %.

2.5 Virus filtration (I10)

[0089] Subsequent to the C30 step the Capto MMC eluate pool (1181 mL) was passed over a Durapore 0.1 µm filter (Millipak 20) prior to the virus filtration. The virus filtration was executed applying an aliquot of the 0.1 µm filtrate (887 mL) on a Planova 15N virus filter (100 cm^2) equilibrated with 50 mM PBS, pH 7.4 (Capto MMC elution buffer) at a working pressure of 0.8 ± 0.1 bar. The post-wash volume was 0.5 mL/cm^2 using equilibration buffer. Filter testing was done prior to filter usage based on detection of pressurized air bubbling. The filtrate flux remained constant during processing (ca. 22 L/m^2*h).

[0090] The virus filtration resulted in 99 % yield.

[0091] Subsequently, the residual 0.1 µm filtrate and the filtrate fraction of the virus filtration was pooled and stored at 5 ± 3 °C until further processing via subsequent UF/DF step.

2.6 Ultrafiltration/Diafiltration (I20)

[0092] Prior to diafiltration the I10 filtrate was concentrated on an ÄKTA Crossflow system to a protein concentration of 25.0 ± 2.0 mg_{APG101}/mL using two Pellicon 3 30 kDa cassettes. Afterwards, a diafiltration was executed to change the buffer system to the following buffer:

50 mM Na-Phosphate, 5 % Sorbitol, pH 6.5

[0093] The material was ultrafiltrated to the above mentioned concentration and diafiltrated by factor 7.0 ± 0.5. The parameters for the ultra- and diafiltration were:

Retentate flow: 450 L/(m^{2*}h)

TMP: 1.2 ± 0.1 bar

[0094] The UF/DF yielded in 97 % product recovery.

2.7 Drug substance concentration adjustment

[0095] For final concentration adjustment a defined volume (107 mL) of the formulation buffer (50 mM Na-Phosphate, 5 % Sorbitol, pH 6.5) was added to the UF/DF retentate pooL. The final drug substance concentration obtained by A280 was 20.4 mg/mL. Finally, the drug substance was 0.22 µm filtered (Sartobran P). Subsequently, aliquots of the drug substance with a volume of 200 µL were bottled in 500 µL vials.

2.8 Total yield

[0096] The step and total yields obtained from ProA-HPLC and A280 analysis are listed in Table 1. The sampling of the target molecule was not taken into account for the calculation of the yields.

Table 1: Overview of step yield

Sample	Total yield (%)	
Protein A capture (C10)	$96^{\#1}$	$101^{\#2}$
Virus inactivation (V10)	90	94
AIEX (C20)	90	94
MMC (C30)	90	94
Virus filtration (I10)	89	93

Sample	Total yield (%)	
UF/DF (I20)	86	90
Formulation (I30)	86	90
#1 Load determined via ProA-HPLC harvest method, Eluate via ProA-HPLC. #2 Load determined via ProA-HPLC harvest method, Eluate via A280.		

[0097] The identity of the mixture of APG101 isoforms was confirmed via nonreducing SDS Page and IEF. The isoform pattern showed additional basic bands compared to the reference material and a slight shift in the isoform distribution towards acidic pI.

[0098] This is shown, for example, by a comparison of an IEF gel of AX fractions obtained by the method of the present invention and an APG101 mixture obtained by the non-inventive method according to Figure 1.

[0099] The APG101 isoform mixture of the present invention further differs in the presence of carbohydrates (N-glycans sialic acid).

Carbohydrates (antennarity/N-glycans)

[0100] In Table 2 the analysis of the carbohydrate structure is summarized. Despite a comparable carbohydrate structure between the reference material and the inventive composition the distribution of carbohydrate structures differs.

Table 2: N-glycans (carbohydrates) analysis result

Peak/Sample	Reference material (Mol-%)	Inventive composition (Mol-%)
cF1GN2	34.1	18.2
cF1GN2G1	19.2	17.8
cF1GN2G3	30.1	51.5
cF1GN2G3	9.1	7.6
Other	7.6	4.9

Carbohydrates (sialic acids)

[0101] The analysis of the amount of sialic acid per mol of APG101 of the inventive composition is summarized in Table 3.

Table 3: Sialic acid (carbohydrate) analysis

Sample	Sialic acid content (mol NeuAc / mol APG101)
Inventive composition	5.1
Reference material	3.9

[0102] The reference material always relates to an APG101 mixture which was not produced by the method of the present invention.

[0103] Finally, an assay was carried out to measure the bioactivity of the mixture according to the present invention comprising APG101 isoforms.

3. Method for the determination of the in vitro potency of APG101 isoforms

[0104] A cellular assay with a Jurkat A3 permanent T-cell line is used for the determination of biological activity of the APG101. This potency assay is schematically shown in Figure 5.

[0105] With this apoptosis assay employing Jurkat A3 cells, EC50 values for the inhibition of APG293 (=CD95L-T4; 250 ng/ml) induced apoptosis by APG101 are determined.

[0106] In brief, Jurkat A3 cells are grown in flasks with RPMI 1640-medium + GlutaMAX (GibCo) supplemented with 10 % FBS, 100 units/ml Penicillin and 100 µg/ml Streptomycin. 100,000 cells are seeded per well into a 96-well microtiter plate. CD95L-T4 (APG293) at a constant concentration of 250 ng/ml is incubated in a separate 96-well microtiter plate for 30 minutes at 37°C with different concentrations of APG101. The addition of the APG101/CD95L-T4 mixture to the cells is followed by 3 hours incubation at 37°C. Cells are lysed by adding lysis buffer (250 mM HEPES, 50 mM $MgCl_2$, 10 mM EGTA, 5 % Triton-X-100, 100 mM DTT, 10 mM AEBSF, pH 7.5) and plates are put on ice for 30 minutes to 2 hours. Apoptosis is paralleled by an increased activity of Caspases (e.g. caspases 3 and 7). Hence, cleavage of the Caspase substrate Ac-DEVD-AFC is used to determine the extent of apoptosis. In fact, caspase activity correlates with the percentage of apoptotic cells determined morphologically after staining the cells with propidium iodide and Hoechst-33342.

[0107] For the caspase activity assay, 20 µl cell lysate is transferred to a black 96-well microtiter plate. After the addition of 80 µl buffer containing 50 mM HEPES, 1 % Sucrose, 0.1 % CHAPS, 50 µM Ac-DEVD-AFC, and 25 mM DTT, pH 7.5, the plate is transferred to a Tecan microtiter plate reader and the increase in fluorescence intensity over a given time frame is monitored (excitation wavelength 400 nm, emission wavelength 505 nm). Employing the GraphPad Prism software, EC50 values for APG101 (i.e. reduction of apoptosis induction of the given concentration of CD95L by 50%) are calculated.

[0108] Determination of the biological activity of APG101 employing the potency assay enables:

- a high specificity. Via its interaction with CD95, CD95L-T4 induces apoptosis on Jurkat A3 cells. The CD95/CD95L-T4 interaction is specifically blocked by the addition of APG101.
- the use of a relevant cellular system; induction of apoptosis is one important physiological feature of the CD95/CD95L signalling and can be monitored in the well characterised human T-cell line Jurkat A3.
- a high sample throughput due to the application of 96 well microtiter plates and short incubation times.

[0109] Figure 6 shows the biological activity (EC50) of APG101 isoforms mixtures obtained by the inventive method (inventive samples) compared to APG101 obtained by non-inventive methods. The activity is comparable.

4. Thermofluorescence assays of APG101 formulation

[0110] The stability of the formulations according to the present invention was confirmed by thermofluorescence assays (cf. Figures 8 and 9).

[0111] The determination of thermal transition points of APG101 were carried out via thermofluorescence (TF) assays. Thereby the fluorescent dye binds to hydrophobic patches of the protein. During temperature increase the protein unfolds and more dye can bind which results in an increase of the fluorescent signal. Therefore higher thermal transition points (melting temperatures, Tm) indicate more stable conditions for APG101. The assay setup is shown in Table 2.

Table 2: Thermofluorescence assay setup

Parameter	Value
Dye	Sypro Orange (Sigma)
Dye concentration	1:1000
Sample volume	50 µL
Sample concentration	100 µg/mL
Temperature gradient	35 °C to 95 °C
Temperature steps	0.2 °C to 0.5 °C
Holding time	10s

[0112] The thermal transition point was defined to be the inflection point of the fluorescent signal increase during increase of temperature. The stability of APG101 in different buffer systems was tested (Figure 8).

[0113] Via thermofluorescence assays the thermal transition points of APG101 were determined with starting material 3 regarding different excipients, namely sugar, poly-alcohol, amino acids and polyglycol. The experimental approach was identical as described above. The results are shown in Figure 9. The Tm values were increased with increasing saccharose, sorbitole and glycine concentrations. Addition of glycylglycine (Gly-Gly) or PEG showed no positive stabilizing effects.

[0114] Further, a thermofluorescence assay was done, to compare a APG101 batches prepared according to the invention with commercially available Fas/Fc sample. A sample concentration (APG101 or Fas/Fc) of 50 µg/ml was employed.

[0115] The thermofluorescence assay clearly reveals the superiority of the two batches APG101 of the invention (batch F10176 and batch 1011626) compared to commercially available Fas/Fc from Sigma with regard to thermal stability (Figure 11).

SEQUENCE LISTING

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Leu Glu Gly Leu His His Asp Gly Gln Phe Cys His Lys Pro Cys Pro
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Pro Gly Glu Arg Lys Ala Arg Asp Cys Thr Val Asn Gly Asp Glu Pro
65                  70                  75                  80

Asp Cys Val Pro Cys Gln Glu Gly Lys Glu Tyr Thr Asp Lys Ala His
                85                  90                  95

Phe Ser Ser Lys Cys Arg Arg Cys Arg Leu Cys Asp Glu Gly His Gly
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Leu Glu Val Glu Ile Asn Cys Thr Arg Thr Gln Asn Thr Lys Cys Arg
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REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

- WO2004085478A [0002]

Patentkrav

1. Farmaceutisk sammensætning omfattende en blanding af APG101-isoformer hvor umodificeret APG101 repræsenteres af SEQ ID NO:1, hvor nævnte blanding omfatter APG101-varianter N-terminalt trunkerede og hvis første aminosyre er aminosyre 17, 21 og 26 i forhold til SEQ ID NO:1 og mindre end 1 molprocent umodificeret APG101 i henhold til SEQ ID NO:1 og fordelt inden for et pI-område på 4,0 - 8,5, og hvor nævnte blanding af APG101-isoformer er opnåelig med en fremgangsmåde omfattende trinnene:

(a) at fremstille en blanding af APG101-isoformer med en fed-batch-fremstillingsfremgangsmåde, som tilvejebringer en cellehøst under anvendelse af et pepton-indeholdende basalmedie, og

(b) at isolere blandingen af APG101-isoformer fra cellehøsten,

hvor trin (a) omfatter en serie af dyrkningstrin af en given master-cellebatch, indtil relevante høstparametre nås, efterfulgt af cellesedimentering og -filtrering af fusionsprotein indeholdende supernatant, hvor de relevante høstparametre er en APG101-titer inden for et område af 0,5 g/l - 5 g/l og en celledensitet på omkring 1 x 10^6 - 1 x 10^8 celler/ml og
trin (b) omfatter indfangningskromatografi, virusinaktivering, en serie af anion- og/eller kationkromatografi, virusfiltrering og justering til en ønsket endelig proteinkoncentration.

2. Den farmaceutiske sammensætning ifølge krav 1, hvor pI-området er 4,5 – 7,8, fortrinsvis 5,0 - 7,5.

3. Den farmaceutiske sammensætning ifølge krav 1 eller 2, omfattende 0,0-5,0 molprocent af APG101-former med høj molekylevægt, såsom dimerer og/eller aggregater.

4. Den farmaceutiske sammensætning ifølge et hvilket som helst af kravene 1-3, omfattende en mængde af sialinsyrer fra 4,0 til 7,0 mol NeuAc/mol APG101.

5. Den farmaceutiske sammensætning ifølge et hvilket som helst af kravene 1-4, omfattende N-terminalt blokerede APG101-varianter, såsom APG101-varianter blokeret ved pyro-Glu modifikation og/eller omfattende APG101-varianter med en fri N-terminus.

6. Den farmaceutiske sammensætning ifølge krav 5, omfattende 80-99 molprocent N-terminalt blokerede APG101-varianter og/eller 1-20 molprocent APG101-varianter med en fri N-terminus.

7. Formulering omfattende en farmaceutisk sammensætning ifølge et hvilket som helst af kravene 1-6.

8. Formuleringen ifølge krav 7,

(a) yderligere omfattende phosphat, fortrinsvis omkring 20 mM til omkring 100 mM phosphat, mere fortrinsvis omkring 50 mM phosphat,

(b) yderligere omfattende et viskositetsfremmende middel, såsom sorbitol, fortrinsvis omkring 0,1-10 vægtprocent viskositetsfremmende middel, mere fortrinsvis omkring 5 vægtprocent viskositetsfremmende middel, og/eller

(c) med en pH-værdi i området på 4-8.

9. Fremgangsmåde til at fremstille en sammensætning ifølge et hvilket som helst af kravene 1-6 omfattende trinnene:

(a) at fremstille en sammensætning ifølge et hvilket som helst af kravene 1-6 med en fed-batch-fremstillingsfremgangsmåde, som tilvejebringer en cellehøst under anvendelse af et pepton-indeholdende basalmedie, og

(b) at isolere af sammensætningen ifølge et hvilket som helst af kravene 1-6 fra cellehøsten,

hvor trin (a) omfatter en serie af dyrkningstrin af en given master-cellebatch, indtil relevante høstparametre nås, efterfulgt af cellesedimentering og -filtrering af fusionsprotein indeholdende supernatant, hvor de relevante høstparametre er en APG101-titer inden for et område af 0,5 g/l - 5 g/l og en celledensitet på

omkring 1 x 10^6 - 1 x 10^8 celler/ml og

trin (b) omfatter indfangningskromatografi, virusinaktivering, en serie af anion- og/eller kationkromatografi, virusfiltrering og justering til en ønsket endelig proteinkoncentration.

DRAWINGS

Figure 1

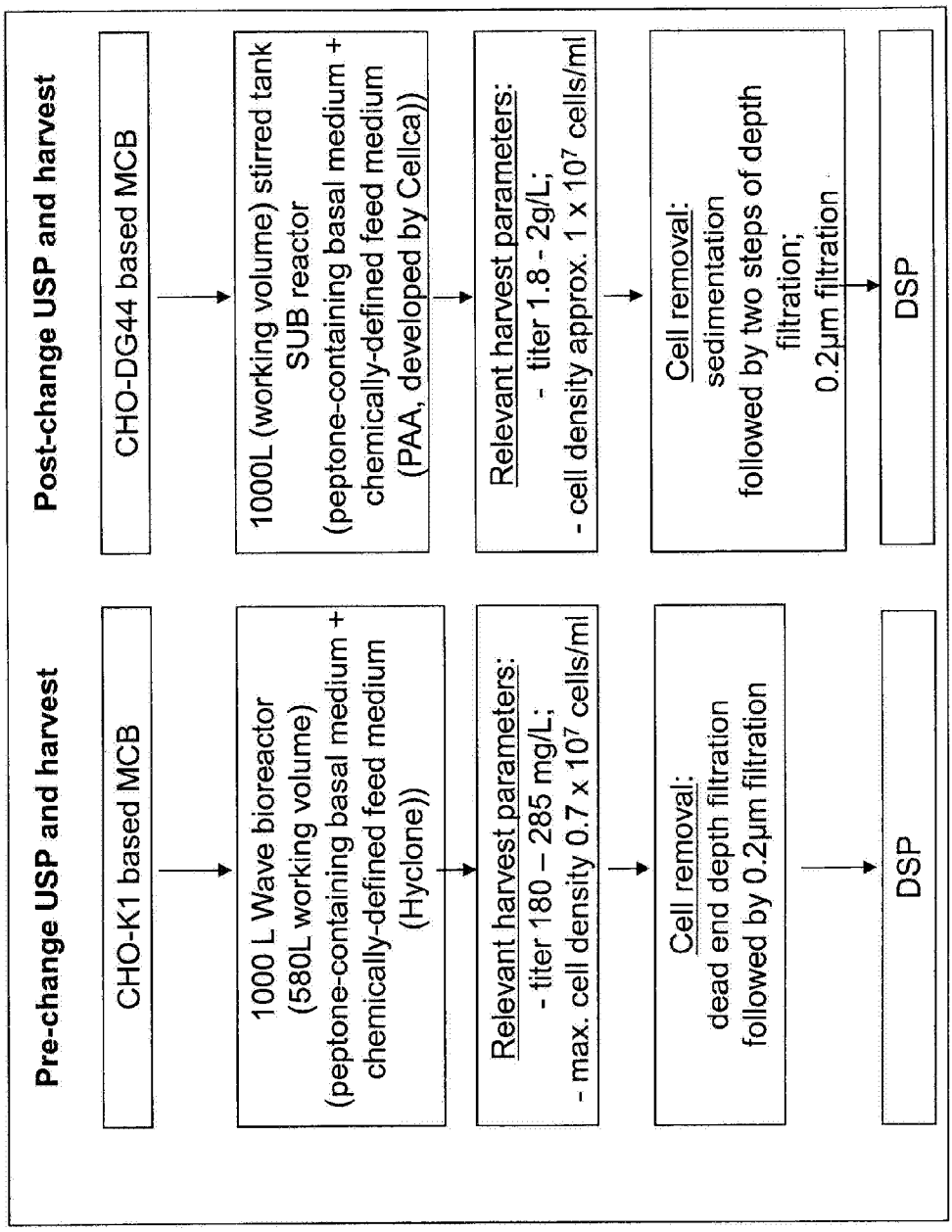


Figure 2

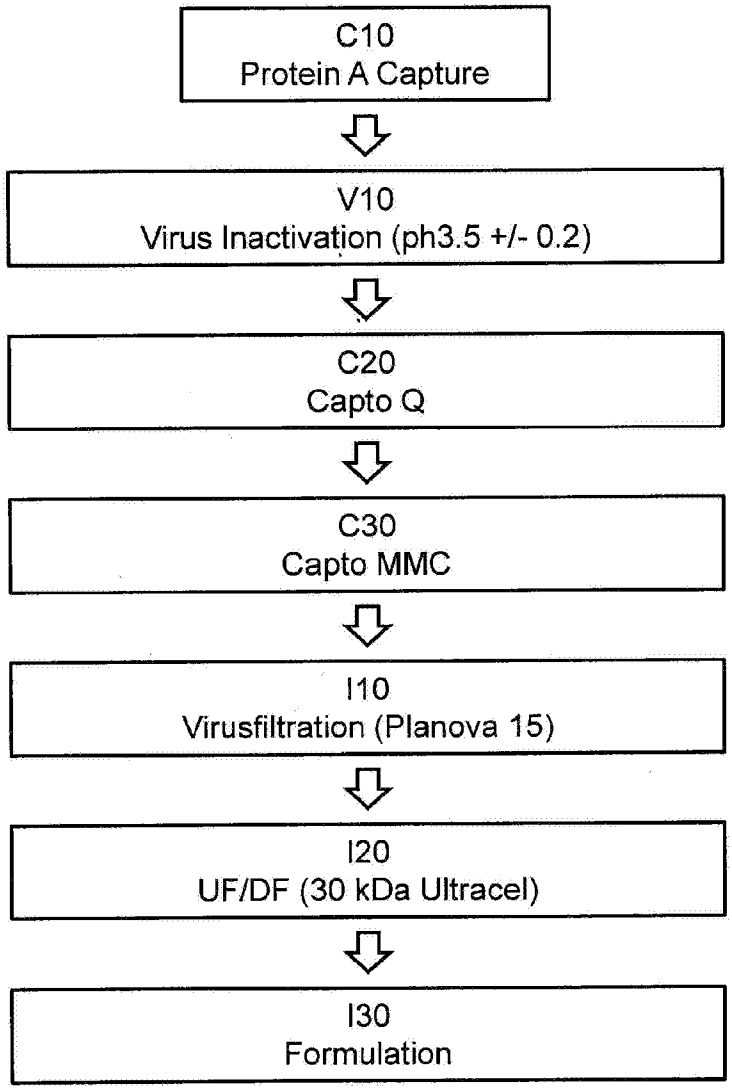

C10
Protein A Capture
V10
Virus Inactivation (ph3.5 +/- 0.2)
C20
Capto Q
C30
Capto MMC
I10
Virusfiltration (Planova 15)
I20
UF/DF (30 kDa Ultracel)
I30
Formulation

Figure 3

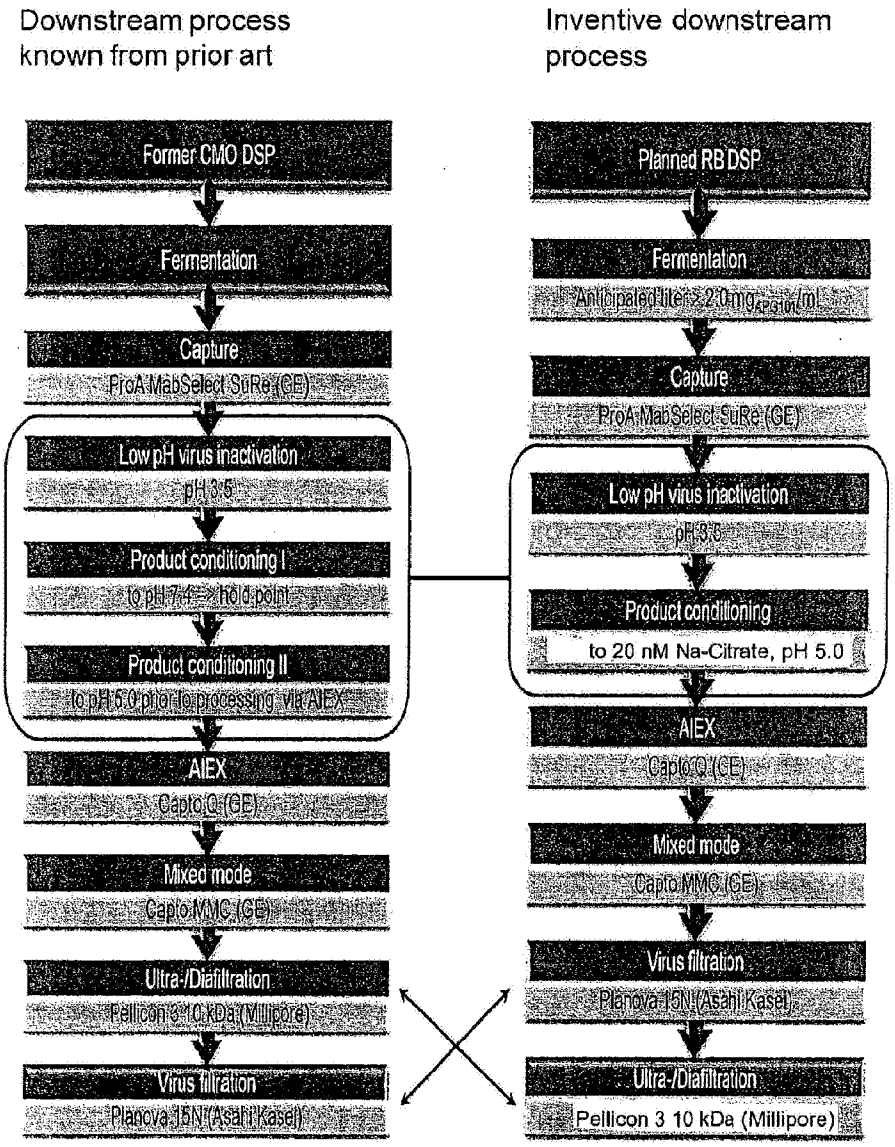

Downstream process known from prior art
Former CMO DSP
Fermentation
Capture
ProA MabSelect SuRe (GE)
Low pH virus inactivation
pH 3.5
Product conditioning I
to pH 7.4 – hold point
Product conditioning II
to pH 5.0 prior to processing via AIEX
AIEX
Capto Q (GE)
Mixed mode
Capto MMC (GE)
Ultra-/Diafiltration
Pellicon 3 10 kDa (Millipore)
Virus filtration
Planova 15N (Asahi Kasei)
Inventive downstream process
Planned RB DSP
Fermentation
Anticipated titer > 2.0 mg$_{AP01/ml}$
Capture
ProA MabSelect SuRe (GE)
Low pH virus inactivation
pH 3.6
Product conditioning
to 20 nM Na-Citrate, pH 5.0
AIEX
Capto Q (GE)
Mixed mode
Capto MMC (GE)
Virus filtration
Planova 15N (Asahi Kasei)
Ultra-/Diafiltration
Pellicon 3 10 kDa (Millipore)

Figure 4a

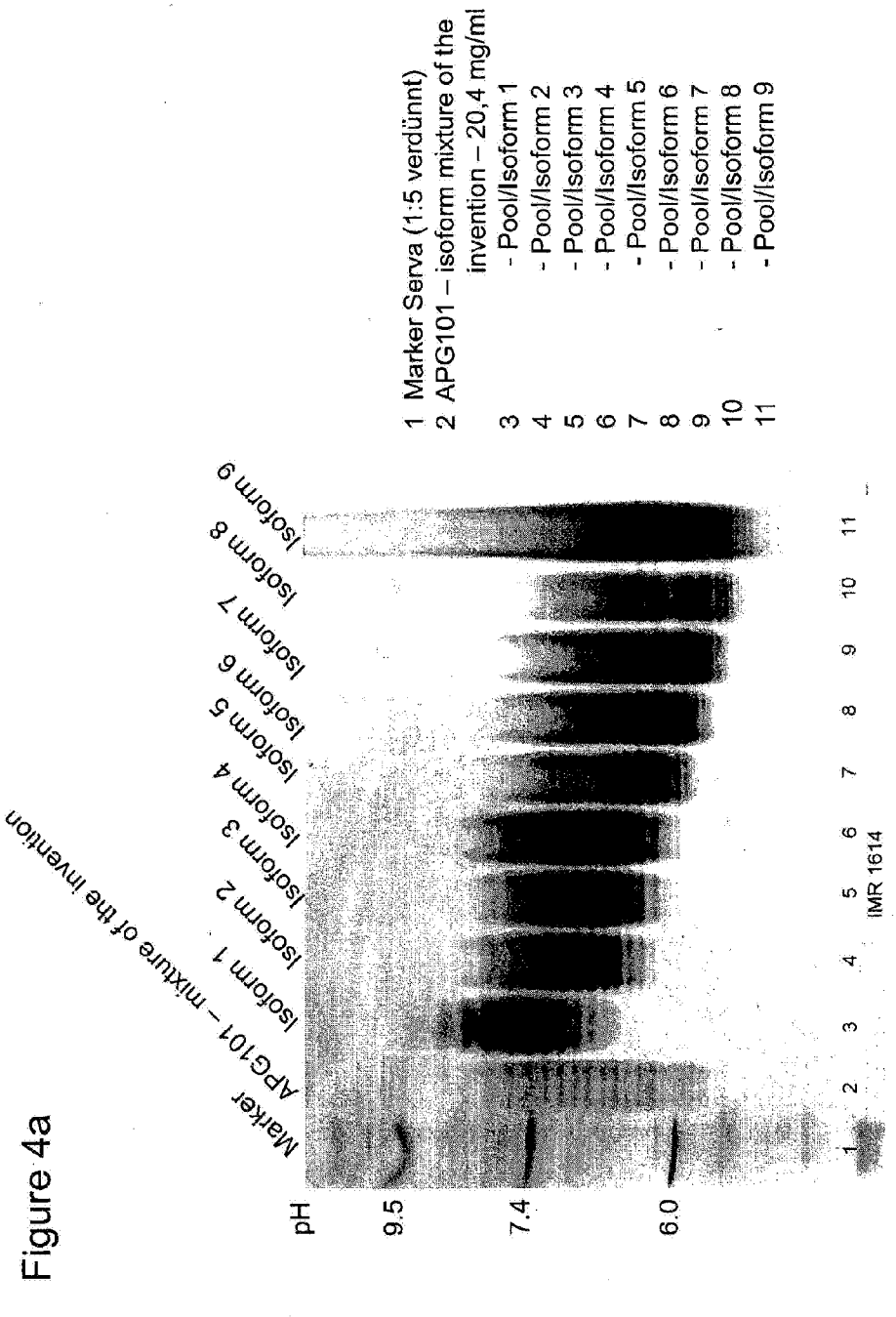

1 Marker Serva (1:5 verdünnt)
2 APG101 – isoform mixture of the invention – 20,4 mg/ml
3 - Pool/Isoform 1
4 - Pool/Isoform 2
5 - Pool/Isoform 3
6 - Pool/Isoform 4
7 - Pool/Isoform 5
8 - Pool/Isoform 6
9 - Pool/Isoform 7
10 - Pool/Isoform 8
11 - Pool/Isoform 9
pH
9.5
7.4
6.0
1 2 3 4 5 6 7 8 9 10 11
IMR 1614
Marker
APG101 – mixture of the invention
isoform 1
isoform 2
isoform 3
isoform 4
isoform 5
isoform 6
isoform 7
isoform 8
isoform 9

Figure 4b

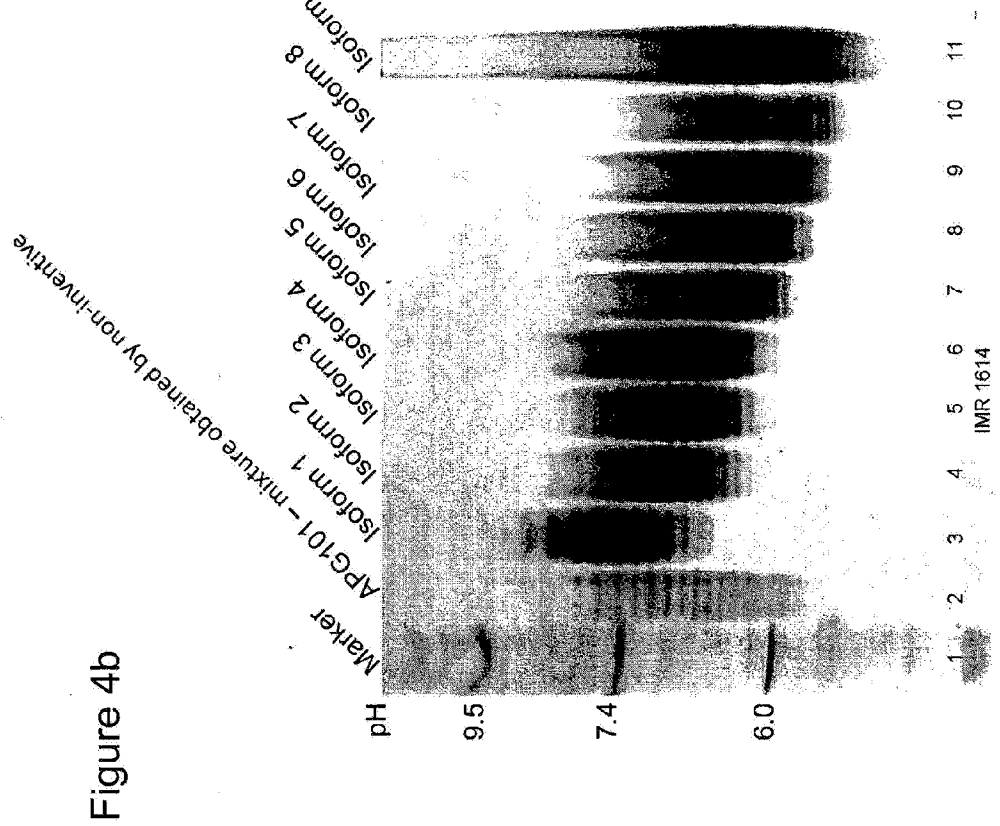
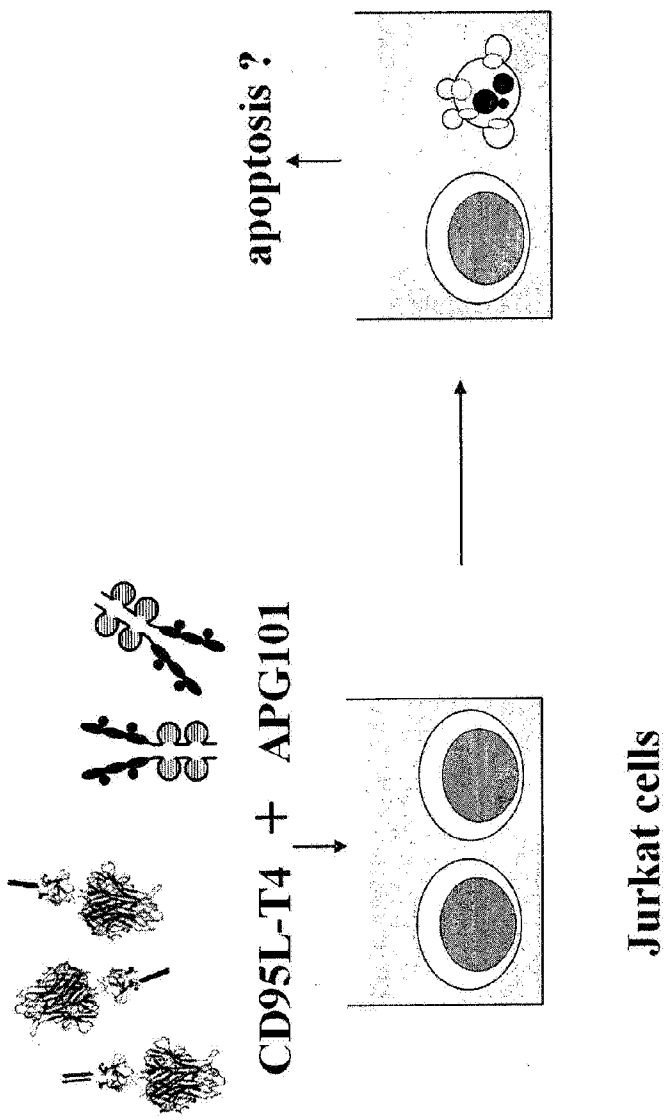

1 Marker Serva (1:5 verdünnt)
2 APG101 – mixture obtained by non-inventive method
3 – Pool / Isoform 1
4 – Pool / Isoform 2
5 – Pool / Isoform 3
6 – Pool / Isoform 4
7 – Pool / Isoform 5
8 – Pool / Isoform 6
9 – Pool / Isoform 7
10 – Pool / Isoform 8
11 – Pool / Isoform 9
pH
9.5
7.4
6.0
Marker
APG101 – mixture obtained by non-inventive
Isoform 1
Isoform 2
Isoform 3
Isoform 4
Isoform 5
Isoform 6
Isoform 7
Isoform 8
Isoform 9
1 2 3 4 5 6 7 8 9 10 11
IMR 1614

Figure 5



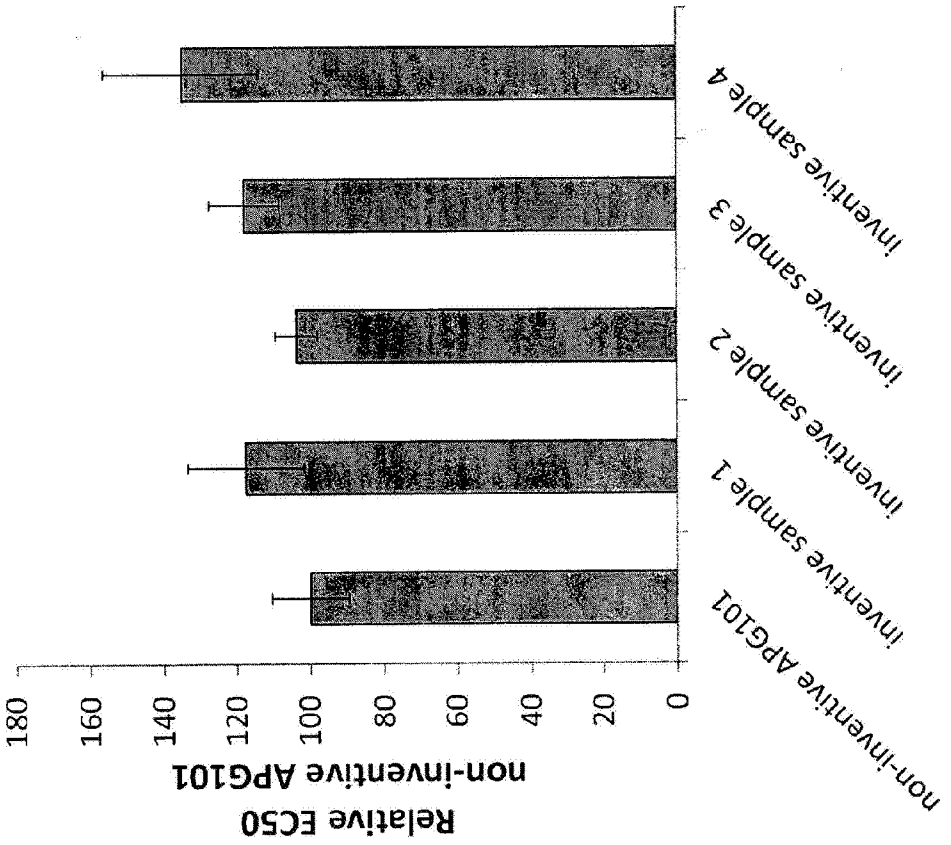

Relative EC50
non-inventive APG101
180
160
140
120
100
80
60
40
20
0
non-inventive APG101
inventive sample 1
inventive sample 2
inventive sample 3
inventive sample 4

Figure 6

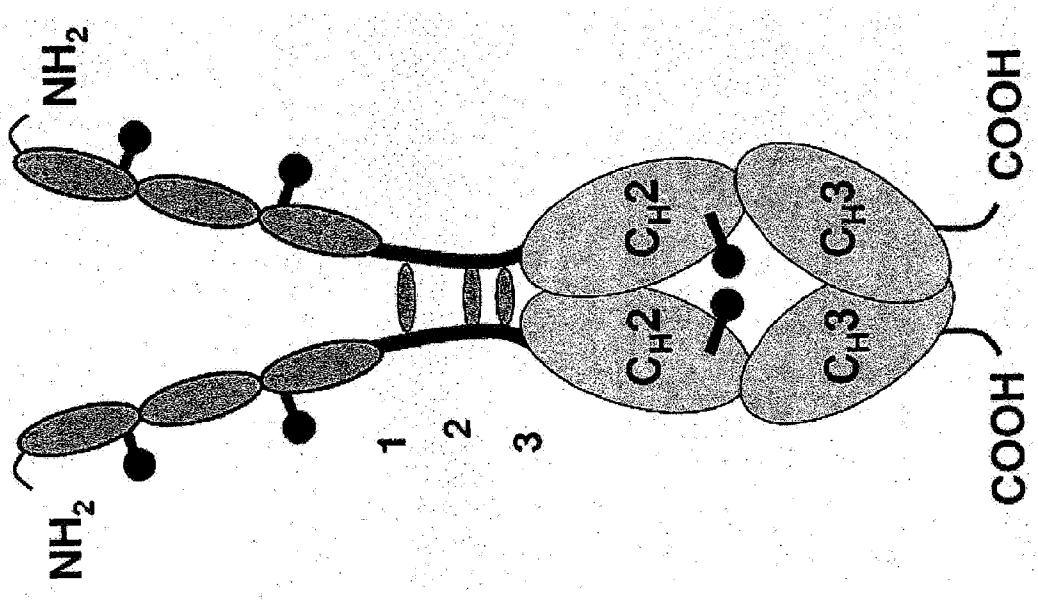


Figure 7

Figure 8

Buffer		pH 7.5	pH 7.0	pH 6.5	pH 6.0	PBS
Histidine / HCl	50 mM	67.3	67.0	66.5	N/A	67.1
	30 mM	66.9	66.9	66.2		
	10 mM	66.9	66.8	66.4		
	5 mM	N/A	66.9	66.5		
50 mM Histidine	25 mM NaCl	67.1	67.1	66.2	N/A	
	50 mM NaCl	67.5	67.0	66.2		
	100 mM NaCl	67.7	67.0	66.3		
L-Histidine / Phosphate	50 mM	67.3	67.0	66.5	N/A	
	30 mM	66.9	66.9	66.2		
	10 mM	66.9	66.8	66.4		
	5 mM	N/A	66.9	66.5		
Na-Acetate	150 mM	67.3	67.5	67.6	N/A	67.3
	50 mM	67.3	67.4	67.5		
	30 mM	67.2	67.4	67.3		
	10 mM	66.9	67.1	67.2		
	5 mM	67.1	67.1	66.9		
Na-Phosphate	50 mM	67.3	67.5	67.5	67.0	67.1
	30 mM	67.4	67.5	67.3	67.0	
	10 mM	67.4	67.3	67.2	66.9	
	5 mM	67.3	67.3	67.1	66.9	
Na- Succinate	50 mM	67.5	67.5	67.4	N/A	67.3
	30 mM	67.5	67.5	67.5		
	10 mM	67.4	67.4	67.3		
	5 mM	67.5	67.5	67.3		
Arginine / Phosphate	50 mM	67.3	67.3	67.2	N/A	
	30 mM	67.3	67.4	67.4		
	10 mM	67.4	67.3	67.4		
	5 mM	67.4	67.5	67.3		
Na-Citrate	50 mM	N/A				67.3
	30 mM	67.0	67.3	66.8	66.1	
	10 mM	67.3	67.5	67.2	66.9	
	5 mM	67.2	67.1	67.1	66.9	

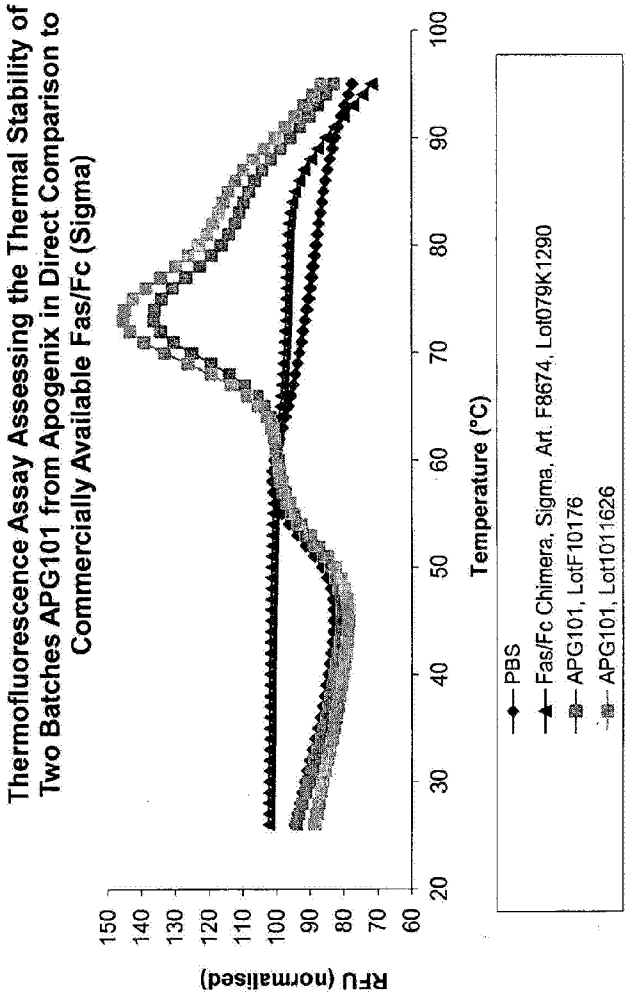
Figure 9

Excipients		Saccharose		Sorbitole		Glycine		Gly-Gly		PEG 6000	
concentration	0.0 %	2.5 %	5.0 %	2.5 %	5.0 %	125 mM	250 mM	5.0 %	10.0 %	2.5 %	5.0 %
150 mM Na-Acetate, pH 6.5	66.6	68.1	N/A	68.1	N/A	67.7	N/A	66.4	N/A	67.3	N/A
50 mM Histidine / 100 mM NaCl, pH 7.5	67.5	68.1	68.3	68.1	68.7	67.9	68.1	66.7	66.4	67.3	66.7
30 mM Na-Succinate, pH 7.0	67.6	68.0	68.5	68.1	68.3	67.8	68.3	66.3	66.1	67.2	66.8
30 mM Na-Phosphate, pH 6.5	67.3	67.8	68.3	67.7	68.3	67.6	68.0	66.7	66.1	67.5	67.2
50 mM Na-Phosphate, pH 6.5	66.7	67.8	68.3	67.9	68.5	67.9	68.1	67.1	66.7	67.5	67.3

Figure 10

	Buffer	SE-HPLC (%)			MFI	SDS-PAGE. red.	IEF
		Aggregates	Homodimer	Fragments	Particles (#/mL)	Colloidal blue	
1	PBS (unstressed control)	4.98	95.02	n.d.	15011		
2	50 mM Histidine, 100 mM NaCl, 5.0 % Sorbitole, pH 7.5	4.13	94.48	1.39	13432	-	-
3	50 mM Histidine, 100 mM NaCl, 5.0 % Sorbitole, 20 µM EDTA, pH 7.5	4.17	94.55	1.28	7051	-	-
4	30 mM Na-Phosphate, 5 % Saccharose, pH 6.5	6.18	92.85	0.96	5386	-	+
5	30 mM Na-Phosphate, 5 % Sorbitole, pH 6.5	5.95	93.22	0.83	9769	+	+
6	50 mM Na-Phosphate, 5 % Sorbitole, pH 6.5	5.72	93.43	0.85	7056	+	+
7	50 mM Na-Phosphate, 250 mM Glycine, pH 6.5	5.08	94.24	0.68	8629	+	-

Figure 11


Thermofluorescence Assay Assessing the Thermal Stability of Two Batches APG101 from Apogenix in Direct Comparison to Commercially Available Fas/Fc (Sigma)
RFU (normalised)
150
140
130
120
110
100
90
80
70
60
20
30
40
50
60
70
80
90
100
Temperature (°C)
PBS
Fas/Fc Chimera, Sigma, Art. F8674, Lot079K1290
APG101, LotF10176
APG101, Lot1011626