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Dosage units and regimen, uses, methods or formulations of compositions comprising a recombinant protein comprising interleukin-12 and an antibody binding the extra-domain B of fibronectin

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M. MATASCI: "Abstract 5553: A novel immunocytokine for the treatment of cancer", CANCER RESEARCH, 1 July 2018 (2018-07-01), XP055720611, Retrieved from the Internet [retrieved on 20200806]
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(54) Title: DOSAGE UNITS AND REGIMEN, USES, METHODS OR FORMULATIONS OF COMPOSITIONS COMPRISING A RECOMBINANT PROTEIN COMPRISING INTERLEUKIN-12 AND AN ANTIBODY BINDING THE EXTRA-DOMAIN B OF FIBRONECTIN

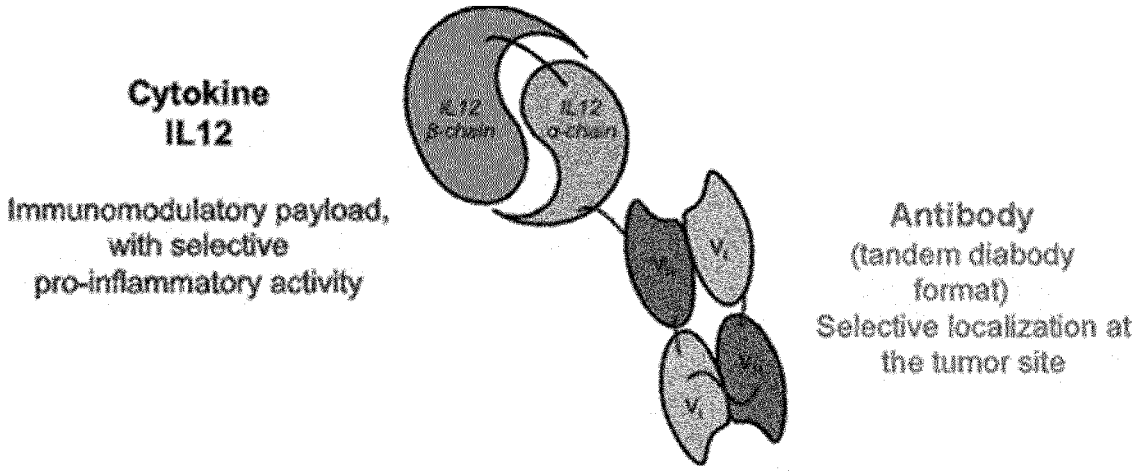

Figure 3

(57) Abstract: The present invention relates to a dosage unit or regimen, recombinant protein for use, method or formulation of a recombinant protein comprising interleukin-12 (IL-12) and an antibody binding the extra-domain B (ED-B) of fibronectin, or a target binding fragment or derivative thereof.

[Continued on next page]

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Dosage units and regimen, uses, methods or formulations of compositions comprising a recombinant protein comprising interleukin-12 and an antibody binding the extra-domain B of fibronectin

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Field of the invention

The present application relates to dosage units and regimen, uses, methods or formulations of compositions comprising a recombinant protein comprising (i) interleukin-12 (IL-12) and (ii) an antibody binding the extra-domain B (ED-B) of fibronectin, or a target-binding fragment or derivative thereof.

Incorporation by Reference

All publications, patents, patent applications and other documents cited in this application are hereby incorporated by reference in their entireties for all purposes to the same extent as if each individual publication, patent, patent application or other document were individually indicated to be incorporated by reference for all purposes. In the event that there are any inconsistencies between the teachings of one or more of the references incorporated herein and the present disclosure, the teachings of the present specification are intended.

Background

IL-12 is a heterodimeric cytokine comprising two disulfide-linked subunits, p35 and p40. IL-12 stimulates the production of IFNγ from T-cells and natural killer cells, and also induces

differentiation of Th1 helper cells. IL-12 is a key mediator of innate and cell-mediated immunity, with the potential for anti-cancer and anti-metastatic activity.

Like many other cytokines, however, the administration of IL-12 is associated with severe toxicity (Car et al., 1999), even at doses as low as 1μg per kg per day, discouraging its development as an anticancer drug.

A number of cytokines have shown beneficial effects in preclinical animal models of cancer and immune disorders and represent promising agents for therapy. However, despite encouraging results, only few cytokines are approved as drugs (e.g., interleukin 2 (IL2, Proleukin®), tumor necrosis factor (TNF, Beromun®), interferon alpha (IFNα, Roferon A® and Intron A®)). (Gutbrodt and Neri, 2012). Current indications in cancer include metastatic renal cell cancer, malignant melanoma, hairy cell leukemia, chronic myeloid lymphoma, sarcoma and multiple myeloma, either as single agents or in combination with chemotherapy. In addition, certain cytokines are used for the treatment of viral and bacterial infections in the clinic and are administered to patients suffering from chronic inflammatory conditions. Unfortunately, considerable toxicities can be observed at low doses, which prevent escalation to therapeutically active regimens.

Finding the right dose or dosage regimen for an immunocytokine which is for human therapy is complicated. The skilled artisan cannot rely on experience made with other biopharmaceutical drugs, like e.g. antibodies, due to the completely different modes of action, and the added toxicity issue. Hence, it is one object of the present invention to find the right dose or dosage regimen for an immunocytokine comprising (i) interleukin-12 (IL-12) and (ii) an antibody binding the extra-domain B (ED-B) of fibronectin.

The formulation of recombinant proteins for therapeutic use, like, *e.g.*, antibodies, is a complex optimization process utilizing unique pharmaceutical additives to address the varying demands of storage and route of administration necessary for the clinical application.

Immunocytokines are recombinant proteins comprising a protein-based binding molecule, mostly an antibody, and a cytokine. On that basis, immunocytokines vary significantly, in their chemico-physical properties, from antibodies. This applies to, for example, the domain structure, the molecular weight, or the number of inter- and intrachain disulfide bridges, and so

on. As a consequence, the solubility, the aggregation behavior, and the pharmacodynamics of immunocytokines may vary significantly from those known from antibodies. Lessons learned from antibodies can hence not simply be transferred to immunocytokines.

All this makes clear that finding the formulation for an immunocytokine which is for human administration is not straightforward. Hence, it is one other object of the present invention to find a formulation for an immunocytokine comprising (i) interleukin-12 (IL-12) and (ii) an antibody binding the extra-domain B (ED-B) of fibronectin.

Brief description of the drawings

The term IL12-L19L19 designates the recombinant protein comprising (i) interleukin-12 (IL-12) and (ii) an antibody binding the extra-domain B (ED-B) of fibronectin, or a target binding fragment or derivative thereof, which is provided in the single chain diabody format (also called "tandem diabody") as explained herein. The recombinant protein comprises the amino acid according to SEQ ID NO: 9.

Table 1. IL12-L19L19 Formulation Study. Storage temperatures, time-points, assays and acceptance criteria are detailed.

Table 2. IL12-L19L19 Formulation Study. Summary of the steps yield (formulation and concentration steps) and of the single chain diabody purity, evaluated by SEC both after formulation and after concentration up to 2mg/ml.

Table 3. IL12-L19L19 Formulation Study. A_{280nm} and SEC results collected for each sample and for each time-point, both after stressing conditions (i.e. 3 repeated cycles of freezing/thawing) and at 2-8°C storage temperature.

Figure 1. IL12-L19L19 Formulation Study. SEC analysis of the different formulated samples, before and after the concentration step.

Figure 2. IL12-L19L19 Formulation Study. SEC analysis of the different formulated samples, before and after the concentration step.

Table 4. IL12-L19L19 Formulation Study. Stability study under stress conditions: evaluation of the loss in A_{280nm} after the 3rd cycle of freezing/thawing and classification of the samples from the best to the worst, based on this loss.

Table 5. IL12-L19L19 Formulation Study. Stability study at 2-8°C: evaluation of the loss in A_{280nm} after 14 days of storage and classification of the samples from the best to the worst, based on this loss.

Figure 3. Schematic drawing of IL12-L19L19. The molecule consists of a single-chain polypeptide consisting of the two subunits of the immunomodulatory payload IL12 fused to a human vascular targeting antibody in a single-chain diabody.

Summary of the Invention

The present invention provides, among other things, dosage units, uses, methods or formulations of compositions comprising a recombinant protein comprising (i) interleukin-12 (IL-12) and (ii) an antibody binding the extra-domain B (ED-B) of fibronectin, or a target binding fragment or derivative thereof.

The invention and general advantages of its features will be discussed in detail below.

According to one aspect of the invention, a dosage unit is provided, comprising ≥ 0.1 µg kg^{-1}, relative to a human patient's body weight, of a recombinant protein comprising (i) interleukin-12 (IL-12) and (ii) an antibody binding the extra-domain B (ED-B) of fibronectin, or a target binding fragment or derivative thereof.

In Rudman *et al.* 2011, AS1409 is disclosed, which is a fusion protein comprising the humanised antibody BC1 in IgG format linked to interleukin-12 (IL-12). It is designed to deliver IL-12 to tumor-associated vasculature using an antibody targeting the ED-B variant of fibronectin. A phase 1 trial of weekly infusional AS1409 was carried out in renal carcinoma and malignant melanoma patients. Safety, efficacy, markers of IL-12-mediated immune response, and pharmacokinetics were evaluated. Doses of 15µg/kg and 25µg/kg were studied. The study demonstrated the safety of this approach, and provided pharmacodynamic support for the proposed mechanism of action. Along with evidence of efficacy against metastatic

melanoma, it was stipulated that the experiments provided a rationale for progression to a phase II trial. However, no phase II trial was ever launched.

Generally, because immunocytokines have a potentiating effect on the immune system – with each cytokine being capable of stimulating two or more immune cells – finding of a safe and efficient dosage is much more difficult than *e.g.* in antibody therapy, where the abundance of a given target – either a ligand that is to be inactivated, or a receptor that is to be blocked – is known, and a clear stochiometric relationship between antibody and target can be calculated.

Strauss *et al.* (2018) disclose a First-In-Human Phase I Trial of a Tumor-Targeted Cytokine (NHS-IL12) in subjects with Metastatic Solid Tumors. The NHS-IL12 immunocytokine is composed of two IL-12 heterodimers, each fused to one of the H chains of the anti-histone antibody NHS76. The maximum tolerated dose (MTD) was determined to be 16.8 μg/kg. Because NHS76 has, via the histones, affinity for both single- and double-stranded DNA, NHS-IL12 targets delivery to regions of tumor necrosis where DNA has become exposed.

The teachings from this study cannot be transferred to the present IL12-anti EDB recombinant protein, because of at least the fact that histones, as targets for the NHS antibody, are intracellular targets, compared to EDB, which is an extracellular target, and because histones have a different abundance in tumor tissue than EDB.

In Puca *et al.* (2019), the cloning and characterization of a novel fusion protein (termed L19-mIL12) is disclosed. The fusion protein consists of murine interleukin-12 in single-chain format, sequentially fused to the anti EDB antibody L19 in a single-chain diabody (also defined as "tandem diabody") format. The authors reported that in mice, L19-mIL12 was very well tolerated at a dose of 12 μg, which is equivalent to the human dose of 2 mg, under consideration of the body surface scaling factor – a dose which is by far higher that what has been reported by Rudman *et al.* and Straus *et al.*

According to another aspect of the invention, a dosage unit or regimen is provided, comprising $\leq$ 100 μg kg^{-1}, relative to a human patient's body weight, of a recombinant protein comprising (i) interleukin-12 (IL-12) and (ii) an antibody binding the extra-domain B (ED-B) of fibronectin, or a target binding fragment or derivative thereof.

In some embodiments, the dosage unit or regimen comprises ≥ 0,1 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≥ 0,25 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≥ 0,5 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≥ 1 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≥ 2 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≥ 3 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≥ 4 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≥ 5 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≥ 6 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≥ 7 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≥ 8 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≥ 9 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≥ 10 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≥ 11 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≥ 12 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≥ 13 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≥ 14 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≥ 15 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≥ 16 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≥ 17 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≥ 18 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≥ 19 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit

or regimen comprises ≥ 20 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≥ 21 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≥ 22 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≥ 23 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≥ 24 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≥ 25 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≥ 26 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≥ 27 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≥ 28 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≥ 29 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≥ 30 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein.

In some embodiments, the dosage unit or regimen comprises ≤ 95 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≤ 90 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≤ 85 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≤ 80 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≤ 75 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≤ 70 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≤ 65 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≤ 60 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≤ 55 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≤ 50 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≤ 45 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit

or regimen comprises ≤ 40 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≤ 35 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≤ 32 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≤ 30 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises ≤ 25 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein.

In some embodiments, the dosage unit or regimen comprises between ≥ 2 and ≤ 20μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 3 and ≤ 25μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 4 and ≤ 30μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 5 and ≤ 35μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 6 and ≤ 40μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 7 and ≤ 45μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 8 and ≤ 50μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 9 and ≤ 55μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 10 and ≤ 60μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 11 and ≤ 65μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 12 and ≤ 70μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 13 and ≤ 75μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 14 and ≤ 80μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 15 and ≤ 85μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises

between ≥ 16 and ≤ 90μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 17 and ≤ 95μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 18 and ≤ 100μg kg^{-1}, relative to a human patient's body weight of the recombinant protein.

In some embodiments, the dosage unit or regimen comprises between ≥ 2 and ≤ 30 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 4 and ≤ 35 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 6 and ≤ 40 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 8 and ≤ 45 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 10 and ≤ 50 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 12 and ≤ 55 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 14 and ≤ 60 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 16 and ≤ 65 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 18 and ≤ 70 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 20 and ≤ 75 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 22 and ≤ 80 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 24 and ≤ 85 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 26 and ≤ 90 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 28 and ≤ 95 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 30 and ≤ 100 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 32 and ≤ 105

μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 34 and ≤ 110 μg kg^{-1}, relative to a human patient's body weight of the recombinant protein.

In some embodiments, the dosage unit or regimen comprises between ≥ 2 and ≤ 30μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 6 and ≤ 35μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 10 and ≤ 40μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 14 and ≤ 45μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 18 and ≤ 50μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 22 and ≤ 55μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 26 and ≤ 60μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 30 and ≤ 65μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 34 and ≤ 70μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 38 and ≤ 75μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 42 and ≤ 80μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 46 and ≤ 85μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 50 and ≤ 90μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 54 and ≤ 95μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 58 and ≤ 100μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 62 and ≤ 105μg kg^{-1}, relative to a human patient's body weight of the recombinant protein. In some embodiments, the dosage unit or regimen comprises between ≥ 66 and ≤ 110μg kg^{-1}, relative to a human patient's body weight of the recombinant protein.

According to one aspect of the invention, a recombinant protein is provided, comprising (i) interleukin-12 (IL-12) and (ii) an antibody binding the extra-domain B (ED-B) of fibronectin, or a target binding fragment or derivative thereof, for use in the treatment of a patient being diagnosed for, or suffering from, cancer, wherein the recombinant protein is administered in a dose according to the above description.

According to one aspect of the invention, a method of treating a patient is provided, the patient being diagnosed for, or suffering from, cancer, comprising administering to the patient a composition comprising a recombinant protein comprising (i) interleukin-12 (IL-12) and (ii) an antibody binding the extra-domain B (ED-B) of fibronectin, or a target binding fragment or derivative thereof, in a dose according to the dosage unit of according to the above description.

According to some embodiments of the recombinant protein for use or the method of treating, the dose is administered to the patient daily. According to some embodiments of the recombinant protein for use or the method of treating the dose is administered to the patient halfweekly. According to some embodiments of the recombinant protein for use or the method of treating the dose is administered to the patient weekly. According to some embodiments of the recombinant protein for use or the method of treating the dose is administered to the patient, biweekly. According to some embodiments of the recombinant protein for use or the method of treating the dose is administered to the patient triweekly. According to some embodiments of the recombinant protein for use or the method of treating the dose is administered to the patient monthly. According to some embodiments of the recombinant protein for use or the method of treating, the dose is administered to the patient bimonthly.

The following dosage regimen of a recombinant protein comprising (i) interleukin-12 (IL-12) and (ii) an antibody binding the extra-domain B (ED-B) of fibronectin, or a target binding fragment or derivative thereof, relative to a human patient's body weight, provide a good compromise between good efficacy and reduced side effects.

µg kg^{-1}	half weekly	weekly	biweekly	triweekly	monthly
0,5	dosage regimen can be administered 1x, 2x, 3x, 4x, 5x, 6x, 7x or 8x				
0,75	dosage regimen can be administered 1x, 2x, 3x, 4x, 5x, 6x, 7x or 8x				
1	dosage regimen can be administered 1x, 2x, 3x, 4x, 5x, 6x, 7x or 8x				

1,25	dosage regimen can be administered 1x, 2x, 3x, 4x, 5x, 6x, 7x or 8x
1,5	dosage regimen can be administered 1x, 2x, 3x, 4x, 5x, 6x, 7x or 8x
1,75	dosage regimen can be administered 1x, 2x, 3x, 4x, 5x, 6x, 7x or 8x
2	dosage regimen can be administered 1x, 2x, 3x, 4x, 5x, 6x, 7x or 8x
2,25	dosage regimen can be administered 1x, 2x, 3x, 4x, 5x, 6x, 7x or 8x
2,5	dosage regimen can be administered 1x, 2x, 3x, 4x, 5x, 6x, 7x or 8x
2,75	dosage regimen can be administered 1x, 2x, 3x, 4x, 5x, 6x, 7x or 8x
3	dosage regimen can be administered 1x, 2x, 3x, 4x, 5x, 6x, 7x or 8x
3,25	dosage regimen can be administered 1x, 2x, 3x, 4x, 5x, 6x, 7x or 8x
3,5	dosage regimen can be administered 1x, 2x, 3x, 4x, 5x, 6x, 7x or 8x
3,75	dosage regimen can be administered 1x, 2x, 3x, 4x, 5x, 6x, 7x or 8x

As shown in the table, any one of the above dosage regimens can be administered 1x, 2x, 3x, 4x, 5x, 6x, 7x or 8x, based on the suggested interval of half weekly to monthly.

Hence, in several embodiments, a dosage unit or regimen comprising between $\geq 0{,}5$ µg kg^{-1} and < 4 µg kg^{-1}, relative to a human patient's body weight, of a recombinant protein comprising (i) interleukin-12 (IL-12) and (ii) an antibody binding the extra-domain B (ED-B) of fibronectin, or a target binding fragment or derivative thereof, is provided.

The following table shows total dosages for patients with different body weights (kg) per each administration in µg, based on the different dosage units/dosage regimen (µg/kg^{-1}).

Dosage µg/kg^{-1}	Body weight kg	40	50	60	70	80	90	100	110	120
0,5		20	25	30	35	40	45	50	55	60
0,75		30	37,5	45	52,5	60	67,5	75	82,5	90
1		40	50	60	70	80	90	100	110	120
1,25		50	62,5	75	87,5	100	112,5	125	137,5	150
1,5		60	75	90	105	120	135	150	165	180
1,75		70	87,5	105	122,5	140	157,5	175	192,5	210
2		80	100	120	140	160	180	200	220	240
2,25		90	112,5	135	157,5	180	202,5	225	247,5	270
2,5		100	125	150	175	200	225	250	275	300
2,75		110	137,5	165	192,5	220	247,5	275	302,5	330
3		120	150	180	210	240	270	300	330	360
3,25		130	162,5	195	227,5	260	292,5	325	357,5	390
3,5		140	175	210	245	280	315	350	385	420

3,75		150	187,5	225	262,5	300	337,5	375	412,5	450

According to one aspect of the invention, a recombinant protein is provided, comprising (i) interleukin-12 (IL-12) and (ii) an antibody binding the extra-domain B (ED-B) of fibronectin, or a target binding fragment or derivative thereof, for (use in) the treatment of a patient being diagnosed for, or suffering from, cancer. In some embodiments, the cancer is advanced/metastatic immunotherapy responsive solid carcinoma or lymphoma.

According to one aspect of the invention, a method of treating a patient is provided, the patient being diagnosed for, or suffering from advanced/metastatic immunotherapy responsive solid carcinoma or lymphoma, the method comprising administering to the patient a composition comprising, in a therapeutically sufficient dose, a recombinant protein comprising (i) interleukin-12 (IL-12) and (ii) an antibody binding the extra-domain B (ED-B) of fibronectin, or a target binding fragment or derivative thereof.

According to some embodiments with regard to the recombinant protein for use or the method of treatment, the carcinoma or lymphoma is malignant melanoma. According to some embodiments with regard to the recombinant protein for use or the method of treatment, the carcinoma or lymphoma is non-small cell lung cancer (NSCLC). According to some embodiments with regard to the recombinant protein for use or the method of treatment, the carcinoma or lymphoma is renal cell carcinoma. According to some embodiments with regard to the recombinant protein for use or the method of treatment, the carcinoma or lymphoma is urothelial carcinoma. According to some embodiments with regard to the recombinant protein for use or the method of treatment, the carcinoma or lymphoma is head and neck squamous cell carcinoma (HNSCC). According to some embodiments with regard to the recombinant protein for use or the method of treatment, the carcinoma or lymphoma is microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR) metastatic colorectal cancer. According to some embodiments with regard to the recombinant protein for use or the method of treatment, the carcinoma or lymphoma is hepatocellular cancer. According to some embodiments with regard to the recombinant protein for use or the method of treatment, the carcinoma or lymphoma is gastric cancer. According to some embodiments with regard to the recombinant protein for use or the method of treatment, the carcinoma or lymphoma is squamous cell carcinoma of the skin. According to some embodiments with regard to the recombinant protein for use or the method of treatment, the carcinoma or lymphoma is cervical cancer. According to some embodiments

with regard to the recombinant protein for use or the method of treatment, the carcinoma or lymphoma is diffuse large B-cell lymphoma (DLBCL).

As part of the present disclosure, the above conditions can as well be combined.

According to one embodiment with regard to the recombinant protein for use or the method of treatment, the carcinoma or lymphoma has progressed on immune checkpoint-blockade therapy,

According to one embodiment with regard to the recombinant protein for use or the method of treatment, the carcinoma or lymphoma has an Eastern cooperative oncology group (ECOG) performance status ≤ 2

According to one embodiment with regard to the recombinant protein for use or the method of treatment, the carcinoma or lymphoma is characterized by at least one unidimensionally measurable lesion either by computed tomography (CT), MRI or PET/CT as defined by RECIST (v. 1.1) for solid tumors or by LUGANO criteria for malignant lymphoma.

As part of the present disclosure, the above conditions can as well be combined.

According to one embodiment with regard to the recombinant protein for use or the method of treatment, the patient has received an immune checkpoint blockade therapy-based regimen as immediate prior treatment.

According to one embodiment with regard to the recombinant protein for use or the method of treatment, the patient has had clinical benefit (CR/PR/SD) while on immune checkpoint blockade therapy defined as ≥ 3 month free from progression from initial imaging documenting metastatic disease followed by radiographic disease progression after immune checkpoint blockade therapy.

According to one embodiment with regard to the recombinant protein for use or the method of treatment, the patient has received ≥ 2 prior systemic therapies, when being diagnosed for, or suffering from, DLCBL.

According to one embodiment with regard to the recombinant protein for use or the method of treatment, the patient has been negatively tested for HIV, HBV and HCV.

As part of the present disclosure, the above conditions can as well be combined.

The terms CR/PR/SD, as used herein, relate to responses as defined by the Response Evaluation Criteria In Solid Tumors (RECIST) criteria. Complete response (CR) = Disappearance of all target lesions, Partial response (PR) = at least a 30% decrease in the sum of the LD of target lesions, taking as reference the baseline sum LD, and Stable disease (SD) = Neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD, taking as reference the smallest sum LD since the treatment started.

The term „ECOG performance status“, as used herein, is a score which describes a patient's level of functioning in terms of their ability to care for themselves, daily activity, and physical ability (walking, working, etc.), as developed by the Eastern Cooperative Oncology Group.

The term „immune checkpoint-blockade therapy“ as used herein, relates to a therapy that uses medications known as immune checkpoint inhibitors to address several types of cancer. Specifically, these medications can help the body's immune system recognize and attack cancerous cells. Immune checkpoint inhibitors include inhibitors or antagonists to inter alia CTLA-4, PD-1, PD-L1, LAG 3, TIM3 and OX40. Some well-established immune checkpoint inhibitors are Ipilimumab, Nivolumab, Pembrolizumab, Atezolizumab, Avelumab, Durvalumab and Cemiplimab.

The term “negatively tested for HIV, HBV and HCV” means that the patient has been tested negatively for infections with, or presence of antibodies against, HIV virus, Hepatitis B virus, or Hepatitis C virus.

According to one aspect of the invention, a pharmaceutical formulation is provided, comprising a recombinant protein comprising (i) interleukin-12 (IL-12) and (ii) an antibody binding the extra-domain B (ED-B) of fibronectin, or a target binding fragment or derivative thereof.

In one embodiment, the formulation can comprise Histidine. In one embodiment, the formulation can comprise Sucrose. In one embodiment, the formulation can comprise EDTA. In one embodiment, the formulation can comprise Histidine and Sucrose and optionally EDTA.

In one embodiment, the formulation can comprise citric acid. In one embodiment, the formulation can comprise sodium citrate. In one embodiment, the formulation can comprise Sucrose. In one embodiment, the formulation can comprise Glycerol. In one embodiment, the formulation can comprise EDTA. In one embodiment, the formulation can comprise citric acid and sodium citrate and optionally at least one of Sucrose, Glycerol and EDTA.

In one embodiment, the formulation can comprise HEPES. In one embodiment, the formulation can comprise NaCl. In one embodiment, the formulation can comprise Mannitol. In one embodiment, the formulation can comprise Glycerol. In one embodiment, the formulation can comprise EDTA. In one embodiment, the formulation can comprise HEPES and NaCl and optionally at least one of Mannitol, Glycerol and, EDTA.

In one embodiment, the formulation can comprise Histidine, Sucrose and EDTA, adjusted to have a pH of 8.0 ± 0.3. In another embodiment, the formulation can comprise citric acid, sodium citrate, Sucrose, Glycerol, EDTA, adjusted to have a pH of 6.0 ± 0.3. In one embodiment, the formulation can comprise Hepes, NaCl, Mannitol, Glycerol, EDTA, adjusted to have a pH of 7.0 ± 0.3.

These formulations satisfy the high demands as regards stability under strass conditions (low protein loss after several cycles of freezing/thawing and low aggregate formation at 2 – 8 °C).

AS1409 (Rudman *et al.*, discussed above), was supplied as a 1mg/ml solution in aqueous buffer at pH 6.0. It was administered to patients following a 1 to 1 dilution with 0.9% sodium chloride. For NHS-IL12 (Strauss *et al.*, discussed above) no data regarding the formulation is given. L19-mIL12 (Puca *et al.*, discussed above) was diluted in phosphate buffer saline.

While these formulations can arguably be used for scientific and clinical trials, they are certainly not suitable for the market, as no considerations have been devoted to issues like shelf life or aggregation. The following table shows six preferred formulations:

Formulation no	2B		4A		1B	
alias	"Histidine buffer"		"Citrate buffer"		"Hepes buffer"	
Histidine mM	5-40	20				
Hepes mM					5-30	15
Sucrose % w/v	4-15	8,5	4-15	8		
Citric acid mM			0.2-4	1		
Na citrate mM			3-20	10		
EDTA	20–100 mg/L	50 mg/L	2–10 mM	5 mM	2–10 mM	5mM
Glycerol % w/v			0.2-4	1	0.2-4	1
Mannitol mM					20-100	50
NaCl mM					10-80	30
pH	7-9	8.0 ± 0.3	5-6	6.0 ± 0.3	6-8	7.0 ± 0.3

Application WO2018011404A1 assigned to the applicant of the present invention discloses a formulation for an immunocytokine comprising the antibody L19 and the cytokine TNF. In example 4, disclosed therein on pages 25 – 27, the following formulations were inter alia investigated:

(i) Histidine buffers prepared at pH 6, 8 and 9:

- Hist-1 comprises 20 mM histidine at pH 6.0, 8.5% Sucrose (w/v), 130 mM EDTA.
- Hist-2 comprises 20 mM histidine at pH 8.0, 8.5% Sucrose (w/v), 130 mM EDTA.
- Hist-3 comprises 20 mM histidine at pH 9.0, 8.5% Sucrose (w/v), 130 mM EDTA.

(ii) Citrate buffer prepared at pH 6 .6

- Citrate-1 comprises 5.6 g/L sodium Citrate, 0.21 g/L citric acid, 70 g/L trehalose dihydrate, 0.2 g/L polysorbate80, 1% (w/v) glycerol, 5mM EDTA, pH 6.6.

In example 1, disclosed therein on pages 21 – 23, the following formulations were inter alia investigated:

(iii) Hepes buffers prepared at pH 7.5 and 8.0:

- Hepes-1 comprises 30 mM Hepes at pH 7.5, 5 mM EDTA, 75 mM mannitol and 1.8% glycerol (w/v)
- Hepes-2 comprises 30 mM Hepes at pH 7.5, 5 mM EDTA, 75 mM mannitol, 1.8% glycerol (w/v) and 0.1% polysorbate20
- Hepes-3 comprises 15 mM Hepes at pH 8.0, 5 mM EDTA, 75 mM mannitol and 1.8% glycerol (w/v)
- Hepes-4 comprises 15 mM Hepes at pH 8.0, 5 mM EDTA, 75 mM mannitol, 1.8% glycerol (w/v) and 0.005% polysorbate20
- Hepes-5 comprises 15 mM Hepes at pH 8.0, 5 mM EDTA, 75 mM mannitol, 1.8% glycerol (w/v) and 0.01% polysorbate20
- Hepes- 6 comprises 15 mM Hepes at pH 8.0, 5 mM EDTA, 75 mM mannitol, 1.8% glycerol (w/v) and 0.05% polysorbate20

For the Histidine buffers, no acceptance criteria were met. For the citrate buffer, both the visual clarity and A280 stability criteria were met but the purity criteria was not met indicating particles in suspension or aggregation of the trimer. Histidine and citrate buffers showed particles in suspension and were not considered for further investigation.

For Hepes formulations comprising < 0.1% polysorbate20, the acceptance criteria were not met either. Only for met. Hepes formulations comprising ≥ 0.1% polysorbate20, the acceptance criteria were met.

On that basis, it is highly surprising that the formulations according to the above table deliver acceptable results, even though they are highly similar to the formulations used in examples 1 and 4 of WO2018011404A1, and even though the recombinant protein of the present invention has structural similarity with L19-TNF of WO2018011404A1.

According to embodiments of the dosage unit, the recombinant protein for use, the method of treatment or the formulation of the invention, the antibody comprised in the recombinant protein comprises at least one single-chain Fv (scFv) antibody fragment, optionally a single chain diabody.

As used herein, the term “single chain diabody” relates to a construct of two single chain Fv (scFv) antibodies with a short linker, preferably 3 - 10 amino acids long, more preferably 5

amino acid long (also known as “diabodies”), joined to one another by a longer linker, preferably 5 - 20 amino acids long, more preferably 15 amino acid long, according to the following scheme (N->C orientation): L19VH-linker-L19VL-linker-L19VH-linker- L19VL.

According to embodiments of the dosage unit, the recombinant protein for use, the method of treatment or the formulation of the invention, the IL-12 comprised in the recombinant protein comprises a p40 subunit and a p35 subunit, linked by a linker.

According to embodiments of the dosage unit, the recombinant protein for use, the method of treatment or the formulation of the invention, the p40 subunit and the p35 subunit comprise the amino acid sequence according to SEQ ID NO: 1 or SEQ ID NO: 3, respectively.

According to embodiments of the dosage unit, the recombinant protein for use, the method of treatment or the formulation of the invention, the antibody comprised in the recombinant protein is the anti-EDB antibody L19, comprising the amino acid sequence according to SEQ ID NO: 5 as VL domain and SEQ ID NO: 7 as VH domain.

According to embodiments of the dosage unit, the recombinant protein for use, the method of treatment or the formulation of the invention, at least one of the antibody, the IL-12 and/or the linker connecting the two is one disclosed in WO2019154986, optionally wherein the recombinant protein is one disclosed in WO2019154986.

WO2019154986 describes technical and physiological properties of the recombinant protein that is subject to the present invention. The content of WO2019154986A1 is incorporated by reference herein.

According to embodiments of the dosage unit, the recombinant protein for use, the method of treatment or the formulation of the invention, the recombinant protein comprises

- a p40 domain linked to a p35 domain by a first linker;
- a first L19 VH domain linked to the p35 domain by a SAD linker;
- a first L19 VL domain linked to the first L19 VH domain by a third linker;
- a second L19 VH domain linked to the first L19 VL domain by a fourth linker;
- a second L19 VL domain linked to the second L19 VH domain by a fifth linker.

According to embodiments of the dosage unit, the recombinant protein for use, the method of treatment or the formulation of the invention, the recombinant protein comprises, optionally consists of, the amino acid sequence according to SEQ ID NO: 9.

According to embodiments of the dosage unit, the recombinant protein for use, the method of treatment or the formulation of the invention, administration is done intravenously or subcutaneously.

According to embodiments of invention, the dosage unit or recombinant protein for use is provided in a formulation according to the above description.

According to embodiments of invention, the formulation according to the above description, comprises a dosage unit or recombinant protein according to the above description.

According to one aspect of the invention, a kit of parts comprising

a) the dosage unit or recombinant protein for use according to the above description
b) an apparatus for administering the dosage unit or recombinant protein, and, optionally
c) instructions for use.

is provided.

Examples

While the invention has been illustrated and described in detail in the drawings and foregoing description, such illustration and description are to be considered illustrative or exemplary and not restrictive; the invention is not limited to the disclosed embodiments. Other variations to the disclosed embodiments can be understood and effected by those skilled in the art in practicing the claimed invention, from a study of the drawings, the disclosure, and the appended claims. In the claims, the word “comprising” does not exclude other elements or steps, and the indefinite article “a” or “an” does not exclude a plurality. The mere fact that certain measures are recited in mutually different dependent claims does not indicate that a combination of these measures cannot be used to advantage. Any reference signs in the claims should not be construed as limiting the scope.

All amino acid sequences disclosed herein are shown from N-terminus to C-terminus.

Example 1: Toxicology Studies in Cynomolgous monkeys

The extradomain B of fibronectin, which is targeted by the L19 antibody in IL12-L19L19, is a very well conserved domain across species and is 100 % identical in man and monkeys.

The homology between human and monkey IL12 is 98% and 95% for p40 and p35, respectively, and human IL12 is active both in man and monkey. The activity of IL12-L19L19 has been tested on human and monkey peripheral blood mononuclear cells (PBMCs). IL12-L19L19 and recombinant human IL12 have comparable activities with regard to IFNγ increase on both human and monkey PBMCs. However, both IL12-L19L19 and recombinant IL12 are 10x less active in monkey than in human PBMCs.

In the toxicity study, monkeys have received eight weekly intravenous administrations of IL12-L19L19 at human equivalent doses (HED) of 13, 51 or 213 μg/kg/week. The tested doses were identified as safe for the respiratory, central nervous, cardiac and renal system since no IL12-L19L19 related observations were reported.

Example 2: Formulation development

The formulation study was performed by screening several buffers, different in salts composition and pH. The following buffers were investigated:

1) Hepes buffer

- 1A. 15mM Hepes, 50mM Mannitol, 1% w/v Glycerol, 5mM EDTA, pH 7.0
- 1B. 15mM Hepes, 30mM NaCl, 50mM Mannitol, 1% w/v Glycerol, 5mM EDTA, pH 7.0

2) Histidine buffer

- 2A. 20mM Histidine, 8,5% w/v Sucrose, 50mg/L EDTA, pH 6.0
- 2B. 20mM Histidine, 8,5% w/v Sucrose, 50mg/L EDTA, pH 8.0

3) Phosphate buffer

- 3A.15mM NaH_2PO_4, 10mM Na_2HPO_4, 30mM NaCl, 50mM Mannitol, 1% w/v Glycerol, 5mM EDTA, pH 6.5
- 3B.15mM NaH_2PO_4, 10mM Na_2HPO_4, 30mM NaCl, 50mM Mannitol, 1% w/v Glycerol, 5mM EDTA, pH 7.5

4) Citrate buffer

- 4A. 1mM citric acid, 10mM sodium citrate, 8% w/v Sucrose, 1% w/v Glycerol, 5mM EDTA, pH 6.0

The study was performed by applying the following operative procedure:

1) The formulation is performed by desalting chromatography.
2) The formulated product is 0,22µm filtered before analysis
3) The concentration of the formulated protein is done by using Amicon device (10kDa cut-off) and centrifugation.
4) The product is formulated at 2mg/ml.

To accept the formulated product, before to start the stability study, a preliminary acceptance criterion related to the visual appearance was evaluated as follows:

- if the solution is cloudy and/or particles in suspension are visible → reject the sample.
- if the solution is clear and free of visible particles in suspension → perform the stability study.

The stability study was performed for each sample as summarized in Table 1, defining that:

- all time-points of the stability study must be analyzed even if the first data are out of specifications.
- A_{280nm} is evaluated after sample centrifugation.
- A_{280nm} pharmacopoeia method is applied (ref. Ph. Eur. curr. ed., paragraph 2.5.33).
- SEC analysis is performed using TSKgel G3000 SWXL column

The material used to perform the formulation study was obtained by preparative SEC of IL12-L19L19: it was expressed by TGE procedure (i.e. Transient Gene expression) and purified on

Protein A resin by eluting with TEA 100mM native pH, then it was desalted in PBS as described in WO2019154896.

Table 2 summarizes the recorded results.

The total yield (i.e., formulation + concentration steps) was between 86.2% and 90.2%, so very comparable data were recorded among the different formulation conditions investigated. The single chain diabody purity was between 95.49% and 96.11% after the formulation step. Considering that the purity of the input sample was 96.06%, the recorded data after the formulation attest that no aggregates formation was induced by the buffer exchange. After concentration up to 2 mg/ml, the SEC results show the % of single chain diabody between 93.15% and 94.89% showing a small increase of the high MW form (< 3%), due to the concentration procedure itself.

Figures 1 and 2 show the SEC profiles of the sample after formulation and after concentration.

The visual appearance of all formulated and concentrated samples was clear and free of visible particles, then the samples were investigated for their stability under stressing conditions (i.e. 3 repeated cycles of freezing at -80°C and thawing at RT) and at 2-8°C storage temperature.

Table 3 reports the recorded results during the stability study, for each formulated sample.

The stability of IL12-L19L19 was investigated in 7 formulation buffers under stress conditions (i.e. 3 cycles of freezing at -80°C and thawing at RT) and after 14 days of storage at 2-8°C. The visual appearance was very good for all samples.

The SEC profile and the % of the single chain diabody form were well comparable among all samples and only very small variations were observed in terms of decrease of diabody and increase of aggregates (reported as “HMW forms” in Table 3).

Based on the comparable results recorded for appearance and SEC, A_{280nm} values were used to compare the different formulations and to define the best conditions.

Table 4 reports the loss in A_{280nm} value after the 3rd cycles of freezing/thawing, recorded for each sample under study. The samples were classified from the best to the worst based on the loss in A_{280nm}.

The loss of protein, evaluated by A_{280nm} reading, seems to depend not only on the pH of the buffer, but, mostly, on the buffer excipients.

In details:

- Comparing the Histidine buffers: pH 8.0 (2B) is better than pH 6.0 (2A).
  In both buffers is 8.5% w/v Sucrose, in this case, the pH makes the difference.
- Comparing Histidine buffer pH 8.0 (2B) to Citrate buffer pH 6.0 (4A) the same stability was recorded.
  In both buffers is Sucrose (8.5% w/v and 8% w/v, respectively): sucrose seems to assure the stability and the pH is irrelevant.
- Comparing Hepes/NaCl buffer (1B) to Hepes buffer without NaCl (1A) → the presence of NaCl seems to help the stability.
- In phosphate buffer pH 6.5 (3A) the product stability is better than at pH 7.5 (3B), same composition buffer composition.

Based on the stress study, the following buffers were selected as good candidates:

2B. 20mM Histidine, 8.5% w/v Sucrose, 50mg/L EDTA, pH 8.0

4A. 1mM citric acid, 10mM sodium citrate, 8% w/v Sucrose, 1% w/v Glycerol, 5mM EDTA, pH 6.0

1B. 15mM Hepes, 30mM NaCl, 50mM Mannitol, 1% w/v Glycerol, 5mM EDTA, pH 7.0

Table 5 reports the loss in A_{280nm} value after 14 days of storage at 2-8°C, recorded for each sample under study. The samples were classified from the best to the worst based on the loss in A_{280nm}.

In detail:

- Histidine buffer pH 8.0 (2B) = Histidine buffer pH 6.0 (2A) = (very similar). Citrate buffer pH 6.0 (4A): also at 2-8°C, the presence of sucrose assures the stability, 2% protein loss was the maximum recorded in these cases.
  However, the buffers listed above showed the highest decrease of single chain diabody form: 2.4%.
- Hepes/NaCl buffer (1B) = Hepes buffer without NaCl (1A) showing 2.4-2.5% of protein loss.
- In phosphate buffer pH 7.5 (3B) the product stability was better than in the phosphate buffer pH 6.5 (3A) (same composition): 0.8% vs. 2.9% of protein loss respectively.

Based on the results recorded during the formulation study, the preferred formulation buffer was the buffer 2B: 20mM Histidine, 8.5% w/v Sucrose, 50mg/L EDTA, pH 8.0.

It showed the best results for the study under stress conditions (3.3% of protein loss after the 3rd cycle of freezing/thawing and no diabody loss in SEC analysis) and good results for the study at 2-8°C (only 2.1% of protein loss and 2.4% of loss of single chain diabody form with aggregates formation).

References

The disclosures of these documents are herein incorporated by reference in their entireties.

Gutbrodt and Neri, Antibodies 2012, 1(1), 70-87

Rudman SM et al, Clin Cancer Res. 2011 April 1; 17(7): 1998–2005

Strauss J et al., Clin Cancer Res. 2019 Jan 1;25(1):99-109. doi: 10.1158/1078-0432.CCR-18-1512. Epub 2018 Aug 21.

Puca et al (2019) Int J Cancer. 2020 May 1;146(9):2518-2530.

Car BD et al., Toxicol Pathol. 1999 Jan-Feb;27(1):58-63.

Sequences

The following sequences form part of the disclosure of the present application. A WIPO ST 25 compatible electronic sequence listing is provided with this application, too. For the avoidance

of doubt, if discrepancies exist between the sequences in the following table and the electronic sequence listing, the sequences in this table shall be deemed to be the correct ones.

SEQ ID NO	qualifier	Sequence
1	P40	IWELKKDVYVVELDWYPDAPGEMVVLTCDTPEEDGITWTLDQSSEVLGSGKT LTIQVKEFGDAGQYTCHKGGEVLSHSLLLLHKKEDGIWSTDILKDQKEPKNK TFLRCEAKNYSGRFTCWWLTTISTDLTFSVKSSRGSSDPQGVTCGAATLSAE RVRGDNKEYEYSVECQEDSACPAAEESLPIEVMVDAVHKLKYENYTSSFFIR DIIKPDPPKNLQLKPLKNSRQVEVSWEYPDTWSTPHSYFSLTFCVQVQGKSK REKKDRVFTDKTSATVICRKNASISVRAQDRYYSSSWSEWASVPCS
2	Linker 1	GGGGSGGGGSGGGGS
3	P35	RNLPVATPDPGMFPCLHHSQNLLRAVSNMLQKARQTLEFYPCTSEEIDHEDI TKDKTSTVEACLPLELTKNESCLNSRETSFITNGSCLASRKTSFMMALCLSS IYEDLKMYQVEFKTMNAKLLMDPKRQIFLDQNMLAVIDELMQALNFNSETVP QKSSLEEPDFYKTKIKLCILLHAFRIRAVTIDRVMSYLNAS
4	Linker 2 (“SAD”)	GSADGGSSAGGSDAG
5	L19VL	EIVLTQSPGTLSLSPGERATLSCRASQSVSSSFLAWYQQKPGQAPRLLIYYA SSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQTGRIPPTFGQGTK VEIK
6	Linker 3/Linker 5	GSSGG
7	L19VH	EVQLLESGGGLVQPGGSLRLSCAASGFTFSSFSMSWVRQAPGKGLEWVSSIS GSSGTTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKPFPYFD YWGQGTLVTVSS
8	Linker 4	SSSSGSSSSGSSSSG
9	Full length SAD variant	IWELKKDVYVVELDWYPDAPGEMVVLTCDTPEEDGITWTLDQSSEVLGSGKT LTIQVKEFGDAGQYTCHKGGEVLSHSLLLLHKKEDGIWSTDILKDQKEPKNK TFLRCEAKNYSGRFTCWWLTTISTDLTFSVKSSRGSSDPQGVTCGAATLSAE RVRGDNKEYEYSVECQEDSACPAAEESLPIEVMVDAVHKLKYENYTSSFFIR DIIKPDPPKNLQLKPLKNSRQVEVSWEYPDTWSTPHSYFSLTFCVQVQGKSK REKKDRVFTDKTSATVICRKNASISVRAQDRYYSSSWSEWASVPCSGGGGSG GGGSGGGGSRNLPVATPDPGMFPCLHHSQNLLRAVSNMLQKARQTLEFYPCT SEEIDHEDITKDKTSTVEACLPLELTKNESCLNSRETSFITNGSCLASRKTS FMMALCLSSIYEDLKMYQVEFKTMNAKLLMDPKRQIFLDQNMLAVIDELMQA LNFNSETVPQKSSLEEPDFYKTKIKLCILLHAFRIRAVTIDRVMSYLNASGS ADGGSSAGGSDAGEVQLLESGGGLVQPGGSLRLSCAASGFTFSSFSMSWVRQ APGKGLEWVSSISGSSGTTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDT AVYYCAKPFPYFDYWGQGTLVTVSSGSSGGEIVLTQSPGTLSLSPGERATLS CRASQSVSSSFLAWYQQKPGQAPRLLIYYASSRATGIPDRFSGSGSGTDFTL TISRLEPEDFAVYYCQQTGRIPPTFGQGTKVEIKSSSSGSSSSGSSSSGEVQ LLESGGGLVQPGGSLRLSCAASGFTFSSFSMSWVRQAPGKGLEWVSSISGSS GTTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKPFPYFDYWG QGTLVTVSSGSSGGEIVLTQSPGTLSLSPGERATLSCRASQSVSSSFLAWYQ QKPGQAPRLLIYYASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQ QTGRIPPTFGQGTKVEIK

STABILITY STUDY TABLE				
Storage Temperature	**Time-point**	**Assay (after each thawing and at each time-point)**	**Assay purpose**	**Acceptance criteria**
-80°C ± 5°C	stress conditions: 3 cycles of freezing at -80°C and thawing at RT	• Visual appearance • A_{280nm} • SEC	• Detection of macro-precipitation • Detection of protein loss • Detection of protein aggregation	• Clear and free of visible particles • Compared to t = 0 value, loss ≤ 10% • Compared to t = 0 value, peak of interest loss ≤ 5%
2-8°C	t = 7 days of storage t = 14 days of storage			

Table 1

Formulation buffer	Formulation yield (1) (after desalting)	Concentration yield (2) (after concentration)	Total yield (1+2)	% single chain diabody in the formulated sample (SEC)	% single chain diabody in the concentrated sample (SEC)
1A	95.0	90.7	86.2	96.08	93.15
1B	96.6	90.9	87.8	95.49	93.72
2A	98.3	88.9	87.4	95.96	94.29
2B	96.6	90.4	87.3	96.11	94.18
3A	93.7	96.3	90.2	96.11	94.89
3B	96.2	93.7	90.1	95.94	94.81
4A	92.6	96.7	89.5	95.95	94.72

Table 2

Sample and time-points	A_{280nm}	A_{280nm} Loss%	SEC: % single chain diabody	SEC: % Loss of diabody	SEC: % HMW forms	SEC: % Total
IL12-L19L19 formulation buffer 1A, t = 0	3.005	-	93.15	-	6.85	100
I freeze/thaw	2.995	0.333	93.92	no loss	6.08	100
II freeze/thaw	2.975	0.998	93.10	0.05	6.90	100
III freeze/thaw	2.840	5.491	93.34	no loss	6.66	100
7 days at 2-8°C	2.965	1.331	93.43	no loss	6.57	100
14 days at 2-8°C	2.930	2.496	91.42	1.73	8.58	100
IL12-L19L19 formulation buffer 1B, t = 0	3.070	-	93.72	-	6.28	100
I freeze/thaw	3.035	1.140	94.05	no loss	5.95	100
II freeze/thaw	3.010	1.954	93.82	no loss	6.18	100
III freeze/thaw	2.950	3.909	94.47	no loss	5.53	100
7 days at 2-8°C	3.020	1.629	93.61	0.11	6.39	100
14 days at 2-8°C	2.995	2.443	92.10	1.62	7.90	100
IL12-L19L19 formulation buffer 2A, t = 0	3.065	-	94.29	-	5.71	100
I freeze/thaw	2.950	3.752	94.15	0.14	5.85	100
II freeze/thaw	2.910	5.057	94.05	0.24	5.95	100
III freeze/thaw	2.820	7.993	93.50	0.79	6.50	100
7 days at 2-8°C	3.040	0.816	93.21	1.08	6.79	100
14 days at 2-8°C	3.010	1.794	91.88	2.41	8.12	100

Sample and time-points	A_{280nm}	A_{280nm} Loss%	SEC: % single chain diabody	SEC: % Loss of diabody	SEC: % HMW forms	SEC: % Total
IL12-L19L19 formulation buffer 2B, t = 0	3.055	-	94.18	-	5.82	100
I freeze/thaw	3.030	0.818	94.11	0.07	5.89	100
II freeze/thaw	2.965	2.946	94.17	0.01	5.83	100
III freeze/thaw	2.955	3.273	94.21	no loss	5.79	100
7 days at 2-8°C	3.045	0.327	93.93	0.24	6.07	100
14 days at 2-8°C	2.990	2.128	91.76	2.42	8.24	100

Table 3

Sample and time-points	A_{280nm}	A_{280nm} Loss%	SEC: % single chain diabody	SEC: % Loss of diabody	SEC: % HMW forms	SEC: % Total
IL12-L19L19 formulation buffer 3A, t = 0	3.225	-	94.89	-	5.11	100
I freeze/thaw	3.190	1.085	94.53	0.37	5.47	100
II freeze/thaw	3.145	2.481	94.28	0.62	5.72	100
III freeze/thaw	3.065	4.961	94.44	0.45	5.56	100
7 days at 2-8°C	3.175	1.550	93.70	1.20	6.30	100
14 days at 2-8°C	3.130	2.946	92.51	2.38	7.49	100
IL12-L19L19 formulation buffer 3B, t = 0	3.200	-	94.81	-	5.19	100
I freeze/thaw	3.095	3.281	94.41	0.40	5.59	100
II freeze/thaw	3.085	3.594	94.42	0.39	5.58	100
III freeze/thaw	2.965	7.344	95.03	no loss	4.97	100
7 days at 2-8°C	3.185	0.469	93.12	1.69	6.88	100
14 days at 2-8°C	3.175	0.781	93.13	1.68	6.87	100

Sample and time-points	A_{280nm}	A_{280nm} Loss%	SEC: % single chain diabody	SEC: % Loss of diabody	SEC: % HMW forms	SEC: % Total
IL12-L19L19 formulation buffer 4A, t = 0	3.195	-	94.72	-	5.28	100
I freeze/thaw	3.125	2.191	95.00	no loss	5.00	100
II freeze/thaw	3.110	2.660	93.59	1.13	6.41	100
III freeze/thaw	3.085	3.443	94.83	no loss	5.17	100
7 days at 2-8°C	3.125	2.191	92.65	2.07	7.35	100
14 days at 2-8°C	3.120	2.347	92.34	2.38	7.66	100

Table 3 ctd'

Stability study under stress conditions				
Classification	**Formulation buffer**	**% loss of A_{280nm} (3° freeze/thaw cycle)**	**Formulation buffer**	**Excipients in the buffer**
1	2B	3.3%	Histidine pH 8.0	8.5% w/v Sucrose
2	4A	3.4%	Citrate pH 6.0	8.0% w/v Sucrose 1% w/v Glycerol
3	1B	3.9%	Hepes/NaCl pH 7.0	50mM Mannitol 1% w/v Glycerol
4	3A	5.0%	Phosphate pH 6.5	50mM Mannitol 1% w/v Glycerol
5	1A	5.5%	Hepes pH 7.0	50mM Mannitol 1% w/v Glycerol
6	3B	7.3%	Phosphate pH 7.5	50mM Mannitol 1% w/v Glycerol
7	2A	8.0%	Histidine pH 6.0	8.5% w/v Sucrose

Table 4

Stability study at 2-8°C				
Classification	**Formulation buffer**	**% loss of A_{280nm} (after 14 days)**	**Formulation buffer**	**Excipients in the buffer**
1	3B	0.8%	Phosphate pH 7.5	50mM Mannitol 1% w/v Glycerol
2	2A	1.8%	Histidine pH 6.0	8.5% w/v Sucrose
3	2B	2.1%	Histidine pH 8.0	8.5% w/v Sucrose
4	4A	2.3%	Citrate pH 6.0	8.0% w/v Sucrose 1% w/v Glycerol
5	1B	2.4%	Hepes/NaCl pH 7.0	50mM Mannitol 1% w/v Glycerol
6	1A	2.5%	Hepes pH 7.0	50mM Mannitol 1% w/v Glycerol
7	3A	2.9%	Phosphate pH 6.5	50mM Mannitol 1% w/v Glycerol

Table 5

What is claimed is:

1. A pharmaceutical formulation comprising a recombinant protein comprising (i) interleukin-12 (IL-12) and (ii) an antibody binding the extra-domain B (ED-B) of fibronectin, or a target binding fragment or derivative thereof, the formulation comprising:

    - Histidine, Sucrose and EDTA, adjusted to have a pH of 8.0 ± 0.3
    - citric acid, sodium citrate, Sucrose, Glycerol, EDTA, adjusted to have a pH of 6.0 ± 0.3; or
    - Hepes, NaCl, Mannitol, Glycerol, EDTA, adjusted to have a pH of 7.0 ± 0.3.

2. A dosage unit or regimen comprising ≥ 0,1 μg kg^{-1}, relative to a human patient's body weight, of a recombinant protein comprising (i) interleukin-12 (IL-12) and (ii) an antibody binding the extra-domain B (ED-B) of fibronectin, or a target binding fragment or derivative thereof.

3. A dosage unit or regimen comprising ≤ 100 μg kg^{-1}, relative to a human patient's body weight, of a recombinant protein comprising (i) interleukin-12 (IL-12) and (ii) an antibody binding the extra-domain B (ED-B) of fibronectin, or a target binding fragment or derivative thereof.

4. A dosage unit or regimen comprising between ≥ 0,5 μg kg^{-1} and < 4 μg kg^{-1}, relative to a human patient's body weight, of a recombinant protein comprising (i) interleukin-12 (IL-12) and (ii) an antibody binding the extra-domain B (ED-B) of fibronectin, or a target binding fragment or derivative thereof.

5. A recombinant protein comprising (i) interleukin-12 (IL-12) and (ii) an antibody binding the extra-domain B (ED-B) of fibronectin, or a target binding fragment or derivative thereof, for use in the treatment of a patient being diagnosed for, or suffering from, cancer, wherein the recombinant protein is administered in a dose according to the dosage unit or regimen of any one of claims 2 - 4.

6. Use of a recombinant protein comprising (i) interleukin-12 (IL-12) and (ii) an antibody binding the extra-domain B (ED-B) of fibronectin, or a target binding fragment or

derivative thereof (in the manufacture of a medicament) for the treatment of a human patient being diagnosed for, or suffering from, cancer, wherein the protein is for administration to a patient at a dosage unit or regimen of any one of claims 2 – 4.

7. A method of treating a patient being diagnosed for, or suffering from cancer, the method comprising administering to the patient a composition comprising a recombinant protein comprising (i) interleukin-12 (IL-12) and (ii) an antibody binding the extra-domain B (ED-B) of fibronectin, or a target binding fragment or derivative thereof, in a dose according to the dosage unit or regimen of any one of claims 2 - 4.

8. The recombinant protein for use of claim 5, the use of claim 6 or the method of claim 7, wherein the dose is administered to the patient daily, halfweekly, weekly, biweely, triweekly, monthly or bimonthly.

9. A recombinant protein comprising (i) interleukin-12 (IL-12) and (ii) an antibody binding the extra-domain B (ED-B) of fibronectin, or a target binding fragment or derivative thereof, for use in the treatment of a patient being diagnosed for, or suffering from, cancer, being selected from advanced/metastatic immunotherapy responsive solid carcinoma or lymphoma.

10. A method of treating a patient being diagnosed for, or suffering from advanced/metastatic immunotherapy responsive solid carcinoma or lymphoma, the method comprising administering to the patient a composition comprising, in a therapeutically sufficient dose, a recombinant protein comprising (i) interleukin-12 (IL-12) and (ii) an antibody binding the extra-domain B (ED-B) of fibronectin, or a target binding fragment or derivative thereof.

11. The recombinant protein for use of claim 5, 8 or 9, the use of claim 6, or the method of claim 7 or 10, wherein the carcinoma or lymphoma is at least one selected from the group consisting of:
    a) malignant melanoma
    b) non-small cell lung cancer (NSCLC)
    c) renal cell carcinoma
    d) urothelial carcinoma
    e) head and neck squamous cell carcinoma (HNSCC)

f) microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR) metastatic colorectal cancer
g) hepatocellular cancer
h) gastric cancer
i) squamous cell carcinoma of the skin
j) cervical cancer, and/or
k) diffuse large B-cell lymphoma (DLBCL).

12. The recombinant protein for use claim 5, 8, 9 or 11, the use of claim 6 or 11, or the method of claim 7 or 10 - 11, wherein the carcinoma or lymphoma is characterized by at least one of:
a) has progressed on immune checkpoint-blockade therapy,
b) Eastern cooperative oncology group (ECOG) performance status ≤ 2, and/or
c) at least one unidimensionally measurable lesion either by computed tomography (CT), MRI or PET/CT as defined by RECIST (v. 1.1) for solid tumors or by LUGANO criteria for malignant lymphoma.

13. The recombinant protein for use of claim 5, 8, 9 or 11 - 12, the use of claim 6 or 11-12, or the method of claim 7 or 10 - 12, wherein the patient is characterized by at least one of:
a) has received an immune checkpoint blockade therapy-based regimen as immediate prior treatment
b) has had clinical benefit (CR/PR/SD) while on immune checkpoint blockade therapy defined as $\geq$ 3 month free from progression from initial imaging documenting metastatic disease followed by radiographic disease progression after immune checkpoint blockade therapy
c) has received ≥ 2 prior systemic therapies, when being diagnosed for, or suffering from, DLCBL; and/or
d) being negatively tested for HIV, HBV and HCV.

14. The dosage unit, recombinant protein for use, method or formulation according to any one of the aforementioned claims, wherein the antibody comprised in the recombinant protein comprises at least one single-chain Fv (scFv) antibody fragment, optionally a single chain diabody.

15. The dosage unit, recombinant protein for use, method or formulation according to any one of the aforementioned claims, wherein the IL-12 comprised in the recombinant protein comprises a p40 subunit and a p35 subunit, linked by a linker.

16. The dosage unit, recombinant protein for use, method or formulation according to any one of the aforementioned claims, wherein at least one of

- the p40 subunit and the p35 subunit comprise the amino acid sequence according to SEQ ID NO: 1 or SEQ ID NO: 3, respectively,
- the antibody comprised in the recombinant protein is the anti EDB antibody L19, comprising the amino acid sequence according to SEQ ID NO: 5 as VL domain and SEQ ID NO: 7 as VH domain,
- at least one of the antibody, the IL-12 and/or the linker connecting the two is one disclosed in WO2019154986, optionally wherein the recombinant protein is one disclosed in WO2019154986, and/or
- the recombinant protein comprises, optionally consists of, the sequence according to SEQ ID NO: 9.

17. The dosage unit, recombinant protein for use, method or formulation according to any one of the aforementioned claims, wherein the recombinant protein comprises

a) a p40 domain linked to a p35 domain by a first linker;
b) a first L19 VH domain linked to the p35 domain by a SAD linker;
c) a first L19 VL domain linked to the first L19 VH domain by a third linker;
d) a second L19 VH domain linked to the first L19 VL domain by a fourth linker; and/or
e) a second L19 VL domain linked to the second L19 VH domain by a fifth linker.

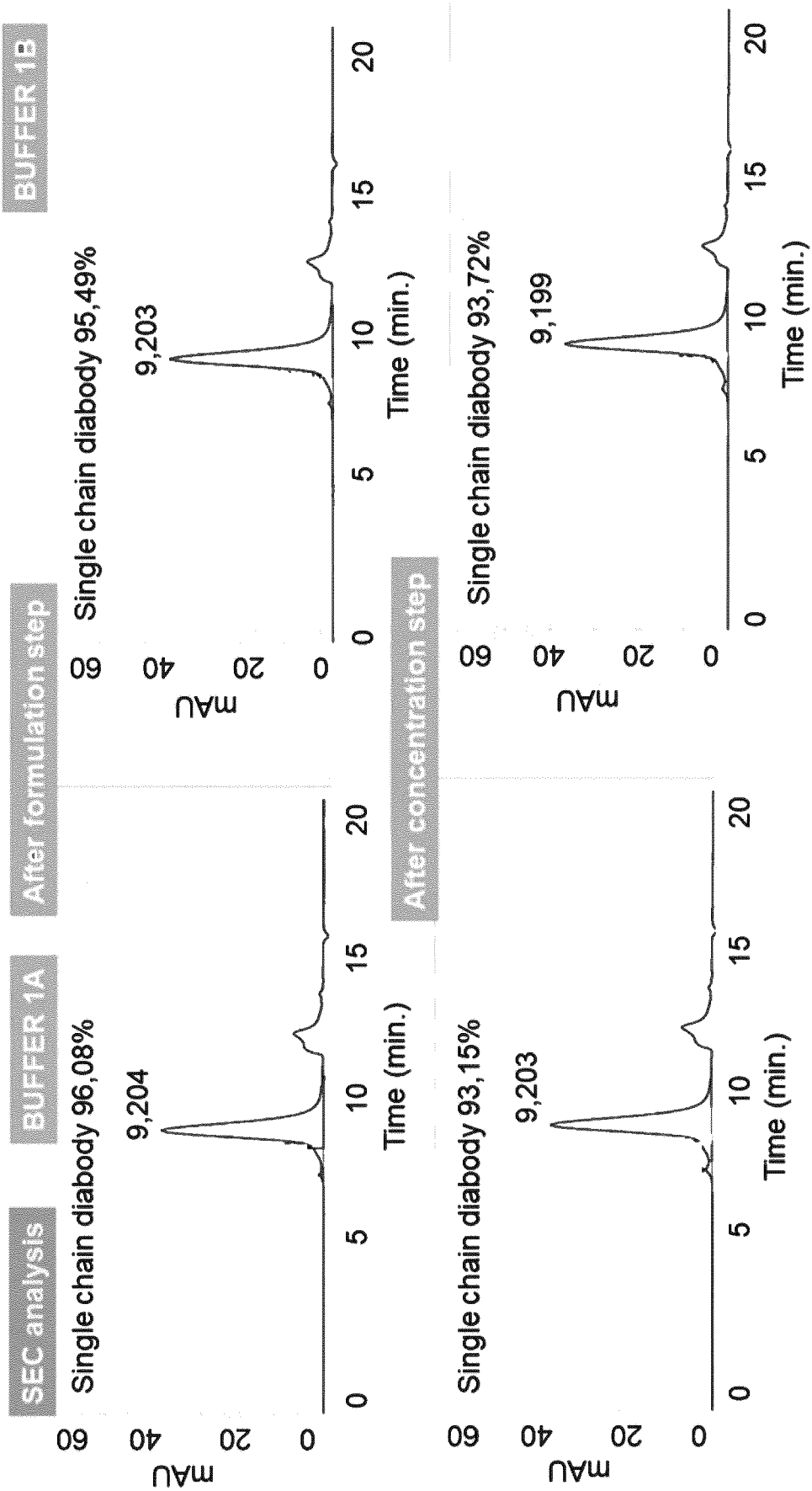
18. The dosage unit or regimen, recombinant protein for use, method or formulation according to any one of the aforementioned claims, where administration is intravenous or subcutaneous.

19. The dosage unit or regimen or recombinant protein for use according to any one of the aforementioned claims, which is provided in a formulation according to claim 1.

20. The formulation according to claim 1, which comprises a dosage unit or regimen or recombinant protein according to any one of claims the aforementioned claims.

21. A kit comprising:

a) the dosage unit or recombinant protein for use according to any one of the aforementioned claims
b) an apparatus for administering the dosage unit or recombinant protein, and, optionally
c) instructions for use.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After concentration step
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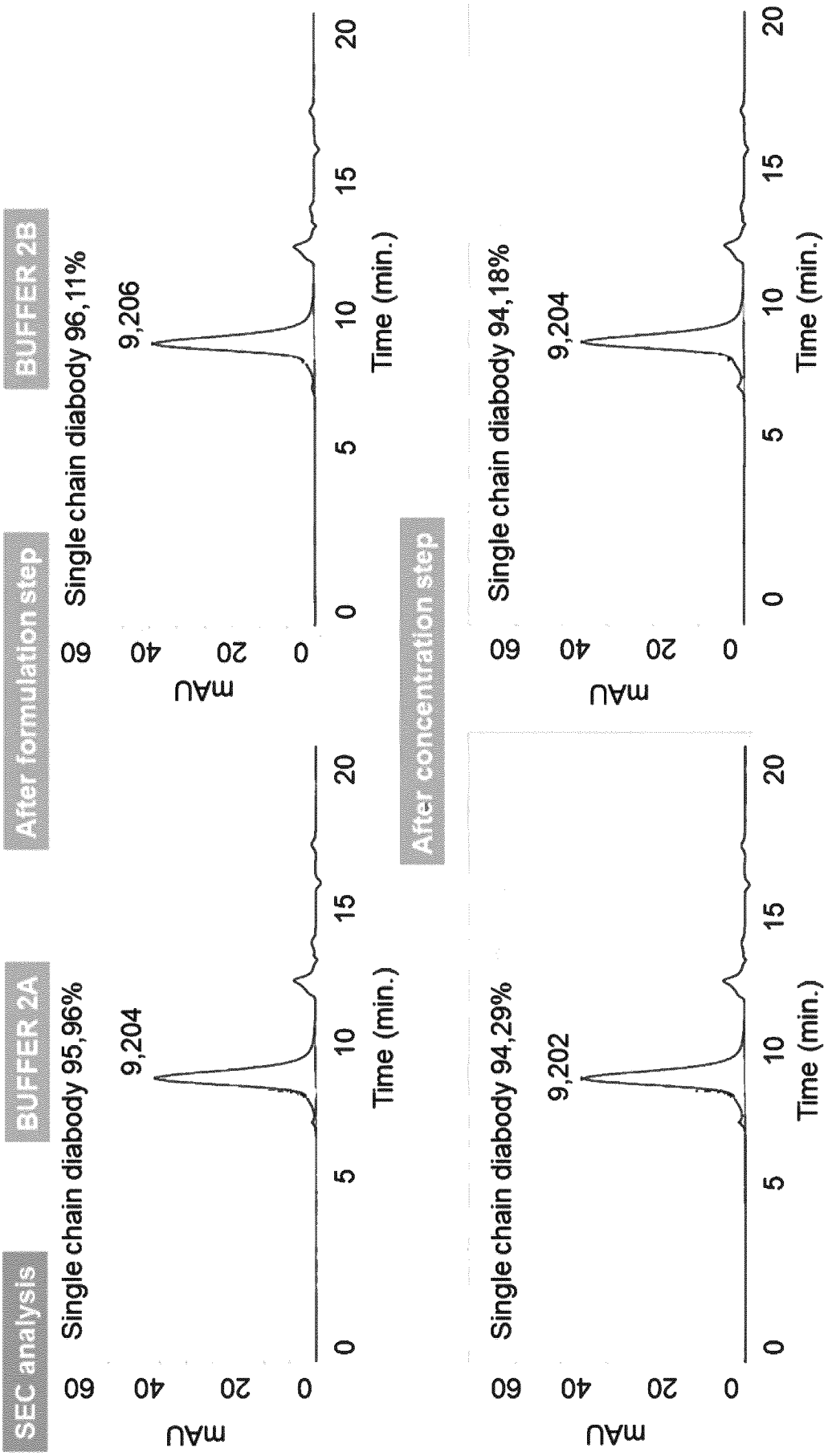
Figure 1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15
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Figure 1 ctd'

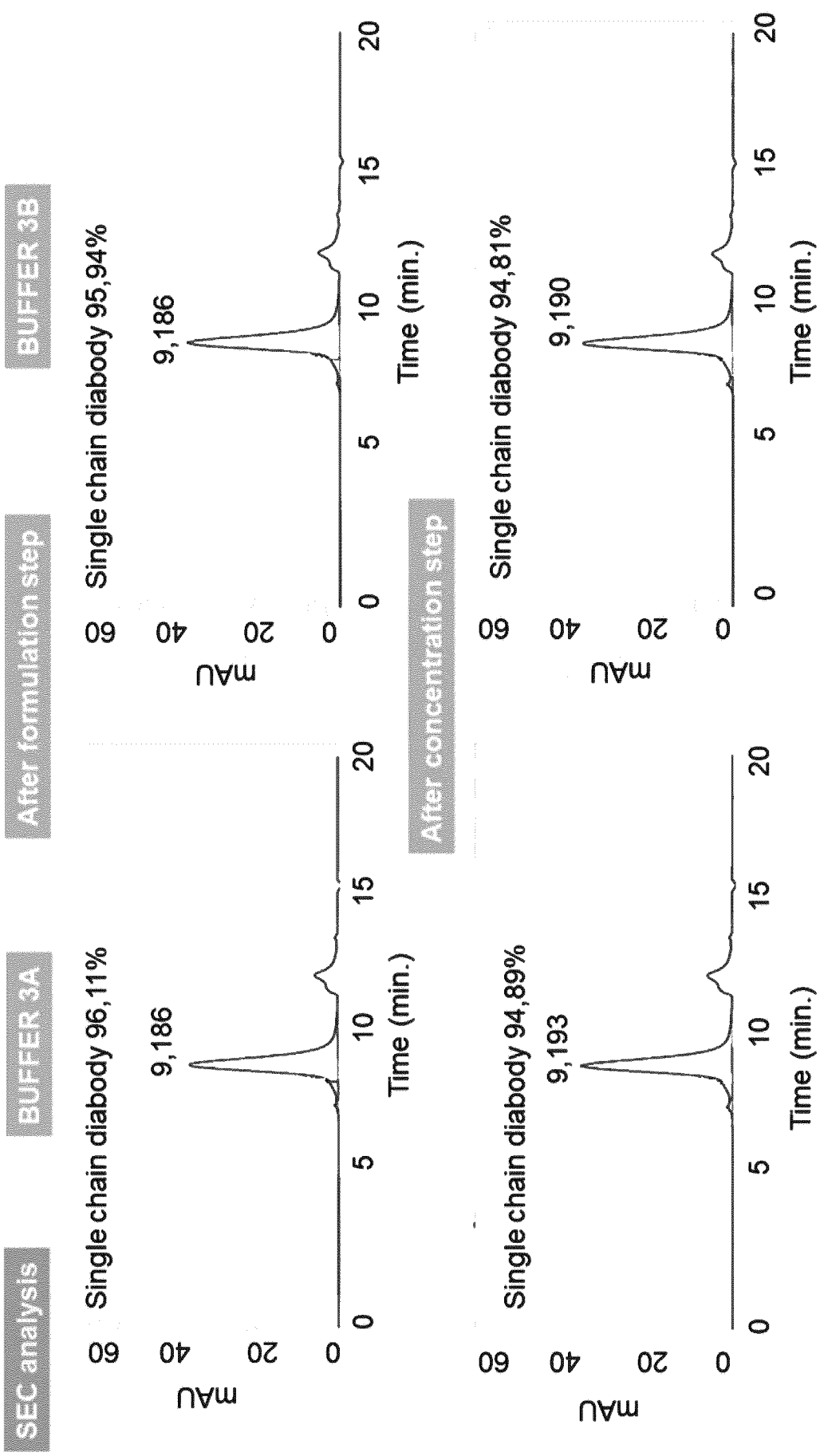
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Figure 2

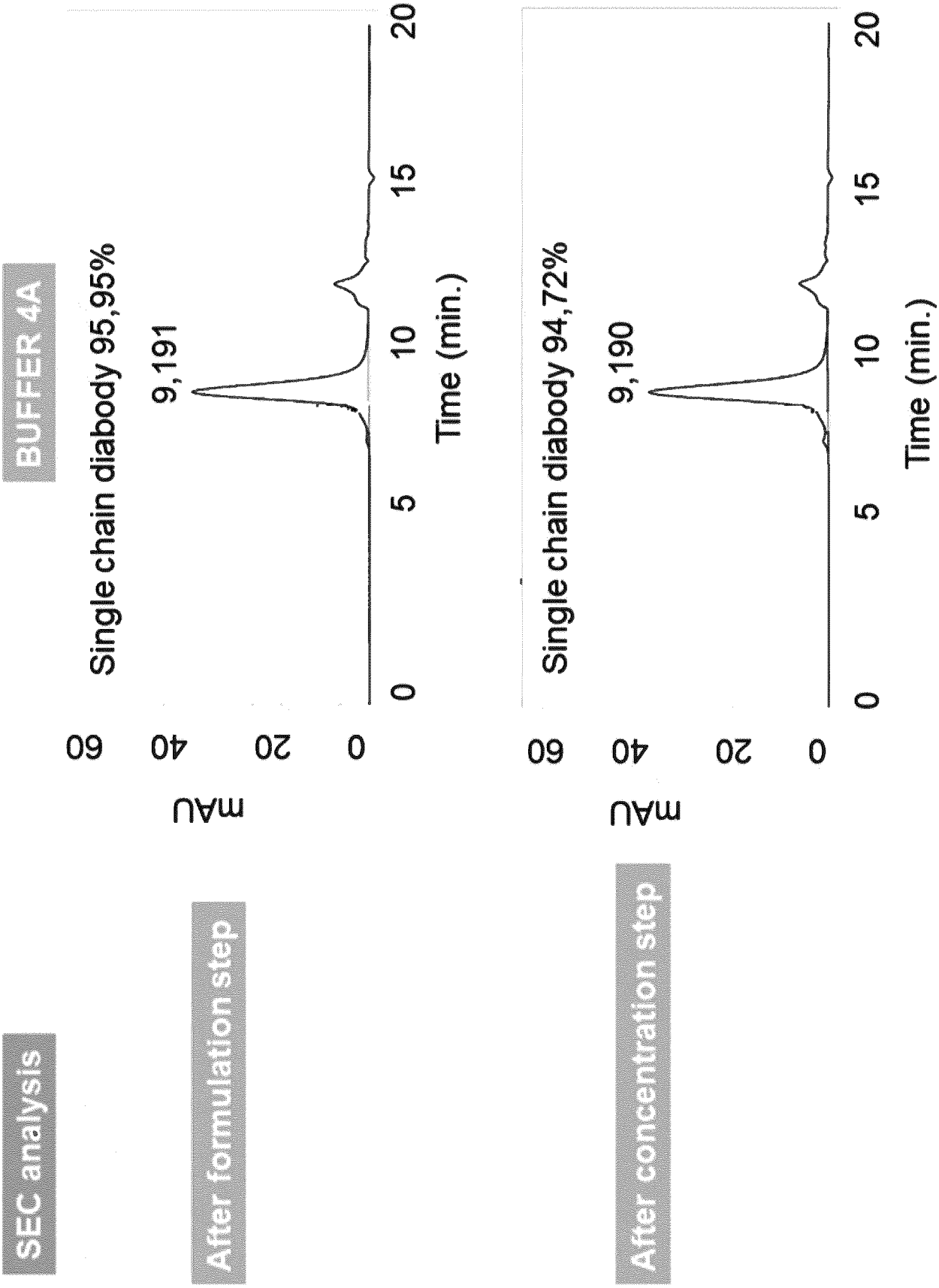
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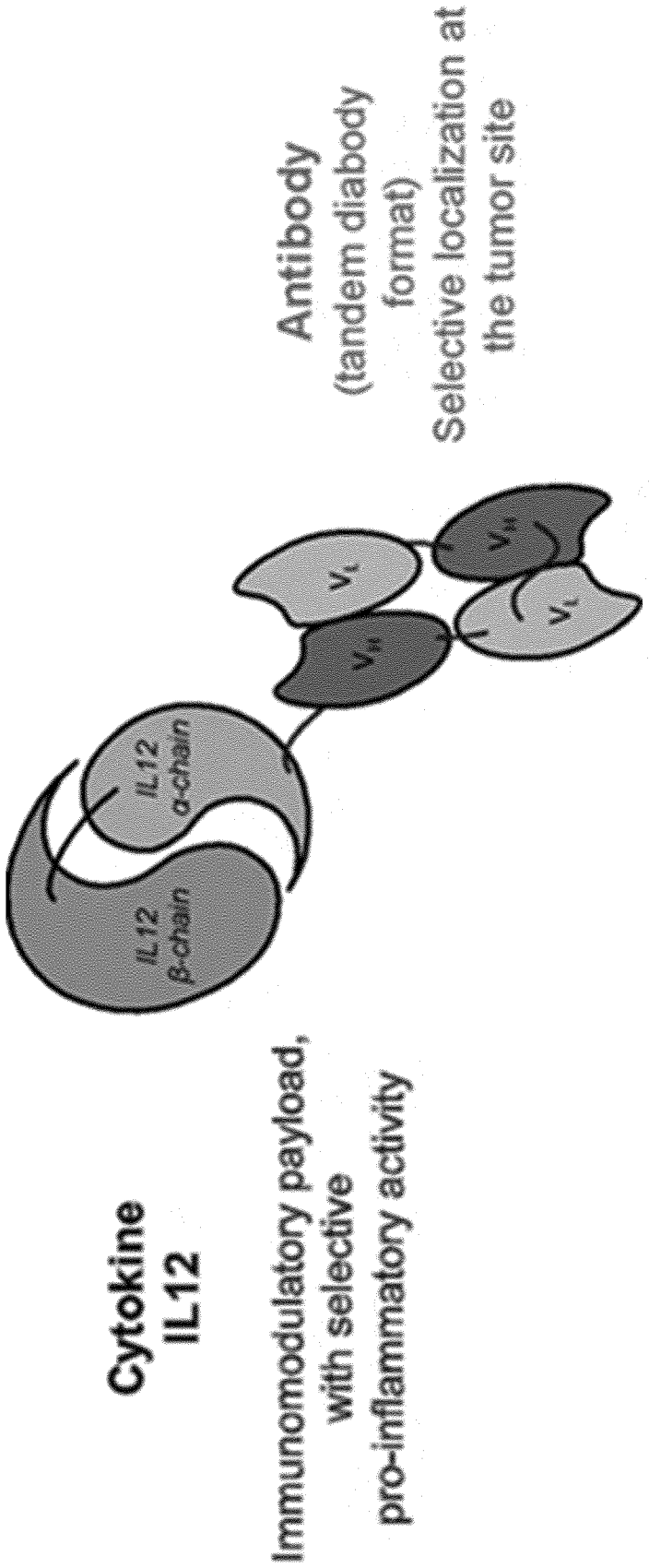
Figure 2 ctd'


Antibody
(tandem diabody format)
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Cytokine
IL12
Immunomodulatory payload, with selective pro-inflammatory activity
IL12 α-chain
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Figure 3

SEQUENCE LISTING

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<223> Immunoconjugate sequence

<400> 9

Ile Trp Glu Leu Lys Lys Asp Val Tyr Val Val Glu Leu Asp Trp Tyr
1 5 10 15

Pro Asp Ala Pro Gly Glu Met Val Val Leu Thr Cys Asp Thr Pro Glu
20 25 30

Glu Asp Gly Ile Thr Trp Thr Leu Asp Gln Ser Ser Glu Val Leu Gly
35 40 45

Ser Gly Lys Thr Leu Thr Ile Gln Val Lys Glu Phe Gly Asp Ala Gly
50 55 60

Gln Tyr Thr Cys His Lys Gly Gly Glu Val Leu Ser His Ser Leu Leu
65 70 75 80

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Leu Leu His Lys Lys Glu Asp Gly Ile Trp Ser Thr Asp Ile Leu Lys
                85                  90                  95

Asp Gln Lys Glu Pro Lys Asn Lys Thr Phe Leu Arg Cys Glu Ala Lys
            100                 105                 110

Asn Tyr Ser Gly Arg Phe Thr Cys Trp Trp Leu Thr Thr Ile Ser Thr
        115                 120                 125

Asp Leu Thr Phe Ser Val Lys Ser Ser Arg Gly Ser Ser Asp Pro Gln
    130                 135                 140

Gly Val Thr Cys Gly Ala Ala Thr Leu Ser Ala Glu Arg Val Arg Gly
145                 150                 155                 160

Asp Asn Lys Glu Tyr Glu Tyr Ser Val Glu Cys Gln Glu Asp Ser Ala
                165                 170                 175

Cys Pro Ala Ala Glu Glu Ser Leu Pro Ile Glu Val Met Val Asp Ala
            180                 185                 190

Val His Lys Leu Lys Tyr Glu Asn Tyr Thr Ser Ser Phe Phe Ile Arg
        195                 200                 205

Asp Ile Ile Lys Pro Asp Pro Pro Lys Asn Leu Gln Leu Lys Pro Leu
    210                 215                 220

Lys Asn Ser Arg Gln Val Glu Val Ser Trp Glu Tyr Pro Asp Thr Trp
225                 230                 235                 240

Ser Thr Pro His Ser Tyr Phe Ser Leu Thr Phe Cys Val Gln Val Gln
                245                 250                 255

Gly Lys Ser Lys Arg Glu Lys Lys Asp Arg Val Phe Thr Asp Lys Thr
            260                 265                 270

Ser Ala Thr Val Ile Cys Arg Lys Asn Ala Ser Ile Ser Val Arg Ala
        275                 280                 285

Gln Asp Arg Tyr Tyr Ser Ser Ser Trp Ser Glu Trp Ala Ser Val Pro
    290                 295                 300
```

```
Cys Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly
305                 310                 315                 320

Ser Arg Asn Leu Pro Val Ala Thr Pro Asp Pro Gly Met Phe Pro Cys
                325                 330                 335

Leu His His Ser Gln Asn Leu Leu Arg Ala Val Ser Asn Met Leu Gln
            340                 345                 350

Lys Ala Arg Gln Thr Leu Glu Phe Tyr Pro Cys Thr Ser Glu Glu Ile
        355                 360                 365

Asp His Glu Asp Ile Thr Lys Asp Lys Thr Ser Thr Val Glu Ala Cys
    370                 375                 380

Leu Pro Leu Glu Leu Thr Lys Asn Glu Ser Cys Leu Asn Ser Arg Glu
385                 390                 395                 400

Thr Ser Phe Ile Thr Asn Gly Ser Cys Leu Ala Ser Arg Lys Thr Ser
                405                 410                 415

Phe Met Met Ala Leu Cys Leu Ser Ser Ile Tyr Glu Asp Leu Lys Met
            420                 425                 430

Tyr Gln Val Glu Phe Lys Thr Met Asn Ala Lys Leu Leu Met Asp Pro
        435                 440                 445

Lys Arg Gln Ile Phe Leu Asp Gln Asn Met Leu Ala Val Ile Asp Glu
    450                 455                 460

Leu Met Gln Ala Leu Asn Phe Asn Ser Glu Thr Val Pro Gln Lys Ser
465                 470                 475                 480

Ser Leu Glu Glu Pro Asp Phe Tyr Lys Thr Lys Ile Lys Leu Cys Ile
                485                 490                 495

Leu Leu His Ala Phe Arg Ile Arg Ala Val Thr Ile Asp Arg Val Met
            500                 505                 510
```

```
Ser Tyr Leu Asn Ala Ser Gly Ser Ala Asp Gly Gly Ser Ser Ala Gly
        515                 520                 525

Gly Ser Asp Ala Gly Glu Val Gln Leu Leu Glu Ser Gly Gly Gly Leu
    530                 535                 540

Val Gln Pro Gly Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe
545                 550                 555                 560

Thr Phe Ser Ser Phe Ser Met Ser Trp Val Arg Gln Ala Pro Gly Lys
                565                 570                 575

Gly Leu Glu Trp Val Ser Ser Ile Ser Gly Ser Ser Gly Thr Thr Tyr
            580                 585                 590

Tyr Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser
        595                 600                 605

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr
    610                 615                 620

Ala Val Tyr Tyr Cys Ala Lys Pro Phe Pro Tyr Phe Asp Tyr Trp Gly
625                 630                 635                 640

Gln Gly Thr Leu Val Thr Val Ser Ser Gly Ser Ser Gly Gly Glu Ile
                645                 650                 655

Val Leu Thr Gln Ser Pro Gly Thr Leu Ser Leu Ser Pro Gly Glu Arg
            660                 665                 670

Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser Val Ser Ser Ser Phe Leu
        675                 680                 685

Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg Leu Leu Ile Tyr
    690                 695                 700

Tyr Ala Ser Ser Arg Ala Thr Gly Ile Pro Asp Arg Phe Ser Gly Ser
705                 710                 715                 720

Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Arg Leu Glu Pro Glu
                725                 730                 735
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```
Asp Phe Ala Val Tyr Tyr Cys Gln Gln Thr Gly Arg Ile Pro Pro Thr
            740                 745                 750

Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Ser Ser Ser Ser Gly Ser
        755                 760                 765

Ser Ser Ser Gly Ser Ser Ser Ser Gly Glu Val Gln Leu Leu Glu Ser
    770                 775                 780

Gly Gly Gly Leu Val Gln Pro Gly Gly Ser Leu Arg Leu Ser Cys Ala
785                 790                 795                 800

Ala Ser Gly Phe Thr Phe Ser Ser Phe Ser Met Ser Trp Val Arg Gln
                805                 810                 815

Ala Pro Gly Lys Gly Leu Glu Trp Val Ser Ser Ile Ser Gly Ser Ser
            820                 825                 830

Gly Thr Thr Tyr Tyr Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser
        835                 840                 845

Arg Asp Asn Ser Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg
    850                 855                 860

Ala Glu Asp Thr Ala Val Tyr Tyr Cys Ala Lys Pro Phe Pro Tyr Phe
865                 870                 875                 880

Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Gly Ser Ser
                885                 890                 895

Gly Gly Glu Ile Val Leu Thr Gln Ser Pro Gly Thr Leu Ser Leu Ser
            900                 905                 910

Pro Gly Glu Arg Ala Thr Leu Ser Cys Arg Ala Ser Gln Ser Val Ser
        915                 920                 925

Ser Ser Phe Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ala Pro Arg
    930                 935                 940
```

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Leu Leu Ile Tyr Tyr Ala Ser Ser Arg Ala Thr Gly Ile Pro Asp Arg
945                 950                 955                 960


Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Arg
                965                 970                 975


Leu Glu Pro Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Thr Gly Arg
            980                 985                 990


Ile Pro Pro Thr Phe Gly Gln Gly  Thr Lys Val Glu Ile  Lys
        995                 1000                1005
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