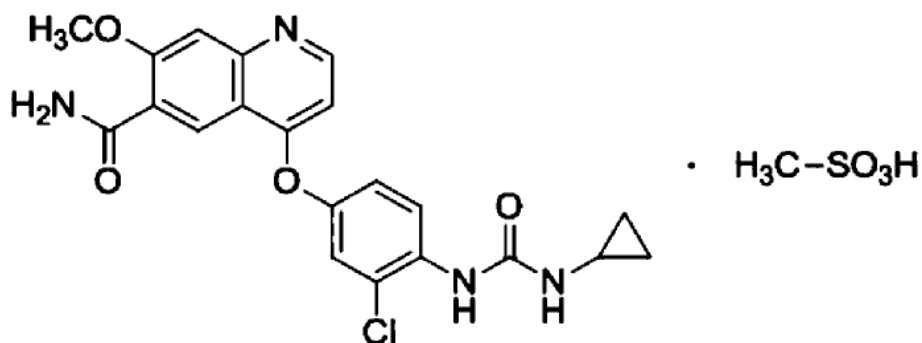




formula II

However, for a variety of tumors, the disease is still uncontrollable for a long term after chemotherapy, and the 5-year survival rate is still very low. Therefore, developing a medication or combination therapy with lower toxicity and higher efficacy is of great meaning.

SUMMARY

By intensive research and creative efforts, the inventor correspondingly modified the Fc fragment of the anti-CTLA4/anti-PD-1 antibody structure to reduce the binding capacity of the Fc region to Fc receptors, thereby reducing ADCC, ADCP and/or CDC effects on immune cells and increasing the efficacy of the anti-CTLA4/anti-PD-1 antibody.

The present disclosure is detailed below.

One example of the present disclosure relates to a bispecific antibody, comprising:

a first protein functional region targeting PD-1, and

a second protein functional region targeting CTLA4;

wherein the first protein functional region is an immunoglobulin, and the second protein functional region is a single chain antibody; or, the first protein functional region is a single chain antibody, and the second protein functional region is an immunoglobulin;

wherein,

for the immunoglobulin, the heavy chain variable region comprises HCDR1–HCDR3 of amino acid sequences set forth in SEQ ID NOs: 27–29 respectively, and the light chain variable region comprises LCDR1–LCDR3 of amino acid sequences set forth in SEQ ID NOs: 30–32 respectively; for the single chain antibody, the heavy chain variable region comprises HCDR1–HCDR3 of amino acid sequences set forth in SEQ ID NOs: 33–35 respectively, and the light chain variable region comprises LCDR1–LCDR3 of amino acid sequences set forth in SEQ ID NOs: 36–38 respectively;

or,

for the immunoglobulin, the heavy chain variable region comprises HCDR1–HCDR3 of amino acid sequences set forth in SEQ ID NOs: 33–35 respectively, and the light chain variable region comprises LCDR1–LCDR3 of amino acid sequences set forth in SEQ ID NOs: 36–38 respectively; for the single chain antibody, the heavy chain variable region comprises HCDR1–HCDR3 of amino acid sequences set forth in SEQ ID NOs: 27–29 respectively, and the light chain variable region comprises LCDR1–LCDR3 of amino acid sequences set forth in SEQ ID NOs: 30–32 respectively;

the immunoglobulin is of human IgG1 subtype;

wherein, according to the EU numbering system, the heavy chain constant region of the immunoglobulin has mutations at any 2 or 3 of positions 234, 235 and 237, and the affinity constant of the bispecific antibody to FcγRIIIa and/or C1q is reduced after the mutation as compared to that before the mutation; preferably, the affinity constant is measured by a Fortebio Octet system.

In one or more examples of the present disclosure, for the bispecific antibody, after the mutation described above, the affinity constant of the bispecific antibody to FcγRIIIa, FcγRI, FcγRIIIa_H131, FcγRIIIa_V158 and/or FcγRIIb is reduced as compared to that before the mutation; preferably, the affinity constant is measured by a Fortebio Octet system.

In one or more examples of the present disclosure, for the bispecific antibody, according to the EU numbering system, the heavy chain constant region of the immunoglobulin has the following mutations at positions 234, 235 and/or 237:

L234A and L235A;

L234A and G237A;

L235A and G237A;

or

L234A, L235A and G237A.

In the present disclosure, letters before the position number represent amino acids before mutation, and letters after the position number represent amino acids after mutation, unless otherwise specified.

In one aspect, the present disclosure provides for a bispecific antibody, comprising: a first protein functional region targeting PD-1; and a second protein functional region targeting CTLA4, wherein the first protein functional region is an immunoglobulin, and the second protein functional region is a single chain antibody; wherein the amino acid sequence of the heavy chain of the immunoglobulin is set forth in SEQ ID NO: 40, and the amino acid sequence of the light chain of the immunoglobulin is set forth in SEQ ID NO: 24;

wherein the amino acid sequence of the heavy chain variable region of the single chain antibody is set forth in SEQ ID NO: 43, and the amino acid sequence of the light chain variable region of the single chain antibody is set forth in SEQ ID NO: 44; wherein the single chain antibody is linked to the C terminus of the heavy chain of the immunoglobulin; wherein the first protein functional region is linked to the second protein functional region via a first linker fragment; and the heavy chain variable region of the single chain antibody is linked to the light chain variable region of the single chain antibody via a second linker fragment; the first linker fragment and the second linker fragment are the same or different; and wherein, the amino acid sequences of the first linker fragment and second linker fragment are independently selected from SEQ ID NO: 25 or SEQ ID NO: 26.

In another example, the present disclosure further relates to a bispecific antibody, comprising:

a first protein functional region targeting PD-1, and

a second protein functional region targeting CTLA4;

wherein the first protein functional region is an immunoglobulin, and the second protein functional region is a single chain antibody; or, the first protein functional region is a single chain antibody, and the second protein functional region is an immunoglobulin;

In one or more examples of the present disclosure, for the bispecific antibody, the linker fragment is (GGGGS)_n, n being a positive integer; preferably, n is 1, 2, 3, 4, 5 or 6.

In one or more examples of the present disclosure, for the bispecific antibody, the numbers of the first protein functional region and the second protein functional region are each independently 1, 2 or more.

In one or more examples of the present disclosure, for the bispecific antibody, the number of the first protein functional region is 1 and the number of the second protein functional region is 2.

In one or more examples of the present disclosure, for the bispecific antibody, the single chain antibody is linked to the C terminus of the heavy chain of the immunoglobulin. Since an immunoglobulin has two heavy chains, two single chain antibody molecules are linked to one immunoglobulin molecule. Preferably, the two single chain antibody molecules are identical. Preferably, the single chain antibody is linked to the C terminus of the heavy chain of the immunoglobulin by forming an amide bond via the aforementioned linker fragment.

In one or more examples of the present disclosure, the constant regions of the immunoglobulin are humanized. For example, the heavy chain constant region is Ig gamma-1 chain C region, **ACCESSION: P01857, and the light chain constant region is Ig kappa chain C region, **ACCESSION: P01834**.**

In one or more examples of the present disclosure, the bispecific antibody binds to CTLA4 protein and/or PD-1 protein with a K_D less than about 10^{-5} M, e.g., less than about 10^{-6} M, 10^{-7} M, 10^{-8} M, 10^{-9} M or 10^{-10} M or less.

In one or more examples of the present disclosure, the bispecific antibody is a monoclonal antibody.

In one or more examples of the present disclosure, the bispecific antibody is a humanized antibody.

In another example of the present disclosure, the bispecific antibody comprises:

a first protein functional region targeting PD-1, and

a second protein functional region targeting CTLA4;

the number of the first protein functional region is 1, and the number of the second protein functional region is 2;

wherein the first protein functional region is an immunoglobulin, and the second protein functional region is a single chain antibody;

the amino acid sequence of the heavy chain of the immunoglobulin is set forth in SEQ ID NO: 40, and the amino acid sequence of the light chain of the immunoglobulin is set forth in SEQ ID NO: 24;

the amino acid sequence of the heavy chain variable region of the single chain antibody is set forth in SEQ ID NO: 43, and the amino acid sequence of the light chain variable region of the single chain antibody is set forth in SEQ ID NO: 44;

the single chain antibody is linked to the C terminus of the heavy chain of the immunoglobulin;

the first protein functional region is linked to the second protein functional region via a first linker fragment; and the heavy chain variable region of the single chain antibody is linked to the light chain variable region of the single chain antibody via a second linker fragment; the first linker fragment and the second linker fragment are the same or different;

preferably, the amino acid sequences of the first linker fragment and second linker fragment are independently selected from SEQ ID NO: 25 and SEQ ID NO: 26;

preferably, the amino acid sequences of the first linker fragment and second linker fragments are set forth in SEQ ID NO: 26.

Another aspect of the present disclosure relates to an isolated nucleic acid molecule encoding the bispecific antibody as described herein.

In another aspect, the present disclosure also relates to a vector comprising the isolated nucleic acid molecule of the present disclosure.

In yet another aspect, the present disclosure also relates to a host cell comprising the isolated nucleic acid molecule of the present disclosure or the vector of the present disclosure.

In another aspect, the present disclosure provides for a conjugate comprising the bispecific antibody as described herein, and a conjugated moiety, wherein the conjugated moiety is a detectable label.

Another example of the present disclosure relates to a conjugate comprising an antibody or antigen-binding fragment thereof, and a conjugated moiety, wherein the immunoglobulin is the bispecific antibody as described herein, and the conjugated moiety is a detectable label; preferably, the conjugated moiety is a radioisotope, a fluorescent substance, a luminescent substance, a colored substance, or an enzyme.

In another aspect, the present disclosure provides for a kit, comprising the bispecific antibody as described herein or the conjugate as described herein.

Another example of the present disclosure relates to a kit comprising the bispecific antibody as described herein or comprising the conjugate of the present disclosure;

wherein preferably, the kit further comprises a second antibody capable of specifically recognizing the immunoglobulin or the antigen binding fragment thereof; optionally, the second antibody further comprises a detectable label, for example, a radioisotope, a fluorescent substance, a luminescent substance, a colored substance, or an enzyme.

Another aspect of the present disclosure relates to use of the bispecific antibody or the conjugate as described herein in preparing a kit for detecting the presence or level of PD-1 and/or CTLA4 in a sample.

In another aspect, the present disclosure provides for a pharmaceutical composition, comprising the bispecific antibody as described herein or the conjugate as described herein.

Another example of the present disclosure relates to a pharmaceutical composition comprising the bispecific antibody or the conjugate as described herein; optionally, the pharmaceutical composition further comprises a pharmaceutically acceptable carrier and/or excipient.

In one or more examples of the present disclosure, the pharmaceutical composition further comprises one or more anti-tumor chemotherapeutics;

preferably, the anti-tumor chemotherapeutic is a tyrosine kinase inhibitor; more preferably, the anti-tumor chemotherapeutic is anlotinib or a pharmaceutically acceptable salt thereof (e.g., hydrochloride salt), or lenvatinib or a pharmaceutically acceptable salt thereof (e.g., mesylate salt).

In one or more examples of the present disclosure, the unit dose of the pharmaceutical composition is 100–1000 mg, 200–800 mg, 200–500 mg, 300–600 mg, 400–500 mg, or 450 mg, based on the mass of the bispecific antibody.

In another aspect, the present disclosure provides for a combination product comprising a first product and a second product in separate packages, wherein, the first product comprises the bispecific antibody as described herein, the conjugate as described herein, or the pharmaceutical composition as described herein, and wherein the second product comprises one or more anti-tumor chemotherapeutics.

Another example of the present disclosure relates to a combination product comprising a first product and a second product in separate packages,

wherein,

the first product comprises the bispecific antibody, the conjugate or the pharmaceutical composition as described herein;

the second product comprises one or more anti-tumor chemotherapeutics; preferably, the anti-tumor chemotherapeutic is a tyrosine kinase inhibitor; more preferably, the anti-tumor chemotherapeutic is anlotinib or a pharmaceutically acceptable salt thereof (e.g., hydrochloride salt), or lenvatinib or a pharmaceutically acceptable salt thereof (e.g., mesylate salt);

preferably, the first product and the second product further independently comprise one or more pharmaceutically acceptable excipients;

preferably, the combination product further comprises a package insert.

In one or more examples of the present disclosure, for the combination product, the unit dose of the first product is 100–1000 mg, 200–800 mg, 200–500 mg, 300–600 mg, 400–500 mg, or 450 mg, based on the mass of the bispecific antibody.

In one or more examples of the present disclosure, for the combination product, the unit dose of the second product is 0.1–100 mg, 0.5–50 mg, 0.5–10 mg, 1–10 mg, 2–8 mg, or 1–5 mg, based on the mass of the active ingredient.

In one or more examples of the present disclosure, for the combination product, the unit dose of the second product is 1–20 mg, 2–15 mg, 4–12 mg, or 8–12 mg, based on the mass of the active ingredient.

In another aspect, the present disclosure provides for a use of the bispecific antibody as described herein, the conjugate as described herein, the pharmaceutical composition as described herein, or the combination product as described herein in preparing a medicament for treating and/or preventing a tumor or anemia, or in preparing a medicament for diagnosing a tumor or anemia.

Another example of the present disclosure relates to use of the bispecific antibody, the conjugate, the pharmaceutical composition or the combination product according to any example of the present disclosure in preparing a medicament for treating and/or preventing a tumor or anemia, or in preparing a medicament for diagnosing a tumor or anemia; preferably, the tumor is selected from one or more of melanoma, renal cancer,

next-generation sequencing, or NGS) can also be used as a method for detecting microsatellite instability. When more microsatellite loci are selected, such as more than 5 loci or other microsatellite loci, for PCR assay, inconsistency in $\geq 30\%$ loci is defined as MSI-H, consistency in all loci is defined as MSS, and inconsistency between 0 and 30% is defined as MSI-L.

Another example of the present disclosure relates to use of the bispecific antibody or the conjugate as described herein in preparing:

a medicament for blocking the binding of PD-1 to PD-L1,

a medicament for down-regulating the activity or level of PD-1,

a medicament for relieving the immunosuppression of PD-1 in an organism, or

a medicament for elevating IFN- γ and/or IL-2 expression in T lymphocytes;

and/or

a medicament for blocking the binding of CTLA4 to B7,

a medicament for down-regulating the activity or level of CTLA4,

a medicament for relieving the immunosuppression of CTLA4 in an organism, or

a medicament for elevating IL-2 expression in T lymphocytes.

Another example of the present disclosure relates to a method for the prevention and/or treatment and/or adjuvant treatment and/or diagnosis of a tumor, comprising: administering to a subject in need an effective amount of the bispecific antibody, the conjugate, the pharmaceutical composition or the combination product according to any example of the present disclosure; preferably, the tumor is selected from one or more of melanoma, renal cancer, prostate cancer, bladder cancer, colon cancer, rectal cancer, gastric cancer, liver cancer, lung cancer, ovarian cancer, leukemia, breast cancer, mesothelioma, cervical cancer, endometrial cancer, lymphoma and nasopharyngeal cancer;

preferably, the lung cancer is selected from one or more of non-small cell lung cancer, small cell lung cancer and squamous cell lung cancer;

preferably, the gastric cancer is gastric adenocarcinoma or gastroesophageal junction adenocarcinoma;

preferably, the tumor is a solid tumor of MSI-H/dMMR phenotype; preferably, the tumor is selected from one or more of the following tumors of MSI-H/dMMR phenotype:

colon cancer, rectal cancer, endometrial cancer, gastric cancer, mesothelioma, sarcoma, adrenocortical carcinoma, malignant melanoma and ovarian germ cell neoplasm.

In one or more examples of the present disclosure, for the method, the administration is before or after a surgical treatment and/or before or after a radiotherapy.

CLAIMS

1. A bispecific antibody, comprising:

a first protein functional region targeting PD-1; and

a second protein functional region targeting CTLA4,

wherein the first protein functional region is an immunoglobulin, and the second protein functional region is a single chain antibody;

wherein the amino acid sequence of the heavy chain of the immunoglobulin is set forth in SEQ ID NO: 40, and the amino acid sequence of the light chain of the immunoglobulin is set forth in SEQ ID NO: 24;

wherein the amino acid sequence of the heavy chain variable region of the single chain antibody is set forth in SEQ ID NO: 43, and the amino acid sequence of the light chain variable region of the single chain antibody is set forth in SEQ ID NO: 44;

wherein the single chain antibody is linked to the C terminus of the heavy chain of the immunoglobulin;

wherein the first protein functional region is linked to the second protein functional region via a first linker fragment; and the heavy chain variable region of the single chain antibody is linked to the light chain variable region of the single chain antibody via a second linker fragment; the first linker fragment and the second linker fragment are the same or different; and

wherein, the amino acid sequences of the first linker fragment and second linker fragment are independently selected from SEQ ID NO: 25 or SEQ ID NO: 26.

2. The bispecific antibody according to claim 1, wherein the amino acid sequences of the first linker fragment and second linker fragments are set forth in SEQ ID NO: 26.

3. The bispecific antibody according to any of claims 1-2, wherein, according to the EU numbering system, the heavy chain constant region of the immunoglobulin further comprises one or more mutations selected from:

N297A, D265A, D270A, P238D, L328E, E233D, H268D, P271G, A330R, C226S, C229S, E233P, P331S, S267E, L328F, A330L, M252Y, S254T, T256E, N297Q, P238S, P238A, A327Q, A327G, P329A, K322A, T394D, G236R, G236A, L328R, A330S, P331S, H268A, E318A and K320A.

4. The bispecific antibody according to any of claims 1-3, wherein the immunoglobulin binds to

FcγRIIIa_F158, FcγRI, FcγRIIa_H131, FcγRIIIa_V158 and/or FcγRIIb with an affinity constant greater than 10^{-7} M, 10^{-6} M, 10^{-5} M, 10^{-4} M, or 10^{-3} M or greater.

5. The bispecific antibody according to any of claims 1-4, wherein the immunoglobulin has no binding signal or a binding signal of less than 0.1 nM to FcγRIIIa_F158, FcγRI, FcγRIIa_H131, FcγRIIIa_V158 and/or FcγRIIb.

6. The bispecific antibody according to any of claims 1-5, wherein the immunoglobulin binds to C1q with an affinity constant greater than 10^{-9} M, 10^{-8} M, 10^{-7} M, 10^{-6} M, or 10^{-5} M or greater.

7. The bispecific antibody according to any of claims 1-6, wherein the immunoglobulin has no binding signal or a binding signal of less than 0.1 nM to C1q.

8. An isolated nucleic acid molecule, encoding the bispecific antibody according to any of claims 1-7.

9. A vector, comprising the isolated nucleic acid molecule according to claim 8.

10. A host cell, comprising the isolated nucleic acid molecule according to claim 8 or the vector according to claim 9.

11. A conjugate comprising the bispecific antibody according to any of claims 1-7, and a conjugated moiety, wherein the conjugated moiety is a detectable label.

12. The conjugate according to claim 11, wherein the conjugated moiety is a radioisotope, a fluorescent substance, a luminescent substance, a colored substance, or an enzyme.

13. A kit, comprising the bispecific antibody according to any of claims 1-7 or the conjugate according to any of claims 11-12.

14. The kit according to claim 13, wherein the kit further comprises a second antibody capable of specifically recognizing the bispecific antibody.

15. The kit according to claim 14, wherein the second antibody further comprises a detectable label.

16. The kit according to claim 15, wherein the detectable label is a radioisotope, a fluorescent substance, a luminescent substance, a colored substance, or an enzyme.

17. Use of the bispecific antibody according to any of claims 1-7 or the conjugate according to any of claims 11-12 in preparing a kit for detecting the presence or level of PD-1 and/or CTLA4 in a sample.

18. A pharmaceutical composition, comprising the bispecific antibody according to any of claims 1-7 or the conjugate according to any of claims 11-12.

19. The pharmaceutical composition according to claim 18, wherein the pharmaceutical composition further comprises a pharmaceutically acceptable carrier and/or excipient.

20. The pharmaceutical composition according to any of claims 18-19, further comprising one or more anti-tumor chemotherapeutics.

21. The pharmaceutical composition according to claim 20, wherein the anti-tumor chemotherapeutic is a tyrosine kinase inhibitor.

22. The pharmaceutical composition according to any of claims 20-21, wherein the anti-tumor chemotherapeutic is anlotinib or a pharmaceutically acceptable salt thereof, or lenvatinib or a pharmaceutically acceptable salt thereof.

23. The pharmaceutical composition according to any of claims 18-22, wherein the unit dose of the pharmaceutical composition is 100–1000 mg, 200–800 mg, 200–500 mg, 300–600 mg, 400–500 mg, or 450 mg, based on the mass of the bispecific antibody.

24. A combination product comprising a first product and a second product in separate packages,

wherein the first product comprises the bispecific antibody according to any of claims 1-7, the conjugate according to any of claims 11-12, or the pharmaceutical composition according to any of claims 18-19, and wherein the second product comprises one or more anti-tumor chemotherapeutics.

25. The combination product according to claim 24, wherein the anti-tumor chemotherapeutic is a tyrosine kinase inhibitor.

26. The combination product according to any of claims 24-25, wherein the anti-tumor chemotherapeutic is anlotinib or a pharmaceutically acceptable salt thereof, or lenvatinib or a pharmaceutically acceptable salt thereof .

27. The combination product according to any of claims 24-26, wherein the first product and the second product further independently comprise one or more pharmaceutically acceptable excipients.

28. The combination product according to any of claims 24-27, wherein the combination product further comprises a package insert.

29. The combination product according to any of claims 24-28, wherein the unit dose of the first product is 100–1000 mg, 200–800 mg, 200–500 mg, 300–600 mg, 400–500 mg, or 450 mg, based on the mass of the bispecific antibody, and wherein the unit dose of the second product is 0.1–100 mg, 0.5–50 mg, 1–20 mg, 2–15 mg, 4–12 mg, or 8–12 mg, based on the mass of the active ingredient.

30. Use of the bispecific antibody according to any of claims 1-7, the conjugate according to any of claims 11-12, the pharmaceutical composition according to any of claims 18-23, or the combination product according to any of claims 24-29 in preparing a medicament for treating and/or preventing a tumor or anemia, or in preparing a medicament for diagnosing a tumor or anemia.

31. The use according to claim 30, wherein the tumor is selected from one or more of melanoma, renal cancer, prostate cancer, bladder cancer, colon cancer, rectal cancer, gastric cancer, liver cancer, lung cancer, ovarian cancer, leukemia, breast cancer, mesothelioma, cervical cancer,

endometrial cancer, lymphoma and nasopharyngeal cancer.

32. The use according to claim 31, wherein the lung cancer is selected from one or more of non-small cell lung cancer, small cell lung cancer and squamous cell lung cancer.

33. The use according to claim 31, wherein the gastric cancer is gastric adenocarcinoma or gastroesophageal junction adenocarcinoma.

34. The use according to any of claims 30-31, wherein the tumor is a solid tumor of MSI-H/dMMR phenotype.

35. The use according to claim 34, wherein the tumor is selected from one or more of the following tumors of MSI-H/dMMR phenotype:

colon cancer, rectal cancer, endometrial cancer, gastric cancer, mesothelioma, sarcoma, adrenocortical carcinoma, malignant melanoma and ovarian germ cell neoplasm.

36. The use according to any of claims 30-35, wherein the medicament is prepared for:

blocking the binding of PD-1 to PD-L1,

down-regulating the activity or level of PD-1,

relieving the immunosuppression of PD-1 in an organism, or

elevating IFN- γ and/or IL-2 expression in T lymphocytes;

and/or

blocking the binding of CTLA4 to B7,

down-regulating the activity or level of CTLA4,

relieving the immunosuppression of CTLA4 in an organism, or

elevating IL-2 expression in T lymphocytes.