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(54) Title: ANTI ROR1 ANTIBODIES AND METHOD OF USE

(57) **Abstract:** The present invention relates to antibodies binding ROR1.

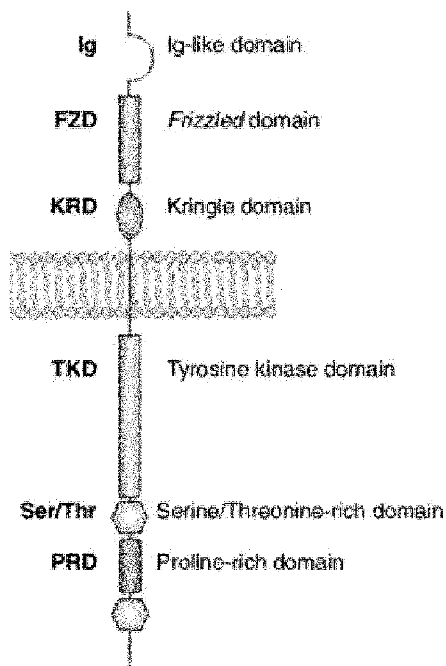

Fig. 9

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Pure Biologics S.A

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anti ROR1 antibodies and method of use

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Pursuant to the EFS-Web legal framework and 37 CFR §§ 1.821-825 (see MPEP § 2442.03(a)), Rule 30 EPC, and § 11 PatV, an electronic sequence listing compliant with WIPO standard ST.26 in the form of an XML 1.0 format file is submitted concurrently with the instant application, and the entire contents of the sequence listing are incorporated herein by reference. For the avoidance of doubt, if discrepancies exist between the sequences mentioned in the specification and the electronic sequence listing, the sequences in the specification shall be deemed to be the correct ones.

Field of the invention

The present invention relates to antibodies and fragments or derivatives thereof that bind to ROR1, and methods of using the same.

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

Receptor tyrosine kinase-like orphan receptor 1 (ROR1) is a member of the ROR family consisting of ROR1 and ROR2. The ROR1 protein contains extracellular immunoglobulin-like (Ig) domain at the amino-terminus, followed by a cysteine-rich domain known as a Frizzled domain (FZD), and then a Kringle domain (KRD). The intracellular part of ROR1 consists of a tyrosine kinase domain, two serine/threonine-rich domains and a proline-rich domain for cell signaling. RORs have been discovered and massively studied in the context of embryogenesis and neurogenesis but lately a growing literature has established ROR1 as a marker for cancer, such as in CLL and in other blood malignancies. More importantly, ROR1 is shown to be critically involved in the progression of a number of blood and solid cancers. For example, ROR1 has been shown to inhibit apoptotic signals, potentiate EGFR signaling, and induce epithelial-mesenchymal transition (EMT).

While ROR1 expression is present during normal embryonic and fetal development, the expression is absent in most of the mature tissues. A low level of ROR1 expression is observed in the adipose tissue and to a lesser degree in the pancreas, lung, thyroid, stomach and a subset of intermediate B cells (Baskar et al., 2008; Hudecek et al., 2010; Bicocca et al., 2012 + others). However, the expression of ROR1 has been seen reported be upregulated in numerous blood and solid malignancies. A strong expression of ROR1 was initially identified in B-Cell chronic lymphocytic leukemia (CLL). Primary CLL cells express high levels of ROR1, but not ROR2 and the expression of ROR1 increases through the progression of CLL. According to CLL research consortium ROR1 is expressed on the surface of CLL cells from nearly all patients with CLL, 94% of them based on the flow cytometry analysis. ROR1 is not only a biomarker for CLL, but it may serve as a potential prognostic indicator (Daneshmanesh et al., 2013). The constitutive phosphorylation of STAT3, a hallmark of CLL, has been shown to bind multiple sites in the ROR1 promoter. In addition, the expression of ROR1 could be induced by IL-6 in a STAT3-dependent and dose-dependent manner (Frank et al., 1997; Li et al., 2010). Since the discovery of the elevated expression of ROR1 in CLL, increased levels of ROR1 have been described in a variety of hematological malignancies, including acute lymphocytic leukemia (ALL), non-Hodgkin lymphomas (NHL), and myeloid malignancies (Daneshmanesh et al., 2008; Barna et al., 2011; Daneshmanesh et al., 2013). In addition, ROR1 expression has been

shown in Ovarian cancer, Breast cancer, especially triple negative breast cancer, and lung cancer.

The importance of ROR1 in cancer cell survival and proliferation is confirmed in clinical trials, where Zilovertamab (also named Cirmtuzumab, UC-961), shows encouraging results in patients suffering from CLL or MCL. Zilovertamab is an ROR1 antagonist inhibiting tumor cell proliferation and migration. However, it does not activate immune cells to induce ADCC (Antibody dependent cellular cytotoxicity).

Although ADCC inducing ROR1 antibodies has been previously reported, the effects of these antibodies are relatively low.

Technical Problem

It is an object of the present invention to provide a new therapeutic approach that increases efficacy of anti ROR1 treatment.

It is an object of the present invention to provide a new and improved therapeutic approach to treat cancer.

These and other problems are solved by the embodiments set forth in the independent claims herein. The dependent claims disclose preferred embodiments.

Brief Description Of The Figures

Table 2 depicts kinetics parameters of interaction with recombinant extracellular hROR1 analyzed by BLI/SPR. Apparent K_D was evaluated from the 1:2 bivalent analyte model: binding EC50 to CHO cells transfected with hROR1 analyzed by flow cytometry, domain-binding analysis by BLI:cross-reactivity analysis to recombinant mouse ROR1, analysed by BLI/SPR and binding to human recombinant ROR-2 analyzed by BLI/SPR and flow cytometry analysis to CHO-ROR2 cells.

Figure 1A-1D depicts binding curves for EC50 assessment of tested antibodies for CHO cell lines overexpressing ROR1, ROR2 and control cell line CHO-EV. (A) PB004.22.0357.aF

afucosylated antibody binds only ROR1 overexpressing cell line with EC50 of 0.933 x 10-9 M. (B) PB004.22.0372.aF afucosylated antibody binds only ROR1 overexpressing cell line with EC50 of 0.617 x 10-9 M (C) PB004.22.0405.aF afucosylated antibody binds only ROR1 overexpressing cell line with EC50 of 0.557 x 10-9 M. (D) PB004.22.0408.aF afucosylated antibody binds only ROR1 overexpressing cell line with EC50 of 0.233 x 10-9 M. Bars represent standard deviation calculated based on two technical replicates.

Table 3 depicts comparison of affinities between standard IgG1 or afucosylated IgG1 and FcyRIIIA receptors.

Figure 2 depicts FcγRIII dependent ADCC**.** (A) ADCC against JeKo-1 cells using Promega ADCC reporter kit measuring FcγRIII interaction. The target cells were incubated for 6 hours in the presence of respective antibody and reporter cells and analyzed by luminescence reader. B-F Flow cytometry-based analysis of NK cell-dependent induction of cancer cell killing (ADCC). NK cells were isolated from healthy donor peripheral blood mononuclear cells (PBMCs). Samples were incubated 4h with different concentrations of Zilovertamab, R12hIgG1 (humanized variant of rabbit monoclonal R12 described in Yang et al, 2011), the anti CD20 antibody Rituximab, afucosylated isotype control and selected antibodies (PB004.22.0357.aF, PB004.22.0372.aF, PB004.22.0405, PB004.22.0405.aF, PB004.22.0408.aF) with the target cells (Jeko-1, ratio NK:Jeko-1; Effector:Target 10:1). (B) Representative ADCC induction for PB004.22.0405 and PB004.22.0405.aF (B) ADCC comparison PB004.22.0405.aF, Rituximab, Zilovertamab and R12hIgG1. (C) Representative ADCC result for PB004.22.0357.aF (D) Representative ADCC result for PB004.22.0372.aF (E) Representative ADCC for PB004.22.0408.aF. Bars represent standard deviation calculated based on two technical replicates.

Figure 3 depicts the potent but safe ADCC induction by the tested antibodies. Either a whole cell PBMC or isolated NK cells isolated from healthy donor peripheral blood mononuclear cells (PBMCs) were used as effector cells. Samples were incubated 4h with different concentrations of PB004.22.0405.aF Zilovertamab, Rituximab, afucosylated isotype control, with the target cells (Jeko-1, primary CLL material, or naive B cells) and NK cells. Combination experiments were perfomed with)N incubation with either venetoclax or ibrutinib A) Representative ADCC induction against JeKo-1 cells using whole cell PBMC (B) Pooled data of ADCC induction against primary CLL patient material , (C) Biomarkor analysis in form of NK cell activation by

PB004.22.0405.aF, Zilovertamab or Rituximab. (D) Additive/ synergistic ADCC effect of PB0004.22.405 with venetoclax (E) Additive/ synergistic ADCC effect of PB0004.22.405 with ibrutinib (F) PB0004.22.405 does not kill naïve B cells compared to rituximab.

Figure 4 depicts additional mode of actions. (A) Flow cytometry-based analysis of Antibody-dependent cellular phagocytosis (ADCP). Samples were incubated 4h with 50 nM isotype control, R12hIgG1 antibody, Zilovertamab, Rituximab and selected leads (PB004.22.0357.aF, PB004.22.0372.aF, PB004.22.0405.aF, PB004.22.0408.aF) with the Jeko-1 cells (Macrophages:Jeko-1, ratio Effector: Target 2:1). Percentage cell phagocytosis was calculated using the formula: number of dual-stain positive target cells (cells engulfed by macrophages; Violet+/CFSE+) divided by the total number of target cells (CFSE+).
(B) C1q binding ELISA was performed for CDC induction capability. The tested antibodies with controls were coated on ELISA plates ON, followed by washing, blocking and recombinant C1q was added in serial dilutions. The C1q binding was detected by anti-C1q-HRP antibodies and after TMB substrate the plates were analysed in a plate reader.

Figure 5 depicts the Anti-tumor efficacy of the tested antibodies against subcutaneous JeKo-1 tumor. (A) CD34$^+$ humanized NCG-hIL15 mice were inoculated subcutaneously with JeKo-1 and were treated with 6 doses of vehicle, PB004.22.0357, PB004.22.0372, PB004.22.0405, PB004.22.0408, Zilovertamab or Rituximab at 10 mg/kg twice weekly starting when the tumor volume was in average around 85 mm^{3,}.The graph represents the mean ± SEM, n=8, Statistical significancy was considered using 2-way ANOVA, with Bonferroni multiple comparison * $p<0.01$, **** $p< 0.0001$. B) Specific activation on tumor NK cells. At the termination of the experiment, the tumors and spleens were removed and processed to single together with terminal bleeding for flowcytometry analysis. The data represents mean ± SD, n=8, Statistical significancy was considered using Kruskal-Wallis, non-parametric ANOVA, ** $p<0.001$.

Table 4 depicts the anti-tumor efficacy against JeKo-1 tumor. CD34$^+$ humanized NCG-hIL15 mice were inoculated subcutaneously with JeKo-1 tumor cells and were treated with 6 doses of different antibodies at 10 mg/kg twice weekly starting when the tumor volume was in average around 85 mm^{3,} At termination the tumor volumes were compared to vehicle. The table decapitates the tumor volume inhibition compared to vehicle treated group.

Figure 6 depicts anti-tumor efficacy in a leukemia model. SCID mice were inoculated intravenously with MEC1-ROR1 cells, a CLL cell line overexpressing human ROR1. After one week the mice received intravenous treatments of vehicle, PB004.22.0405.aF or Zilovertamab at 10 mg/kg twice a week for total of 6 doses. Four weeks after inoculation the mice were terminated, and the bone marrow and spleen were analyzed for CD19 and ROR1 positive CLL cells. The graph represents the mean ± SD, n=4-5, Statistical significancy was considered using one-way analysis of variance (ANOVA), with Kruskal-Wallis multiple comparison *p<0.01

Figure 7 depicts the *in vivo* safety assessment. HuHSC-NCG-IL15 mice were inoculated with subcutaneous JeKo-1 cells and established tumor-bearing mice were treated intravenously biweekly with 6 injections at 10 mg/kg. Safety of the antibodies was assessed by (A) weight measurements (B) GvHD score (measuring weight, posture, fur texture and skin integrity) (C). serum cytokines in plasma 6 h or 24 h after treatment. The graph represents pooled 3 exemplary cytokines measured. The graphs present mean ± SEM for A and B and ± SD for C. No statistical significance was observed in any parameters measured, (Kruskal-Wallis).

Figure 8 depict the pharmacokinetic profile of PB004.22.0405 measured by mouse serum in ELISA. BALB/cJRj mice were injected with a single IV bolus injection of PB004.22.0405 at 10 mg/kg, bled at different time-points and the plasma was analyzed in ELISA using ROR1 coated plates. Detection was performed by anti-FC-HRP antibodies. Data represents mean ± SD.

Figure 9 depicts the domain structure of ROR1.

DETAILED DESCRIPTION OF THE INVENTION

According to a first aspect of the invention, an antibody binding ROR1, or a target-binding fragment or derivative thereof retaining target binding capacities, is provided, which comprises the heavy chain/light chain variable domain (HCVD/LCVD) pairs set forth in the following:

- SEQ ID NO: 1 and SEQ ID NO: 2,
- SEQ ID NO: 3 and SEQ ID NO: 4,
- SEQ ID NO: 5 and SEQ ID NO: 6, and/or
- SEQ ID NO: 7 and SEQ ID NO: 8.

The antibody having the VH/VL sequences of SEQ ID NOs: 1 and 2 is called PB004.22.0357 herein.
The antibody having the VH/VL sequences of SEQ ID NOs: 3 and 4 is called PB004.22.0372 herein.
The antibody having the VH/VL sequences of SEQ ID NOs: 5 and 6 is called PBA0405, AF0405, PB0405 or PB004.22.0405 herein.
The antibody having the VH/VL sequences of SEQ ID NOs: 7 and 8 is called PB004.22.0408 herein.

Afucosylated variants of these antibodies carry the tag “af”.

Tyrosine-protein kinase transmembrane receptor ROR1 (UniProt: Q01973), also known as neurotrophic tyrosine kinase, receptor-related 1 (NTRKR1), is an enzyme that in humans is encoded by the ROR1 gene. ROR1 is a member of the receptor tyrosine kinase-like orphan receptor (ROR) family. The protein encoded by this gene is a receptor tyrosine kinase that modulates neurite growth in the central nervous system. It is a type I membrane protein and belongs to the ROR subfamily of cell surface receptors. ROR1 is currently under investigation for its role in the metastasis of cancer cells.

ROR1 has recently been shown to be expressed on ovarian cancer stem cell, on which it seems to play a functional role in promoting migration/invasion or spheroid formation in vitro and tumor engraftment in immune-deficient mice.

Human ROR1 consists of an immunoglobulin-like domain (IG), two cysteine-rich domain, frizzled (FZD) and kringle domain (KRD). On the intracellular side, ROR1 possesses a tyrosine kinase domain (TKD), two serine/threonine-rich domains (Ser/Thr), and a proline-rich domain (PRD).

In one embodiment, said antibody is a monoclonal antibody.

As used herein, the term “monoclonal antibody (mAb)” shall refer to an antibody composition having a homogenous antibody population, i.e., a homogeneous population consisting of a whole immunoglobulin, or a fragment or derivative thereof retaining target binding capacities.

Particularly preferred, such antibody is selected from the group consisting of IgG, IgD, IgE, IgA and/or IgM, or a fragment or derivative thereof retaining target binding capacities.

As used herein, the term “fragment” shall refer to fragments of such antibody retaining target binding capacities, e.g.

- a CDR (complementarity determining region)
- a hypervariable region,
- a variable domain (Fv)
- an IgG or IgM heavy chain (consisting of VH, CH1, hinge, CH2 and CH3 regions)
- an IgG or IgM light chain (consisting of VL and CL regions), and/or
- a Fab and/or $F(ab)_2$.

As used herein, the term “derivative” shall refer to protein constructs being structurally different from, but still having some structural relationship to, the common antibody concept, e.g., scFv, Fab and/or $F(ab)_2$, as well as bi-, tri- or higher specific antibody constructs, and further retaining target binding capacities. All these items are explained below.

Other antibody derivatives known to the skilled person are Diabodies, Camelid Antibodies, Nanobodies, Domain Antibodies, bivalent homodimers with two chains consisting of scFvs, IgAs (two IgG structures joined by a J chain and a secretory component), shark antibodies, antibodies consisting of new world primate framework plus non-new world primate CDR, dimerized constructs comprising CH3+VL+VH, and antibody conjugates (e.g. antibody or fragments or derivatives linked to a toxin, a cytokine, a radioisotope or a label). These types are well described in the literature and can be used by the skilled person on the basis of the present disclosure, without adding further inventive activity.

Methods for the production of a hybridoma cell are disclosed in Köhler & Milstein (1975).

Methods for the production and/or selection of chimeric or humanised mAbs are known in the art. For example, US6331415 by Genentech describes the production of chimeric antibodies, while US6548640 by Medical Research Council describes CDR grafting techniques and US5859205 by Celltech describes the production of humanised antibodies.

Methods for the production and/or selection of fully human mAbs are known in the art. These can involve the use of a transgenic animal which is immunized with the respective protein or peptide, or the use of a suitable display technique, like yeast display, phage display, B-cell display or ribosome display, where antibodies from a library are screened against human iRhom2 in a stationary phase.

In vitro antibody libraries are, among others, disclosed in US6300064 by MorphoSys and US6248516 by MRC/Scripps/Stratagene. Phage Display techniques are for example disclosed in US5223409 by Dyax. Transgenic mammal platforms are for example described in EP1480515A2 by TaconicArtemis.

IgG, IgM, scFv, Fab and/or $F(ab)_2$ are antibody formats well known to the skilled person. Related enabling techniques are available from the respective textbooks.

As used herein, the term "Fab" relates to an IgG/IgM fragment comprising the antigen binding region, said fragment being composed of one constant and one variable domain from each heavy and light chain of the antibody.

As used herein, the term "$F(ab)_2$" relates to an IgG/IgM fragment consisting of two Fab fragments connected to one another by disulfide bonds.

As used herein, the term "scFv" relates to a single-chain variable fragment being a fusion of the variable regions of the heavy and light chains of immunoglobulins, linked together with a short linker, usually serine (S) or glycine (G). This chimeric molecule retains the specificity of the original immunoglobulin, despite removal of the constant regions and the introduction of a linker peptide.

According to one embodiment, the antibody or fragment or derivative thereof has an enhanced potential to induce ADCC, relative to a naturally occurring antibody or fragment or derivative.

The main strategy to enhance the capacity of an IgG to induce ADCC is to alter the Fc portion of the antibody to increase binding affinity to the activating FcγRIIIA via site-directed mutagenesis, changing Fc domain glycosylation, and/or preventing Fc domain fucosylation. Creation of IgG variants with improved binding to activating FcγR by mutagenesis has been an

effective strategy for increasing ADCC efficiency of IgG antibodies (Shields et al 2000, Tang et al 2007, Zahavi et al 2018, the contents of all of which are incorporated herein by reference for enablement purposes). In addition to modification of Fc residues, asymmetrical engineering of the Fc portion to create heterodimers of different heavy chains yielded more stable antibodies with enhanced ADCC functionality (Liu et al 2013, the content of which is incorporated herein by reference for enablement purposes).

According to one embodiment, the antibody or fragment or derivative thereof comprises an afucosylated Fc domain.

Afucosylated antibodies are monoclonal antibodies engineered such that the oligosaccharides in the Fc region of the antibody do not have any fucose sugar units. When antibodies are in afucosylated IgG format, their capacity to induce antibody-dependent cellular cytotoxicity (ADCC) is increased.

Technically, afucosylation can be accomplished by either

- inhibiting addition of fucose to the already existing sugar chain, e.g., by overexpressing th enzyme GnTIII (see e.g. Davies et al 2001), or by knocking out FUT8 Y(Fucosyltransferase 8, Yamane-Ohnuki et al 2004))
- removing fucoses that has already been added to the existing sugar chain chemically or enzymatically,
- redirecting fucose synthesis by a heterologous enzyme that depletes the fucose pool inside the cell (GlyMaxx technology by ProBioGen, see also Chung et al, 2012), and/or
- using an expression system that does not add fucose residues into the sugar chains, like e.g. a Ciliate base expression system (see e.g., EP2542575A1).

In one embodiment, the antibodies according to the invention were produced by expression in FUT8-KO CHO (Chinese hamster ovary) cell line containing a knock-out of the FUT8 gene. The antibodies thus produced lack fucose, and are, as such, afucosylated. In the examples shown herein, the afucosylated antibodies according to the invention were produced in that way. It should be noted, however, that similar effects are to be expected for antibodies according to the present invention afucosylated by other approaches, as e.g. described above.

According to one embodiment, the antibody or target-binding fragment or derivative thereof, is in the IgG format, preferably having the heavy chain/light chain variable domain (HCVD/LCVD) pairs of SEQ ID NO: 1 and 2, SEQ ID NO: 3 and 4, SEQ ID NO: 5 and 6 and/or SEQ ID NO: 7 and 8,

According to one embodiment the antibody or target-binding fragment or derivative thereof evokes increased ADCC relative to Zilovertamab. Zilovertamab is an anti-ROR1 antibody supplied by Oncternal Therapeutics.

Similar findings apply to the antibody R12hIgG1 (Yang et al (2021), which is currently used as scFv in CAR-T cells but not as a naked antibody.

Table 1: summary of some characteristics of the antibody according to the invention and prior art

Feature	PBA0405	Zilovertamab	R12hIgG1
Binding to ROR1	Yes	Yes	Yes
Binding to Frizzled domain of ROR1	Yes	No	No
Binding to IgG-like domain of ROR1	No	Yes	Yes
ADCC on cancer cells	Strong	No/weak	No/weak
ADCP-CDC	Strong	Moderate	N/A
in vivo efficacy - Leukemic cells in spleen and bone marrow	Strong	Moderate	N/A

In one embodiment, the antibody or target-binding fragment or derivative thereof is administered to the same or another subject under comparable conditions to demonstrate increased ADCC. As used herein, the term “administered to a subject under comparable

conditions shall refer to comparable subject parameters (size, weight, sex, age, disease history) as well as to comparable administration conditions (dosage, timing, intervals).

In one embodiment, the antibody or target-binding fragment or derivative thereof is tested *in vitro* relative to Zilovertamab to demonstrate increased ADCC. Suitable cell based ADCC assays are commercially available at commercial laboratory suppliers, Principles of such assays are described in Parekh et al (2012) or Alpert et al (2012), and also elsewhere herein.

According to another aspect of the invention, a target binding molecule is provided that

(i) competes for binding to ROR1 with the antibody or target-binding fragment or derivative thereof, according to the above description,

(ii) or binds to the same epitope of ROR1 as the antibody or target-binding fragment or derivative thereof, according to the above description.

In one embodiment, said target binding molecule is an antibody or target-binding fragment or derivative thereof, as defined elsewhere herein.

As used herein, the term "competes for binding" is used in reference to target binding molecule with an activity which binds to the same substrate as does the antibody or target-binding fragment or derivative thereof. The efficiency (e.g., kinetics or thermodynamics) of binding the binding molecule may be the same as or greater than or less than the efficiency substrate binding by the antibody or target-binding fragment or derivative thereof. For example, the equilibrium binding constant (Kj)) for binding to the substrate may be different. The term "K_m" as used herein refers to the Michaelis-Menton constant for an enzyme and is defined as the concentration of the specific substrate at which a given enzyme yields one-half its maximum velocity in an enzyme catalysed reaction.

As used herein, the term "binds to the same epitope" with reference to two or more binding molecules means that the molecules bind to the same segment of amino acid residues, as determined by a given method. Techniques for determining whether an antibody binds to the same epitope as another antibody include, for example, epitope mapping methods, such as, x-ray analyses of crystals of antigen: antibody complexes which provides atomic resolution of the epitope and hydrogen/deuterium exchange mass spectrometry (HDX-MS). Other methods monitor the binding to antigen fragments or mutated variations of the antigen where loss of

binding due to a modification of an amino acid residue within the antigen sequence is often considered an indication of an epitope component. In addition, computational combinatorial methods for epitope mapping can also be used. These methods rely on the ability of the antibody of interest to affinity isolate specific short peptides from combinatorial phage display peptide libraries.

According to another aspect of the invention, a pharmaceutical composition comprising the antibody or target-binding fragment or derivative thereof or the target binding molecule according to the above description, and optionally one or more pharmaceutically acceptable excipients, is provided.

According to another aspect of the invention, a combination comprising (i) the antibody or target-binding fragment or derivative thereof or the target binding molecule according to the above description, and (ii) one or more further therapeutically active compounds, is provided.

According to embodiments, such combination comprises at least one of

- a Bcl-2 inhibitor, and/or
- an inhibitor of Bruton's tyrosine kinase (BTK)

Bcl-2 (B-cell lymphoma 2), encoded in humans by the BCL2 gene, is a member of the Bcl-2 family of regulator proteins that regulate cell death (apoptosis), by either inhibiting (anti-apoptotic) or inducing (pro-apoptotic) apoptosis.

BCL-2 is localized to the outer membrane of mitochondria, where it plays an important role in promoting cellular survival and inhibiting the actions of pro-apoptotic proteins. BCL-2 is further known to regulate mitochondrial dynamics, and is involved in the regulation of mitochondrial fusion and fission.

Damage to the Bcl-2 gene has been identified as a cause of a number of cancers, including melanoma, breast, prostate, chronic lymphocytic leukemia, and lung cancer. It is also a cause of resistance to cancer treatments.

Inhibitors of Bcl-2 are *inter alia*

- Oblimersen (antisense oligonucleotide) (G3139)

- ABT-737
- Navitoclax (ABT-263)
- Venetoclax (ABT-199)
- Sonrotoclax (BGB-11417)

Bruton's tyrosine kinase (abbreviated Btk or BTK), also known as tyrosine-protein kinase BTK, is a tyrosine kinase that is encoded by the BTK gene in humans. BTK plays a crucial role in B cell development. BTK plays a crucial role in B cell development as it is required for transmitting signals from the pre-B cell receptor that forms after successful immunoglobulin heavy chain rearrangement. It also has a role in mast cell activation through the high-affinity IgE receptor. Mutations in the BTK gene are implicated in the primary immunodeficiency disease X-linked agammaglobulinemia (Bruton's agammaglobulinemia); sometimes abbreviated to XLA and selective IgM deficiency.

Patients with XLA have normal pre-B cell populations in their bone marrow but these cells fail to mature and enter the circulation. The Btk gene is located on the X chromosome (Xq21.3-q22). At least 400 mutations of the BTK gene have been identified. Of these, at least 212 are considered to be disease-causing mutations.

Inhibitors of BTK are, *inter alia*,

- Ibrutinib (Imbruvica)
- Acalabrutinib (Calquence)
- Zanubrutinib (Brukinsa)
- Tirabrutinib (Velexbru)
- Pirtobrutinib (Jaypirca)
- Orelabrutinib
- Evobrutinib
- Tolebrutinib
- Remibrutinib
- Fenebrutinib (RG7845)
- ABBV-105
- Fenebrutinib (GDC-0853)

- Tirabrutinib (GS-4059)
- Spebrutinib (AVL-292, CC-292)
- HM71224, and/or
- Luxeptinib

According to embodiments of such combination,

- the Bcl-2 inhibitor is venetoclax, and/or
- the inhibitor of Bruton’s tyrosine kinase is ibrutibnib.

Venetoclax (Cas number 1257044-40-8) is a drug which attaches to a protein called Bcl-2. This protein is present in high amounts in CLL cancer cells, where it helps the cells survive for longer in the body and makes them resistant to cancer medicines. By attaching to Bcl-2 and blocking its actions, venetoclax causes the death of cancer cells and thereby slows down progression of the disease. Venetoclax is used, *inter alia,* to treat adults with chronic lymphocytic leukemia (CLL), small lymphocytic lymphoma (SLL), or acute myeloid leukemia. The inventors of the present invention have investigated coadministration of venetoclax and the anti-ROR1 antibodies according to the present invention, and demonstrated that there is a synergistic effect derivable from that combination.

Ibrutinib (Cas number 936563-96-1 is a small molecule drug that inhibits B-cell proliferation and survival by irreversibly binding the protein Bruton's tyrosine kinase (BTK). Blocking BTK inhibits the B-cell receptor pathway, which is often aberrantly active in B cell cancers. Ibrutinib is therefore used to treat such cancers, including mantle cell lymphoma, chronic lymphocytic leukemia, and Waldenström's macroglobulinemia.[6][7] Ibrutinib also binds to C-terminal Src Kinases. These are off-target receptors for the BTK inhibitor. Ibrutinib binds to these receptors and inhibits the kinase from promoting cell differentiation and growth. This leads to many different side effects like left atrial enlargement and atrial fibrillation during the treatment of Chronic Lymphocytic Leukemia. Ibrutinib is indicated for the treatment of mantle cell lymphoma (MCL), chronic lymphocytic leukemia (CLL)/small lymphocytic lymphoma (SLL), Waldenström's macroglobulinemia (WM), marginal zone lymphoma (MZL), and chronic graft versus host disease (cGVHD). The inventors of the present invention have investigated coadministration of ibrutinib and the anti-ROR1 antibodies according to the present invention, and demonstrated that there is a synergistic effect derivable from that combination.

According to another aspect of the invention, the use of the antibody or target-binding fragment or derivative thereof, the target binding molecule, the pharmaceutical composition according, or the combination according to the above description (for the manufacture of a medicament) is provided in the treatment of a human or animal subject

- being diagnosed for,
- suffering from or
- being at risk of developing

a neoplastic disease, or for the prevention of such condition.

This language is deemed to encompass both the swiss type claim language accepted in some countries (in this case, brackets are deemed absent) and EPC2000 language (in this case, brackets and content within the brackets is deemed absent).

According to another aspect of the invention, a method for treating or preventing a neoplastic disease is provided, which method comprises administration, to a human or animal subject, of the antibody or target-binding fragment or derivative thereof, the target binding molecule, the pharmaceutical composition according, or the combination according to the above description, in a therapeutically sufficient dose 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.

Materials & Methods

Binding to CHO-cell lines (barcoding procedure) – lead candidates

Materials and Methods

CellTrace™ CFSE stock solution (Invitrogen, # C34554) of concentration 5 mM was thawed prior to analysis at RT, 1 µL CellTrace™ CFSE was added to 10 mL of DPBS for a working stock solution 0.5 µM, 10-fold serial dilutions of the CFSE were made by transferring 10 mL from 0.5 µM solution to 90 mL of DPBS to achieve 0.05 and 0.005 µM working concentrations.

80 million of CHO cells (overexpressing ROR1, overexpressing ROR2 or transfected with empty vector) were washed twice with 20 mL of DPBS and resuspended in respective solution: CHO-EV (empty vector) in 90 mL of DPBS; CHO-ROR1 in 90 mL of 0.05 µM CFSE solution; CHO-ROR2 in 90 mL of 0.005 µM CFSE solution. Cells were incubated for 30 min at RT with agitation and protected from light.

Following incubation 10 mL of cell culture medium with 10% FBS was added and cells were incubated for 10 min at RT with agitation. Cells were centrifuged (450 rcf, 5 min), washed twice with 20 mL DPBS and resuspended in 22.5 mL staining medium (DPBS + 5% FBS) at 4 mln cells/mL density. CHO-ROR1, CHO-ROR2 and CHO-EV cell lines were mixed in 1:1:1 ratio (22 mL each). Mixed cells were aliquoted (150 µL - 0.6 million cells per well) to 96-well plates.

For staining with tested antibodies serial dilutions in DPBS + 5% FBS were made to achieve final concentrations of: 50, 10, 1, 0.1, 0.01, 0.001, 0.0001 and 0.00001 µg/mL. Aliquoted cells were centrifuged (450 rcf, 5 min), supernatant was discarded, and cells were resuspended in 100 µL of appropriate tested antibody dilution and incubated for 30 min on ice.

For secondary antibody staining cells were washed twice with 200 µL of DPBS + 0.5% FBS, resuspended in 100 µL of DPBS + 0.5% FBS containing Allophycocyanin (APC) AffiniPure Goat Anti-Human IgG, Fcγ fragment specific (Jackson ImmunoResearch, #109-135-098)

antibody (0.4 µL per sample) and incubated for 30 min on ice. Following incubation cells were washed twice with 200 µL of DPBS + 0.5% FBS, resuspend in 200 µL DPBS with Sytox Blue (Invitrogen, #S34857), 1 µL per 1 mL of DPBS, and stored on ice until analysis.

Samples were measured on Attune™ NxT Focusing Cytometer equipped with CytKick autosampler (Thermo Fisher Scientific), analysis was done using Attune™ Software. Prior to analysis performance test was performed. 20000 events were captured for tested samples in "population of interest" gate after discarding dead cells and doublets.

Results and conclusions

Tested afucosylated antibodies bind target ROR1 on CHO cells in a dose dependent manner. All of the antibodies exhibited very low EC50 values in sub-nanomolar range.

KD evaluation – BLI (hROR1-Fc, mROR1-Fc, hROR2-Fc)

The avidity of antibodies was determined using the Octet RED384 instrument (Sartorius). Antibodies were captured on FAB2G (Anti-hIgG CH1 Capture) biosensors (Sartorius) at the concentration 5 µg/mL. Two-fold serial dilution of recombinant hROR1/Fc, hROR2/Fc, mROR1/Fc (R&D Systems) and mROR2/Fc (Acro Biosystems) (200 – 3.13 nM) was prepared in 10× Kinetics Buffer (Sartorius). Association of analytes were measured for 200 s followed by 500 s of dissociation in 10× Kinetics Buffer. Biosensors were regenerated after each binding cycle with 10 mM glycine, pH 1.7 (Cytiva). Sensorgrams were referenced by blank (buffer) subtraction and the association (k_{on}) and dissociation (k_{off}) rate constants were evaluated from global fitting based on a 1:2 (bivalent analyte) binding model using Octet Analysis Studio 12.2 software (Sartorius). The apparent dissociation constant was calculated from the equation: $K_D = k_{off1} / k_{on1}$.

KD evaluation – SPR (FcγR) (CAP or SA)

The affinities of antibodies were determined using surface plasmon resonance in a BIACORE 8K instrument (Cytiva). Recombinant FcγR proteins (R&D Systems) were biotinylated and immobilized on the CAP chip, according to the supplier's instruction. Molecules were injected over the chip surface to a capture level of ~70 RU. Serial dilutions of antibodies as analytes were prepared in PBS-P^+ buffer (Cytiva) and injected at the flow rate of 30 µL/min. Generated data with double reference subtraction (reference cell subtracted and blank subtracted) were analyzed using BIAevaluation software (Cytiva). Association (k_{on}) and dissociation (k_{off}) rate

constants were evaluated based on a 1:1 Langmuir binding model and the equilibrium dissociation constant was calculated from the equation: $K_D = k_{off} / k_{on}$.

Epitope binning – BLI

Epitope binning was performed using the Octet RED384 instrument (Sartorius). Antibodies (5 µg/mL in 10 mM acetate buffer pH 6.0) were immobilized on AR2G sensors. The resulting antibody sensors were incubated in 1000 nM ROR1-His (100s) followed by 1000 nM ROR1-His premixed with 500 nM of a competing antibody (100s), and dissociation was measured for 200s. Sensorgrams were evaluated with the Octet Analysis Studio 12.2 software (Sartorius).

Results and conclusion

Lead compounds bind to distinguished epitopes (Frizzled domain) from Zilovertamab and R12hIgG1 (IgG domain).

ADCC – materials and methods

Cell culturing

Raji (CCL-86) was purchased from ATCC, Jeko-1 (ACC 553) and MEC-1 (ACC 497) were purchased from DSMZ, MDA-MB-231 (92020424) was purchased from ECACC. Raji and MEC-1 were cultured in RPMI 1640 medium (Gibco™, 31870074) with 10% Fetal Bovine Serum (Sigma Aldrich, F9665-500ML), 1% L-Glutamine (Gibco™, 35050061) and 1% Penicillin/Streptomycin (Gibco™, 15140130). Jeko-1 was cultured in RPMI 1640 medium with 20% Fetal Bovine Serum, 1% L-Glutamine and 1% Penicillin/Streptomycin. MDA-MB-231 was cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Gibco™, 11965092) with 10% Fetal Bovine Serum. All cell lines were kept at 37°C, 5% CO_2 and cultured up to 20 passages.

Isolation of PBMC and enrichment of human NK cells

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy donor buffy coat samples derived by leukapheresis, described as HIV-, HCV- and HBV-. The density centrifugation was performed with SepMate™-50 PBMC Isolation Tubes (Stemcell™, #85460). Isolation tubes were filled with 15 mL of Lymphoprep (Stemcell™, #07811). Buffy coats were transferred to a sterile PETG media bottle (Fisherbrand™, PBMB125) and diluted with equal volume of DPBS (Gibco™, 14190144). 17 mL of diluted buffy coat was gently layered on Lymphoprep and the SepMate™ tubes were centrifuged (1200*g*, 30 min, acc/brake

on). After centrifugation, the layer of PBMCs, visible above the plastic membrane of each SepMate™ tube was collected and transferred to a fresh 50 mL conical tube. Tubes with PBMCs were diluted with equal volume of fresh DPBS and centrifuged (800*g*, 8 min). Supernatant was discarded and cell pellets were resuspended in 5 mL of 1X Red Blood Cell Lysis Buffer (Biolegend, 420302) and incubated at RT for 10 minutes. Tubes were filled up with DPBS and centrifuged (350*g*, 5 min). Supernatant was discarded, the cell pellets were washed three times with 30 mL portions of fresh DPBS. PBMCs were cultured overnight in Iscove's Modified Dulbecco Medium (IMDM) (Gibco™, 31980030) with 10% Fetal Bovine Serum at 37°C, 5% CO_2 or subjected to NK cell isolation.

NK cells were isolated from PBMCs by immunomagnetic negative selection with NK Cell Isolation Kit (Miltenyi Biotec, 130-092-657) according to manufacturer's protocol. NK cells were cultured overnight in IMDM (Gibco™, 31980030) with 10% Fetal Bovine Serum at 37°C, 5% CO_2 and used the next day.

For NK cells which were used as effector cells in ADCC assay, phenotyping and isolation purity characterization by flow cytometry (Cytek Northern Lights (NL-00020)) was performed. Following markers were used: viability (LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit, Invitrogen™, L34966), CD3 (APC-H7 Mouse Anti-Human CD3, BD Bioscience, 560176), CD16 (Alexa Fluor® 647 Mouse Anti-Human CD16, BD Biosciences, 557710), CD56 (Brilliant Violet 605™ anti-human CD56(NCAM), BioLegend®, 362538), CD69 (BV711 Mouse Anti-Human CD69, BD Biosciences, 563836), CD107a (PE Mouse Anti-Human CD107a, BD Pharmingen™, 555801), NKG2D (BV421 Mouse Anti-Human CD314 (NKG2D), BD Biosciences, 743558).

ADCC assay

Target cells were collected, washed twice with DPBS and labeled with 0.1 µM of CellTrace™ CFSE Proliferation Kit (Invitrogen™, C34554) at 37°C for 20 min. After incubation, cells were washed twice with assay medium (IMDM with 10% FBS) and resuspended in assay medium at 0.2×10^6 cells/mL. Target cells (10,000 cells per well) in 50 µL volume were seeded into conical bottom 96-well plate (Thermo Scientific™, Nunc™, 249935). Afterwards, serial concentrations of antibodies in 50 µL of IMDM indicated for each experiment were added. Wells selected for *NK background control* and *target viability control* were supplemented with 50 µL of assay medium. Target cells were incubated with antibodies for 30 min at 37C°, 5% CO_2 for pre-

coating. In the meantime, NK cells (effector cells) isolated on the previous day were centrifuged (350*g*, 5 min) and resuspended in fresh assay medium at 1×10^6 cells/mL. After pre-coating step, NK cells were added to each well in 100 µL giving 100,000 cells/well. Wells selected for *target viability control* were supplemented with 100 µL of assay medium. Then, the plate was centrifuged at 100*g* for 1 min and incubated at 37C°, 5% CO_2 for 4 hours. The final ratio of effector cells to target cells was 10:1.

After 4 hours, the plate was centrifuged (350*g*, 5 min) and supernatants were discarded by vigorous inverting of the plate. Cell pellets were washed with DPBS and centrifuged (350*g*, 5 min) and resuspended in 100 µL of Live/Dead™ Fixable Violet Dead Cell Stain Kit solution (1:1000 in DPBS, Invitrogen™, L34964) followed by incubation at 4°C for 20 min. After that time, 100 µL of FACS buffer (DPBS with 2% of Fetal Bovine Serum) was added and the plate was centrifuged (350*g*, 5 min). Cell pellets were resuspended in 100 µL fixing solution of 4% paraformaldehyde (Thermo Scientific™, J19943.K2) for 10 min at 4°C. Following fixation, 100 µL of FACS buffer was added and plate was centrifuged (500*g*, 5 min). Cell pellets were resuspended in 80 µL of FACS buffer and subjected for flow cytometry analysis.

The plate was then read on Cytek Northern Lights (NL-00020) or BD FACSCelesta™ (660344) flow cytometers. Relative percentage of cell lysis was calculated by subtracting *target viability control* from experimental lysis values. Data were presented as relative percentage lysis against indicated antibody concentration (nM) on a $\log_{10}$ scale. Graphs presenting dose-response curves were fitted by 4-parameters nonlinear regression equation. For determination of half maximal effective concentration (EC_{50}) and other EC values (EC_{10}, EC_{20}, EC_{30}, EC_{50}, EC_{70} and EC_{90}), antibodies dilution range was selected to cover upper and lower asymptotes. EC values were calculated including at least 3 biological repetitions. Effective concentration values other than EC50 were calculated as follows: $EC_F = (F/(100 - F))^{1/H} \times EC_{50}$; where F is percentage change in the response and H is hill slope value. Data were analyzed using FlowJo™ v10.8.1 Software (BD Life Sciences) and GraphPad Prism 7.05 software (GraphPad Software, Inc.).

Promega ADCC reporter assay

ADCC was performed according to manufacturers instructions.

Combination ADCC

In Combination ADCC venetoclax or ibrutinib was incubated on target cell ON before used in the ADCC assay.

Cytokine release and degranulation assays

CD107a degranulation assay and measurement of cytokine release

Fresh PBMC were purified from buffy coat and incubated overnight at 37°C in IMDM supplemented with 10% FBS and 55μM of β-mercaptoethanol. Jeko-1 cell line was used as a target cell. 500 000 of Jeko-1 cells per well were preincubated 30min with 50nM of selected binders PB004.22.0357.aF, PB004.22.0372.aF, PB004.22.0405.aF, PB004.22.0408.aF, Zilovertamab, R12hIgG1 and Rituximab, washed, added to target cells and co-cultured for 4 hours at 1:1 (PBMC: target cells) ratio in the presence of APC-conjugated anti-CD107a antibody and protein transport inhibitors (BD GolgiStop and BD GolgiPlug). Thereafter, cells were labelled with aqua viability dye, BV605 anti-CD56, BV421 anti-CD16, APC-H7 anti-CD3 (from BD Biosciences or Biolegend) for 25 minutes at 37°C, fixed with BD Cytofix/Cytoperm™ Kit for 15min at 4°C, followed by intracellular staining for IFNγ (BB700 anti-IFNγ) and TNFα (BV750 anti-TNFα) for 60min at RT. Isotype-matched antibodies from the same manufacturer were used to assess background fluorescence. Cells were run through a FACS Cytek® flow cytometer with standard equipment. Data were analyzed using FlowJo v10.

ADCP

Monocyte-derived macrophages for phagocytosis assay – cell culture and preparation

Monocytes were enriched from frozen samples of Peripheral blood mononuclear cells (PBMCs) using the human Pan Monocyte Isolation Kit (Miltenyi Biotech, 130-096-537) according to manufacture instructions. Purification was verified by phenotypic analysis of surface markers: anti-CD14 and anti- CD16. The enriched monocyte from PBMCs were 7 days in culture in IMDM 10% FBS in the presence of 50 ng/ml M-CSF + 50ng/ml IL-4 + 10% human serum. The adherent population of differentiated macrophages were stained with 0,1uM Violet Dye (CellTrace™ Violet Cell Proliferation Kit, for flow cytometry, C34557) and washed 2 times before ADCP experiment. To verify the phenotype of differentiated macrophages the following surface markers were used: anti-CD163, anti-CD16, anti-CD32, anti-CD64.

Target cell culture and preparation

Jeko-1 cells were cultured as a suspension in 75 cm2 flasks at 37 °C in 5% CO2 and maintained between 0.5-1.0 106 cells/mL by adding fresh complete growth medium every 3 days. Before the experiment the Jeko-1 cells were counted and stained with 0,1uM CFSE Dye (CellTrace™ CFSE Cell Proliferation Kit, for flow cytometry, C34554) and washed 2 times before the ADCP experiment.

3-Color flow-cytometric ADCP assay

Stained target cells (Jeko-1, stock: 1M/ml, 50ul per well) were preincubated 30min with 50nM concentration of selected binders: Isotype ctrl, PB004.22.0357.aF, PB004.22.0372.aF, PB004.22.0405.aF, PB004.22.0408.aF, Zilovertamab, R12hIgG1 and Rituximab. The fluorescently labeled effector monocyte-derived macrophages differentiated (Violet) from PBMCs were co-cultured with fluorescently labeled Jeko-1 cells (CFSE) and incubated at 2:1 (effector: target ratio) for 4 h at 37°, then stained 15min with viability dye (LIVE/DEAD Fixable Near-IR (780) stain) and analyzed using FACS Attune to measure phagocytosis. Percentage cell phagocytosis was calculated using the formula: number of dual-stain positive target cells (cells engulfed by macrophages; Violet+/CFSE+) divided by the total number of target cells (CFSE+). Data were analyzed using FlowJo v10.

CDC/C1q Binding

ELISA plates were coated with either with lead compounds or controls at 5 µg/mL followed by blocking and washing and serially diluted recombinant C1q protein. The detection was performer with anti-C1q-HRP antibodies and after adding TMB substrate, the plates were read at 450 nm and 650 nm wavelength.

Results and conclusions

PB004.22.0405.aF demonstrated a potent Cq1 binding, thus proving the CDC-inducing potential of ROR1-antibodies.

In vivo antitumor efficacy

huHSC-NCG-hIL15 JeKo-1 model

Fifty-six (56) females + surplus huHSC-NCG-hIL15 mice were subcutaneously injected with Jeko-1 cells resuspended in DPBS. The huHSC-NCG-hIL15 model was generated by

reconstitution of CD34+ huHSC from 5 independent donors in irradiated female NCG-hIL15 mice. The immune reconstitution level was confirmed by Flow cytometry and humanization of the mice model was identified as hCD45+/live single >25% in the peripheral blood before the inoculation of tumor cells. When the average tumor size reaches about 82 mm^3, the mice were randomly grouped based on the table above. HuHSC-NCG-hIL15 tumor bearing mice from different donors were evenly distributed into each experimental group, and each group contained mice from all the huHSC donors. The mice were treated biweekly with intravenous injections with vehicle, the compounds, Zilovertamab or Rituximab at 10mg/kg/. Blood was collected from mice 6 hours and 24 hours post dose, processed as serum for cytokine analysis using CBA detection using BD CBA Human Th1/Th2 kit. At the study endpoint, tumors, spleens and blood was collected and processed for flow cytometry and analyzed for cell markers: Live, mCD45, hCD45, hCD3, hCD8, hCD56, hCD69, hCD25, hCD107a, hGranzyme B, hIFN-γ.

Results and conclusions

Tested afucosylated antibodies demonstrate anti-tumor efficacy against JeKo-1 cells *in vivo* with similar or more potent efficacy than Zilovertamab. In contrary to Zilovertamab, PB004.22.0405.aF was able to activate tumor infiltrating NK cells to secrete IFN-γ, even more potently than Rituximab. PB004.22.0405.aF demonstrates safety as the activation was only in the tumor environment, and not systematically, as the activation was not seen in the blood or spleen NK cells. In addition, the compounds were as safe as Rituximab and Zilovertamab as no weight loss, no GvDH score or increases in serum cytokines were observed.

MEC-ROR1 leukemia model

CB-17/Icr-Prkdcscid/scid/Rj immunodeficient female mice were injected intravenously with MEC-1-ROR-1 cells (1X106) and the biweekly intravenous treatments were performed a week after inoculation for 6 injections at 10mg/kg, After four weeks of tumor cell inoculation, the spleens and bone marrows from femur and tibia of both hind legs were isolated from each transplanted mouse. A cell suspension was prepared either from the homogenized spleen with a flat plunger end of a syringe or the flushed cells from BM after passing through a cell strainer (70mM) in a complete RPMI and red blood cells were lyzed and cells were counted. Flow cytometry analysis was performed on splenic and BM derived cells ($1X10^6$) using commercially

available anti-human ROR1 (ROR1-PE) and anti-human CD19 (CD19 APC) antibodies, as well as the corresponding isotype controls. Cells were subjected for data acquisition and multiparametric FACS assessment by using a four-laser BD FACSMelody instrument. Data were analyzed using FlowJo software and both the mean or median readout was recorded for $CD19^+$ or $ROR\text{-}1^+$ cells after the background was set using the respective isotype controls.

Results and conclusions

PB004.22.0405.aF showed potent anti-tumor efficacy in the leukemia model, demonstrating less CD19+ROR1+ cells in the spleen and bone marrow. PB004.22.0405.aF demonstrated more potent anti-tumor efficacy than Zilovertamab.

Tables

Table 2: Parameters of interaction with hROR1 analyzed by BLI/SPR

	hROR1				
Sample	**K_D (M)**	**EC50 (M)**	**Domain**	**mROR1**	**hROR-2**
PB004.22.0357.aF	1.98E-09	9.33E-10	Frizzle	2.88E-9	±
PB004.22.0372.aF	6.85E-08	6.17E-10	Frizzle	1.72E-7	-
PB004.22.0405.aF	5.60E-08	5.57E-10	Frizzle	2.42E-7	-
PB004.22.0408.aF	5.17E-09	2.33E-10	Frizzle	7.73E-9	±
Zilovertamab	1.42E-08	5.01E-10	IgG	-	-
R12 scFV	1.43E-09	3.30E-09	IgG	-	-

Table 3: comparison of affinities between standard IgG or afucosylated IgG and FcγRIIIA receptors.

Sample	Affinity K_D (M) FcγRIIIA CD16a (176V)	fold increased affinity	FcγRIIIA CD16a (176F)	fold increased affinity
PB004.22.0357	9.20E-08	21x	5.37E-07	14x
PB004.22.0357.aF	4.30E-09		3.80E-08	
PB004.22.0372	2.94E-07	10x	1.50E-06	11x
PB004.22.0372.aF	3.00E-08		1.39E-07	
PB004.22.0405	2.17E-07	18x	6.90E-07	12x
PB004.22.0405.aF	1.22E-08		5.70E-08	
PB004.22.0408	7.30E-08	15x	4.66E-07	15x
PB004.22.0408.aF	4.90E-09		3.20E-08	
Zilovertamab	5.51E-07	N/A	3.40E-06	N/A

Table 4: Anti-tumor efficacy against JeKo-1 tumor

Compound	Tumor volume inhibition cf vehicle
PB004.22.0357.aF	30,3%
PB004.22.0372.aF	30,4%
PB004.22.0405.aF	46,5%
PB004.22.0408.aF	38,1%
Zilovertamab	38,9%

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Sequences

The following sequences form part of the disclosure of the present application. A WIPO ST26 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.

In some cases, the signal peptides may be encompassed in the reproduced sequences. In such case, the sequences shall be deemed disclosed with and without signal peptides. A readily available tool to identify signal peptides in a given protein sequence is SignalP - 6.0 provided by Dansk Technical University under https://services.healthtech.dtu.dk/service.php?SignalP. The same applies for sequences that comprise a His tag, which shall be deemed disclosed with and without His tag.

Table 5: Sequences

SEQ NO	Name	format	chain/ domain	AA Sequence

1	PB004. 22.0357	antiROR1 (optionally with afucosylated Fc domain)	VH	EVQLLESGGGLVQPGGSLRLSCAASGFTFSNYGMSWVRQAPGKGLEWVSMISSSGLWTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARSYGGGFDYWGQGTLVTVSS
2			VL	DIQMTQSPSSLSASVGDRVTITCRTSQDISNYLNWYQQKPGKAPKLLIYKKRPSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQNGHSFPLTFGQGTKVEIK
3	PB004. 22.0372	antiROR1 (optionally with afucosylated Fc domain)	VH	EVQLLESGGGLVQPGGSLRLSCAAPGFSFSNYAMSWVRQVPGKGLEWVSAISGSGGGTFYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARQLRYFDPFDYWGQGTLVTVSS
4			VL	DIQMTQSPSSLSASVGDRVTITCRASQSVSTFLNWYQQKPGKAPKLLIYGASNLYSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSFTIPRTFGGGTKVEIK
5	PB004. 22.0405	antiROR1 (optionally with afucosylated Fc domain)	VH	EVQLLESGGGLVQPGGSLRLSCAASGFTFHKYGMAWVRQAPGKGLEWVSSIDDRGRYTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARGSYIIWSALDYWGQGTLVTVSS
6			VL	DIQMTQSPSSLSASVGDRVTITCRPNQNIATYINWYQQKPGKAPKLLIYHDNKRPSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYEKLPWTFGQGTKVEIK
7	PB004. 22.0408	antiROR1 (optionally with afucosylated Fc domain)	VH	EVQLLESGGGLVQPGGSLRLSCAASGFTFSEYAMGWVRQAPGKGLEWVGVIWGGGGTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCALGGSYFGYWGQGTLVTVSS
8			VL	DIQMTQSPSSLSASVGDRVTITCRTSQSLSSYLHWYQQKPGKAPKLLIYHTSRLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCGQSYRYPLTFGQGTKVEIK

What is claimed is:

1. An antibody binding ROR1, or a target-binding fragment or derivative thereof retaining target binding capacities, which comprises the heavy chain/light chain variable domain (HCVD/LCVD) pairs set forth in the following:

    - SEQ ID NO: 1 and SEQ ID NO: 2,
    - SEQ ID NO: 3 and SEQ ID NO: 4,
    - SEQ ID NO: 5 and SEQ ID NO: 6, and/or
    - SEQ ID NO: 7 and SEQ ID NO: 8.

2. The antibody or fragment according to claim 1, which is or is derived from a monoclonal antibody.

3. The antibody or fragment according to any one of the aforementioned claims, which antibody or fragment or derivative has an enhanced potential to induce ADCC.

4. The antibody or fragment or derivative thereof, which comprises an afucosylated Fc domain.

5. The antibody or target-binding fragment or derivative thereof according to any one of the aforementioned claims, which is in the IgG format, preferably having the heavy chain/light chain variable domain (HCVD/LCVD) pairs of SEQ ID NO: 1 and 2, SEQ ID NO: 3 and 4, SEQ ID NO: 5 and 6 and/or SEQ ID NO: 7 and 8.

6. The antibody or target-binding fragment or derivative thereof according to any one of the aforementioned claims, which evokes increased ADCC relative to Zilovertamab.

7. A target binding molecule that competes for binding to ROR1 with the antibody or target-binding fragment or derivative thereof according to any one of the aforementioned claims, or binds to the same epitope of ROR1 as the antibody or target-binding fragment or derivative thereof according to any one of the aforementioned claims.

8. A pharmaceutical composition comprising the antibody or target-binding fragment or derivative thereof according to any one of claims 1 – 6, or the target binding molecule according to claim 7, and optionally one or more pharmaceutically acceptable excipients.

9. A combination comprising (i) the antibody or target-binding fragment or derivative thereof according to any one of claims 1 – 6, or the target binding molecule according to claim 7, and (ii) one or more further additional therapeutically active compounds.

10. The combination according to claim 8, which comprises at least one of
    - a Bcl-2 inhibitor, and/or
    - an inhibitor of Bruton’s tyrosine kinase (BTK)

11. The combination according to claim 9, wherein
    - the Bcl-2 inhibitor is venetoclax, and/or
    - the inhibitor of Bruton’s tyrosine kinase is ibrutibnib

12. Use of the antibody or target-binding fragment or derivative thereof according to any one of claims 1 – 6, the target binding molecule according to claim 7, the pharmaceutical composition according to claim 8, or the combination according to any one of claims 9 - 11 (for the manufacture of a medicament) in the treatment of a human or animal subject
    - being diagnosed for,
    - suffering from or
    - being at risk of developing

    a neoplastic disease, or for the prevention of such condition.

13. A method for treating or preventing a neoplastic disease, which method comprises administration, to a human or animal subject, of the antibody or target-binding fragment or derivative thereof according to any one of claims 1 – 6, the target binding molecule according to claim 7, the pharmaceutical composition according to claim 8, or the combination according to any one of claims 9 – 11, in a therapeutically sufficient dose.

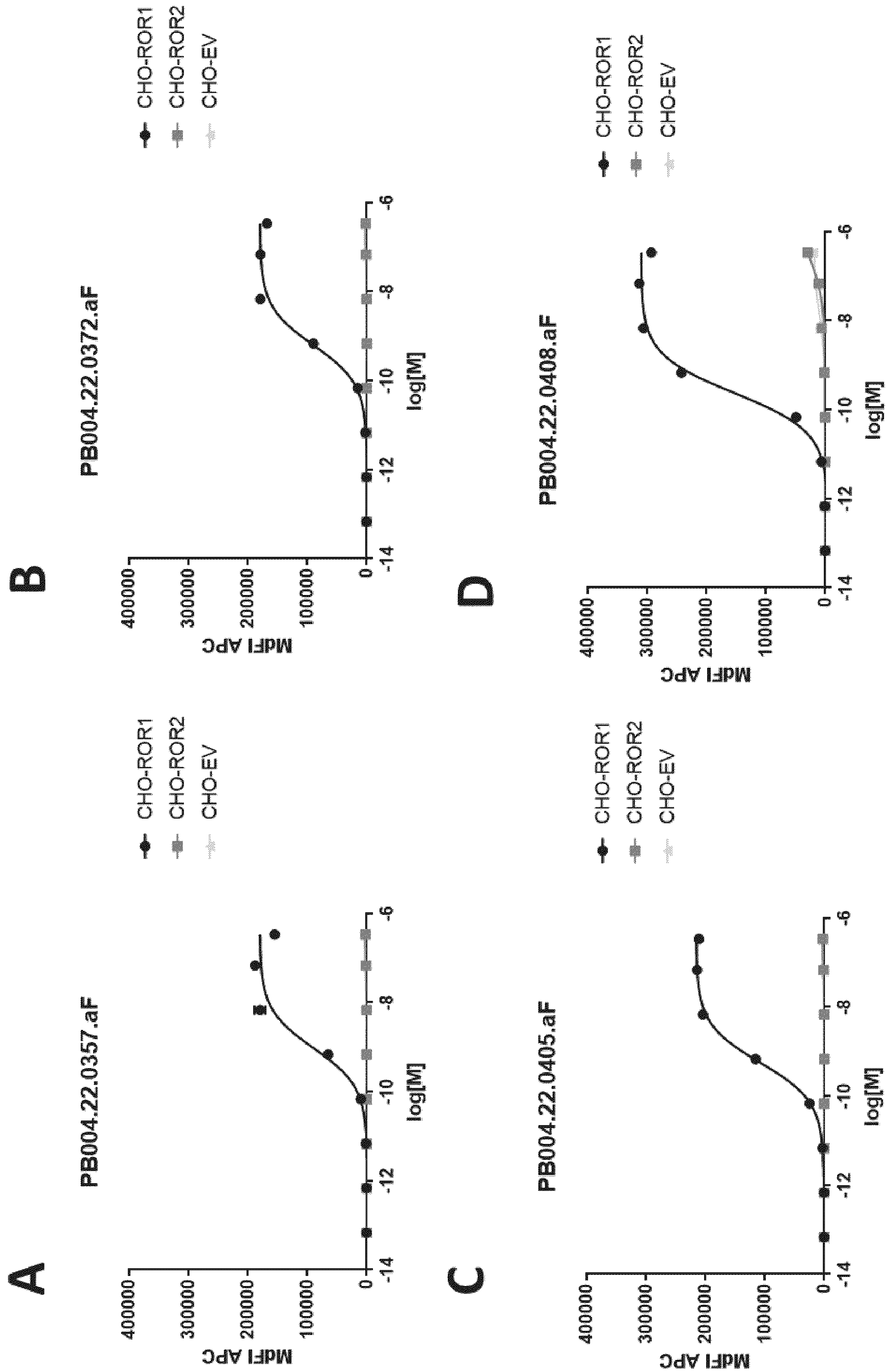

Fig. 1

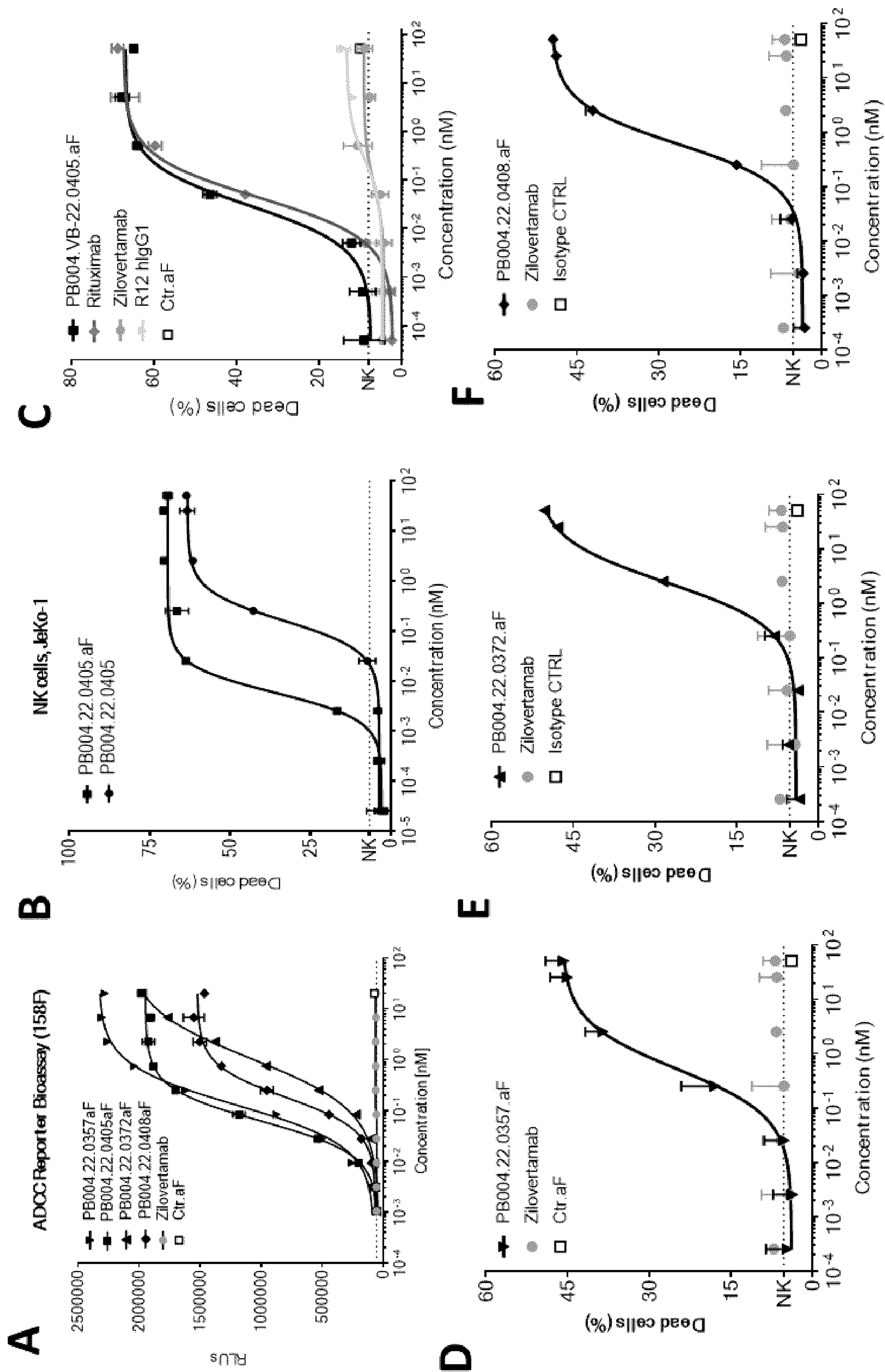

Fig. 2

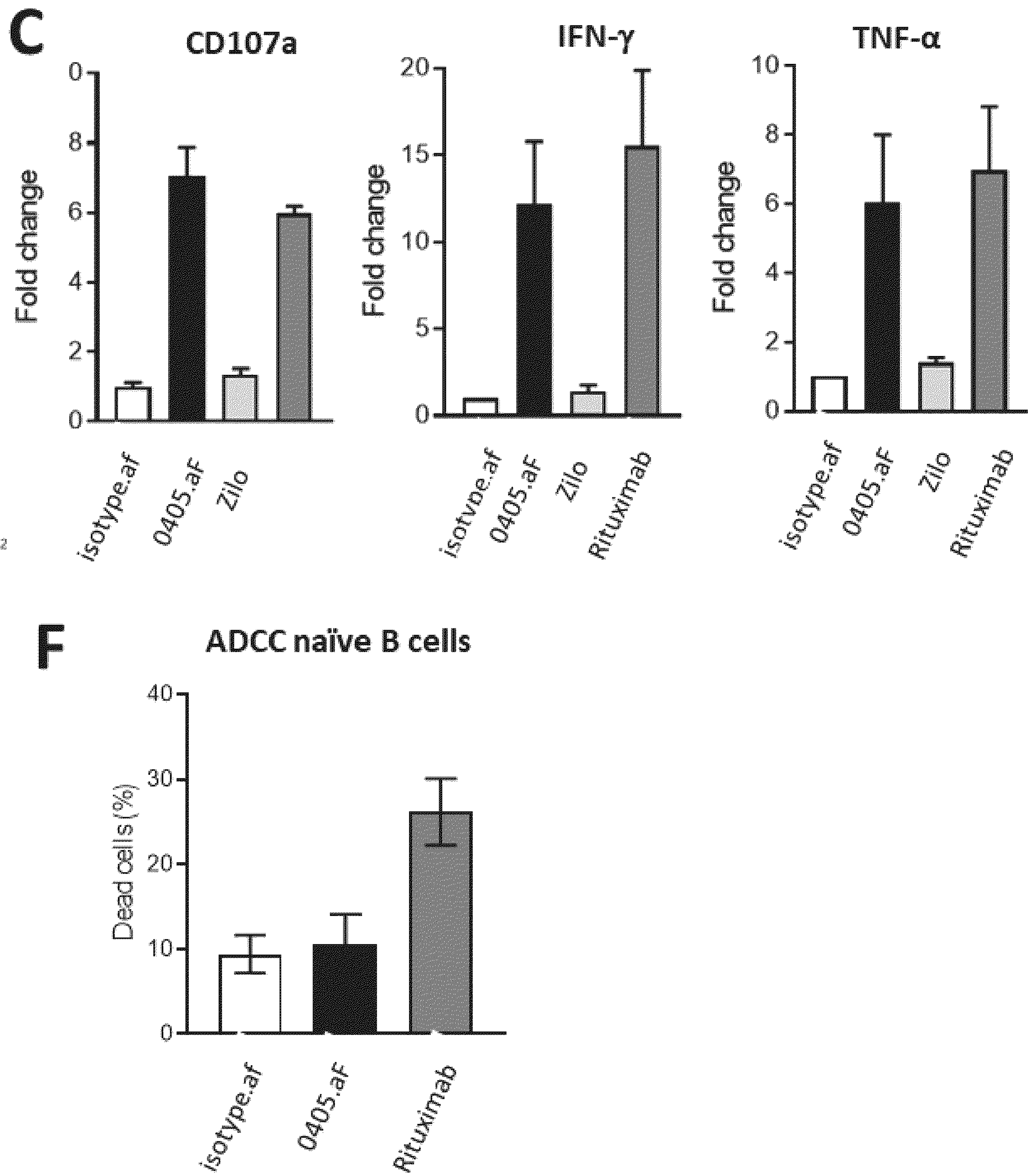

Fig. 3

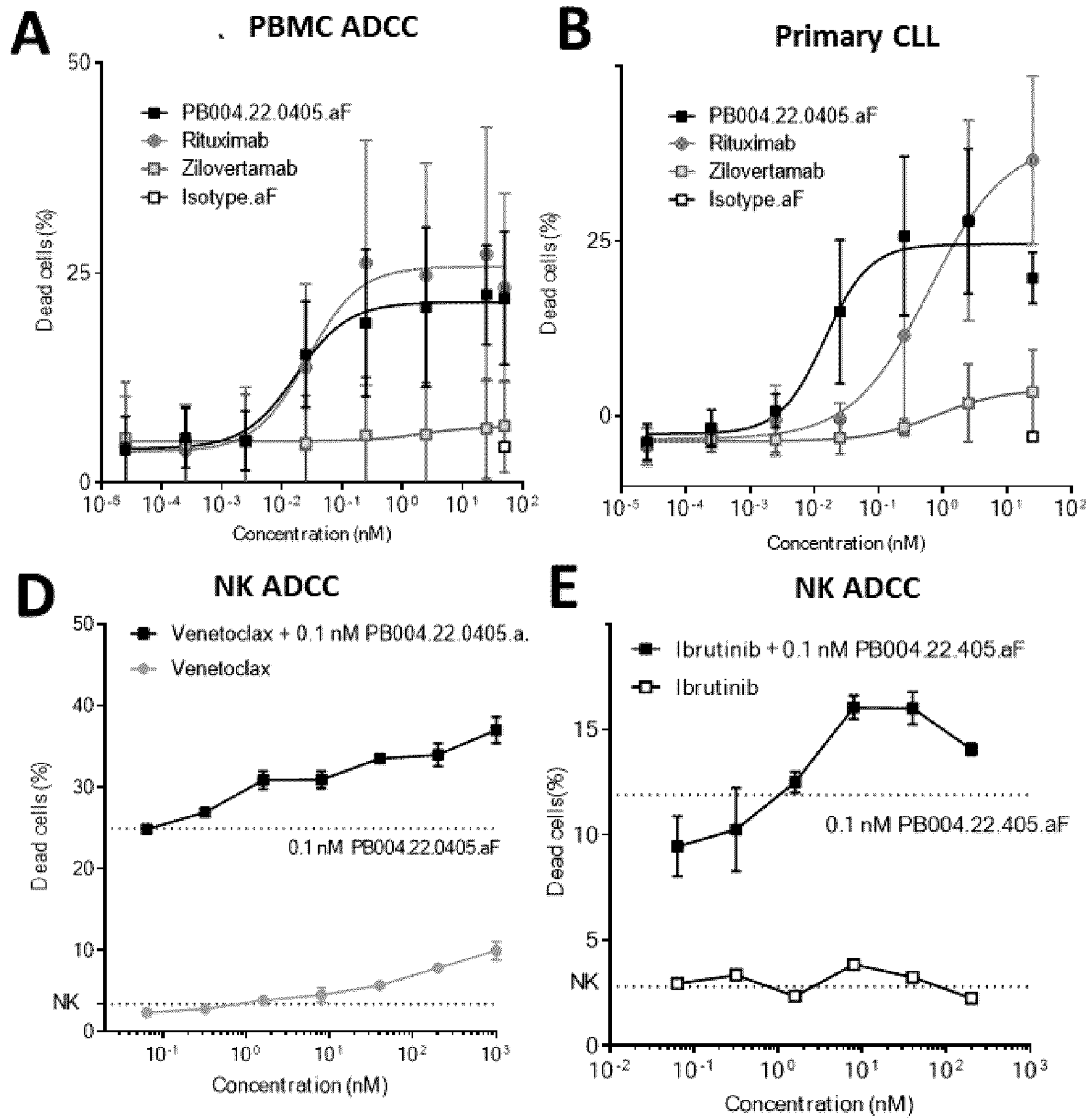

Fig. 3 ctd'

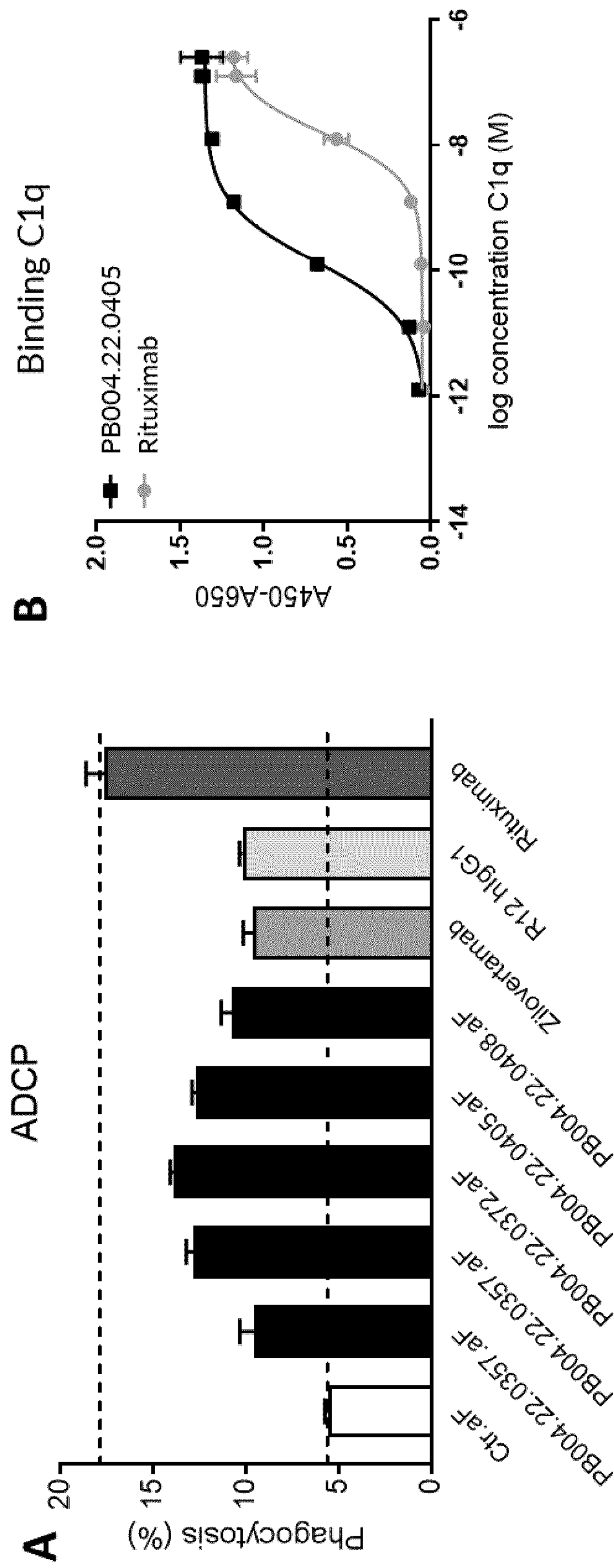

Fig. 4

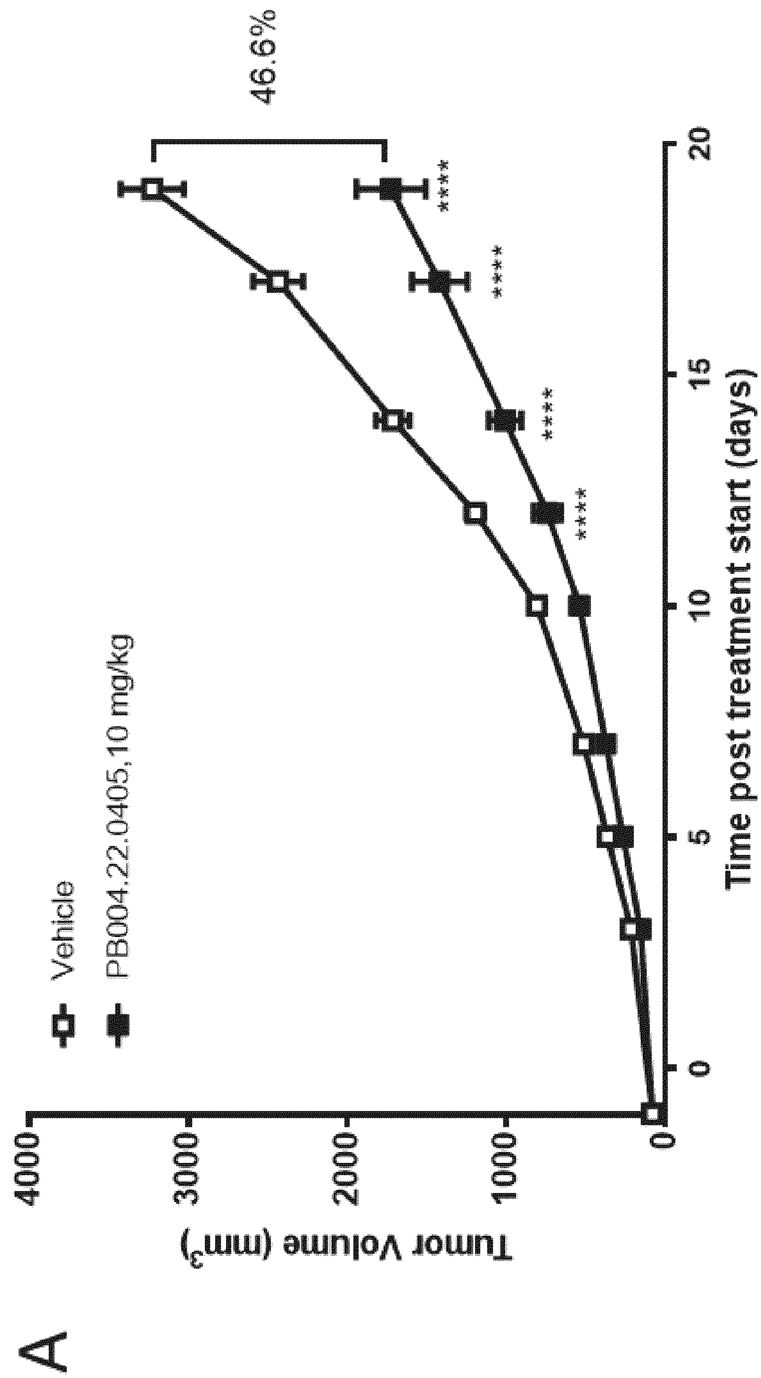

Fig. 5

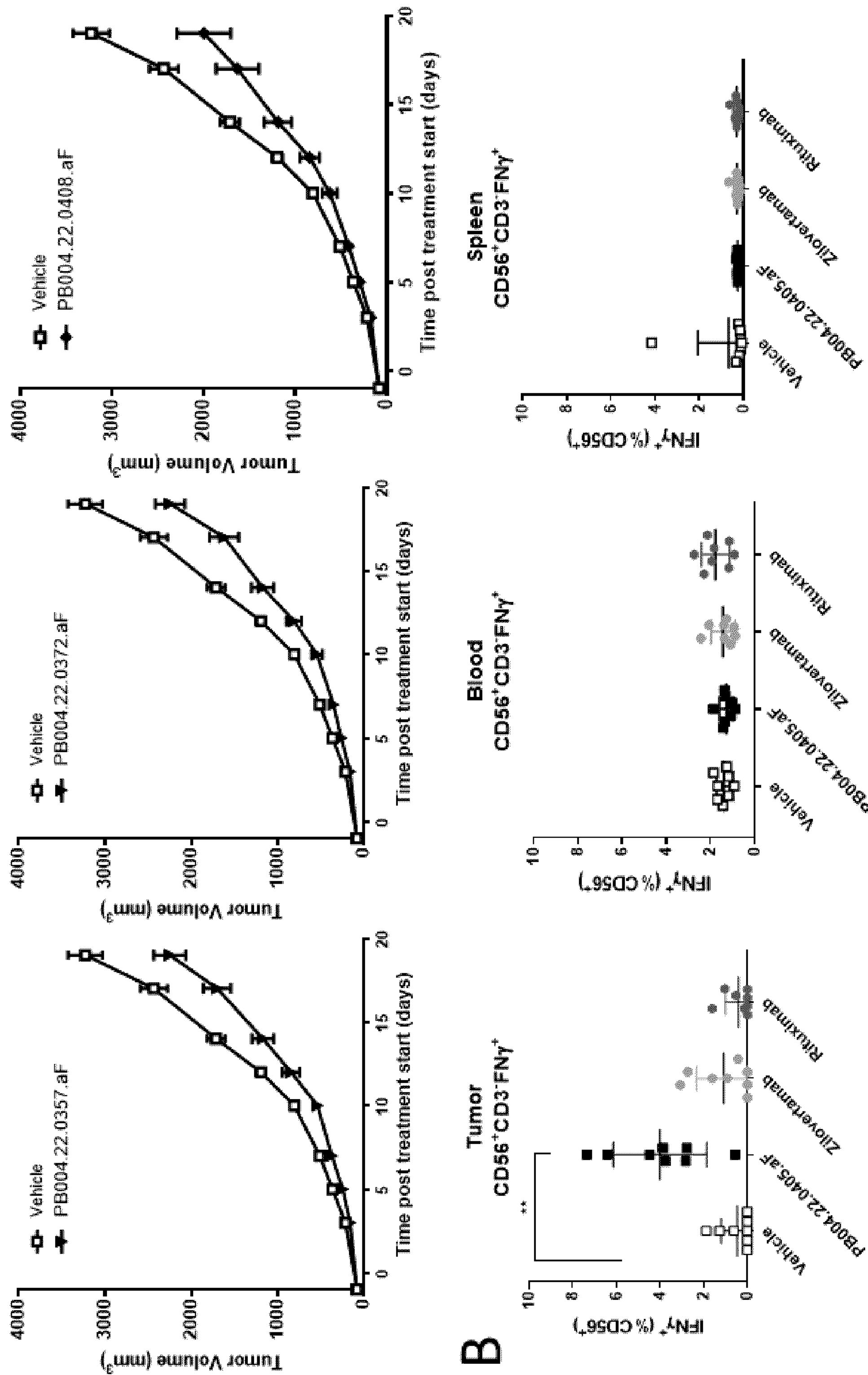

Fig. 5 ctd’

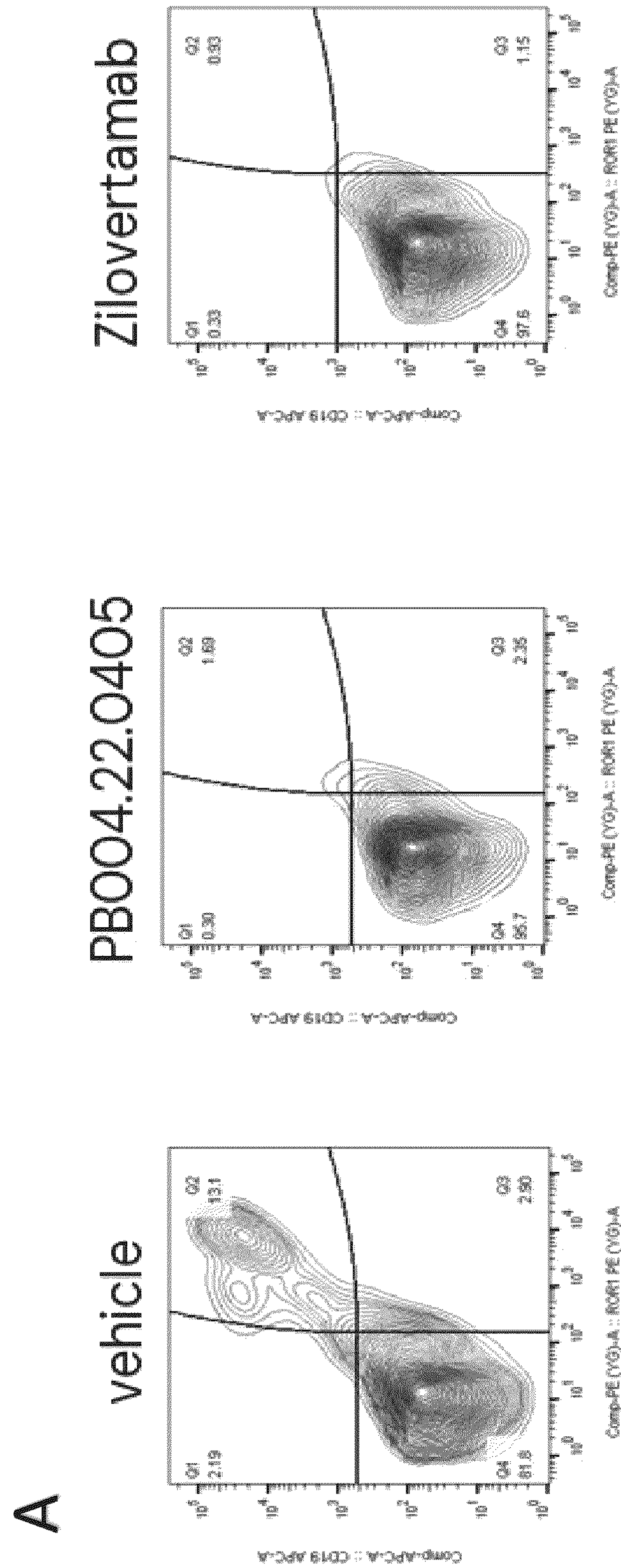

Fig. 6

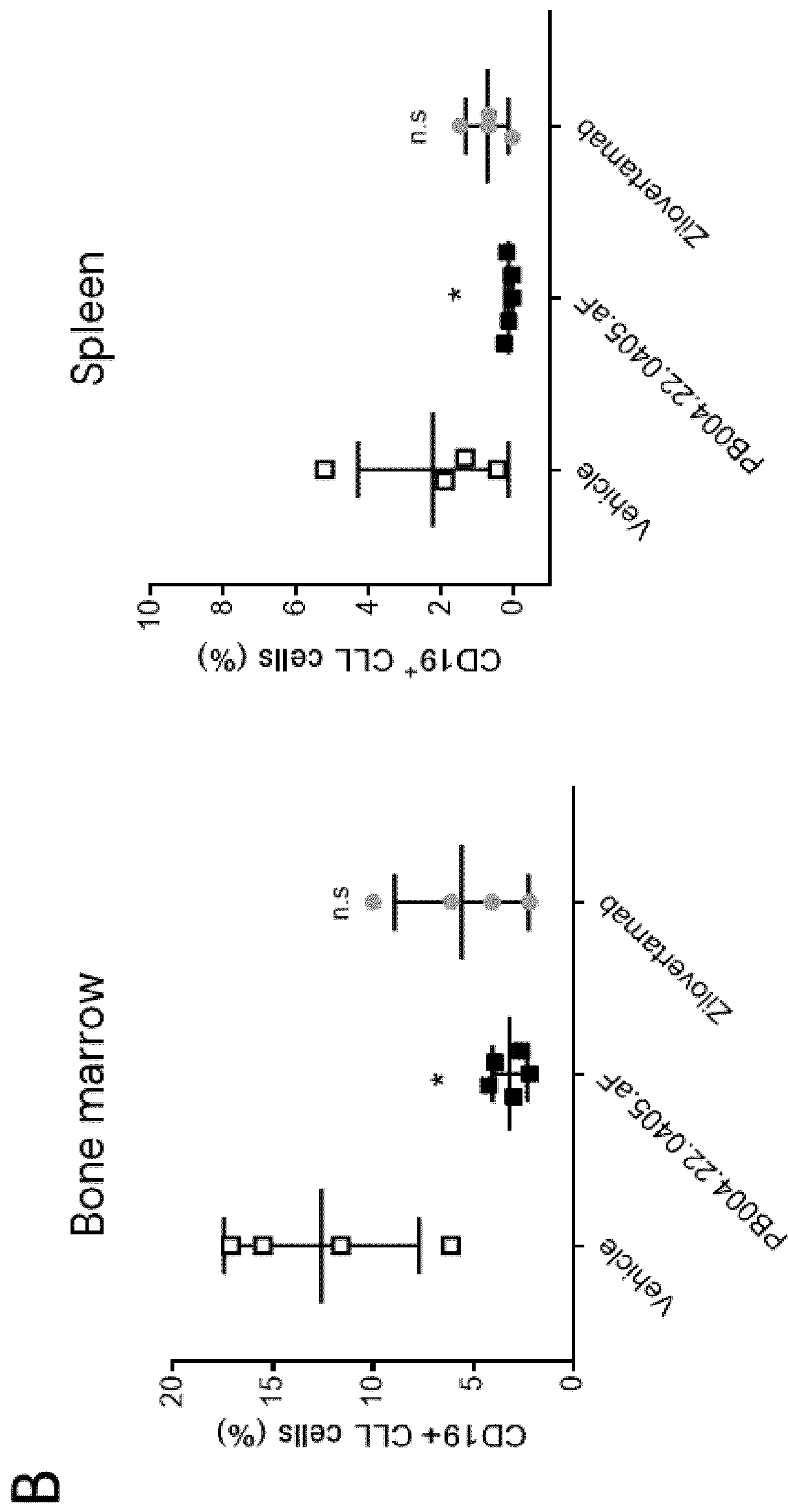

Fig. 6 ctd'

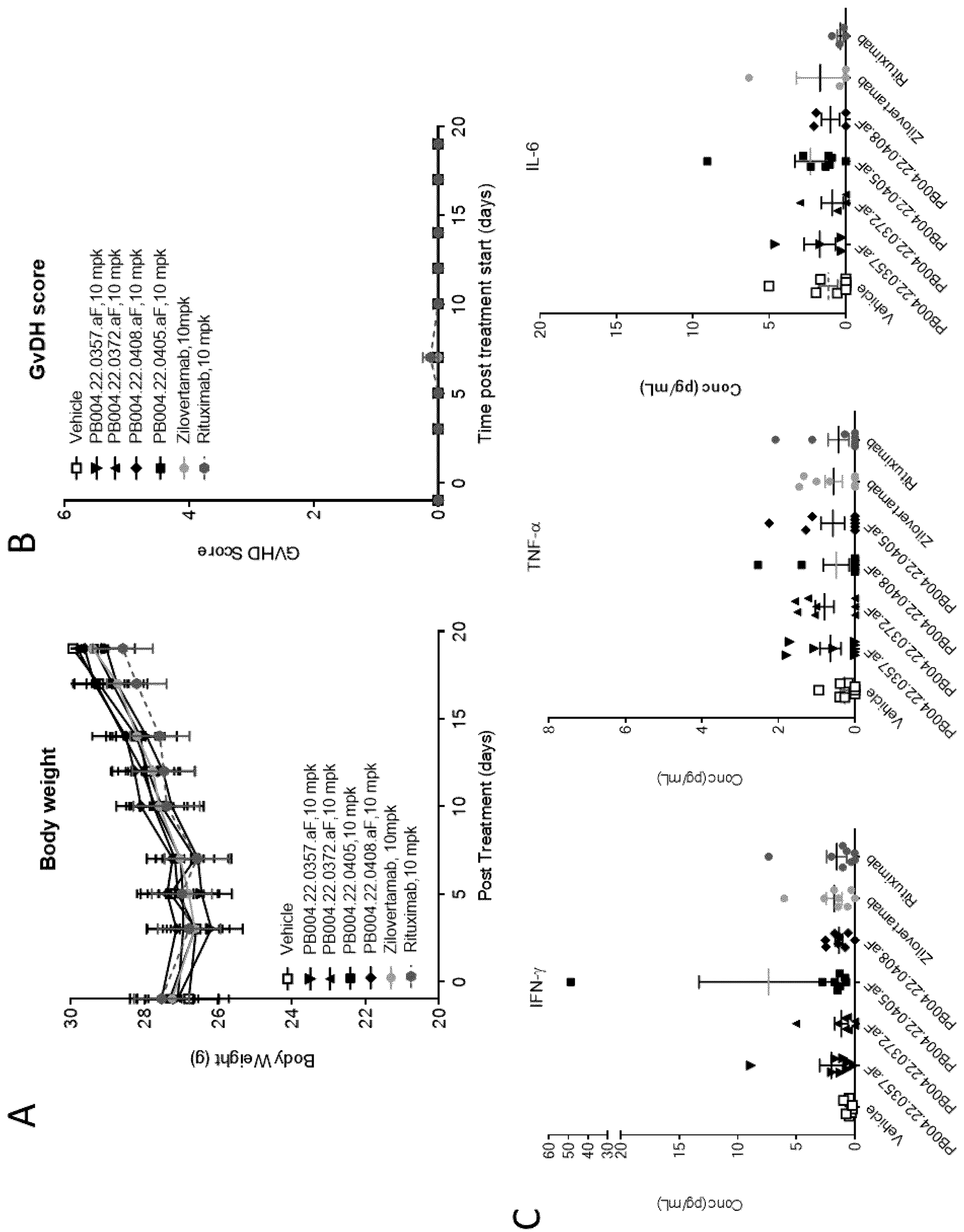

Fig. 7

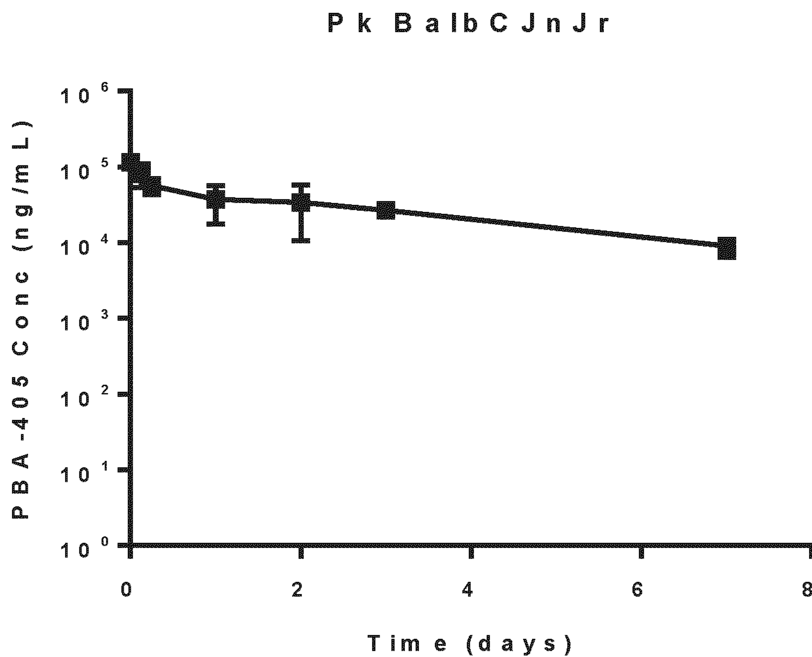

Fig. 8

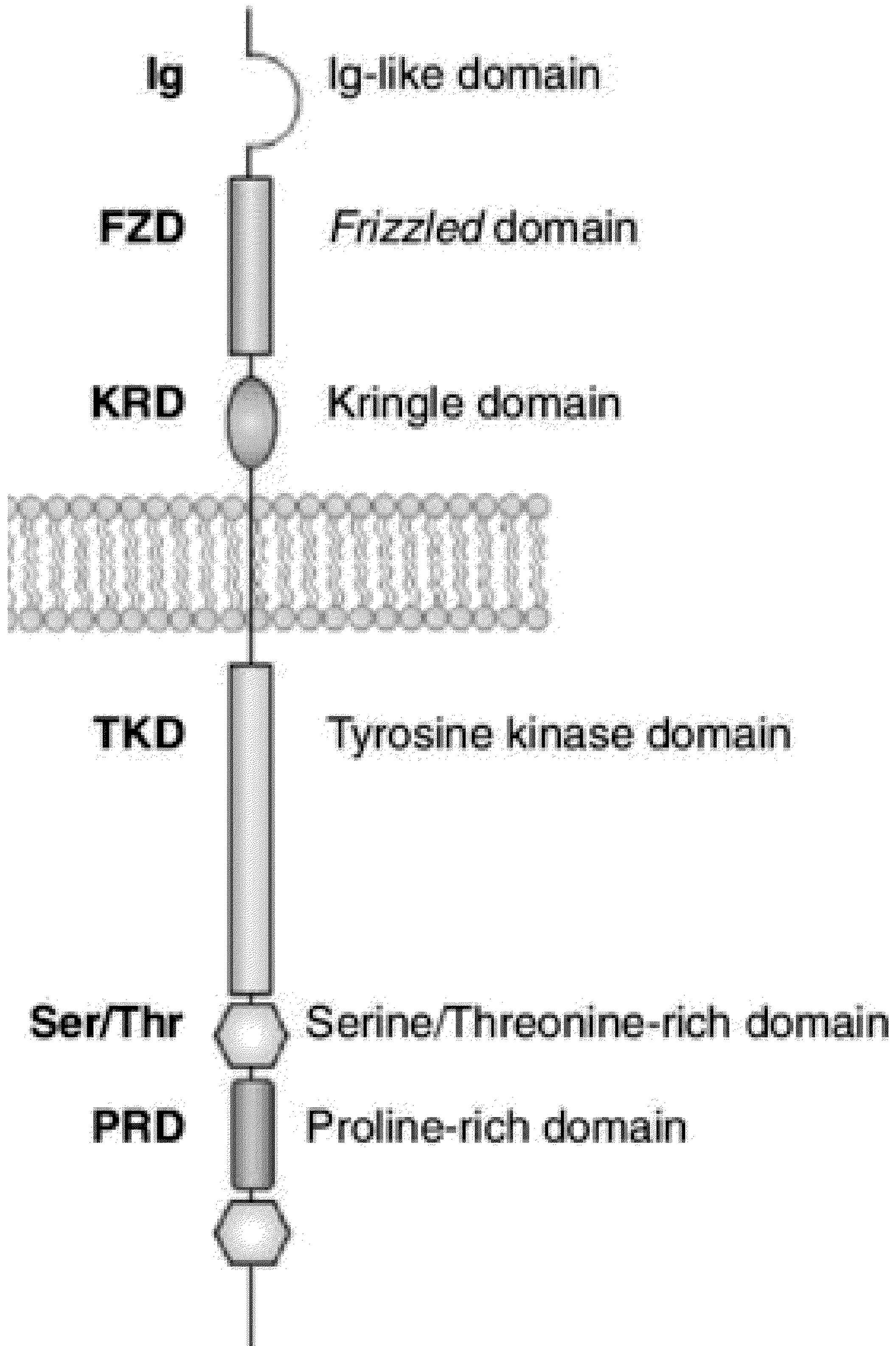

Fig. 9

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2024/065428

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/00 C07K16/28 A61P35/00
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K A61K

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

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

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KIPPS THOMAS J: "ROR1: an orphan becomes apparent", BLOOD, AMERICAN SOCIETY OF HEMATOLOGY, US, vol. 140, no. 14, 18 May 2022 (2022-05-18), pages 1583-1591, XP087193279, ISSN: 0006-4971, DOI: 10.1182/BLOOD.2021014760 [retrieved on 2022-05-18] page 1587, column 1 - page 1588, column 1 -----	1-13

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[x] Further documents are listed in the continuation of Box C.

[x] See patent family annex.

* Special categories of cited documents :

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

"E" earlier application or patent but published on or after the international filing date

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 October 2024	17/10/2024

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Covone-van Hees, M

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2024/065428

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

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

   a. [X] forming part of the international application as filed.

   b. [ ] furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

      [ ] accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. [ ] With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2024/065428

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J YU ET AL: "Cirmtuzumab inhibits Wnt5a-induced Rac1 activation in chronic lymphocytic leukemia treated with ibrutinib", BLOOD CANCER JOURNAL, vol. 31, no. 6, 1 December 2016 (2016-12-01), pages 1333-1339, XP055656723, London ISSN: 0887-6924, DOI: 10.1038/leu.2016.368 the whole document -----	1-13
X	WO 2018/005519 A2 (UNIV CALIFORNIA [US]) 4 January 2018 (2018-01-04) the whole document -----	1-13

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2024/065428

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2018005519 A2	04-01-2018	AR 108889 A1	03-10-2018
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		CA 3029003 A1	04-01-2018
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