

Description

Title of Invention

PHARMACEUTICAL COMPOSITION FOR CANCER TREATMENT COMPRISING FUSION PROTEIN INCLUDING IL-2 PROTEIN AND CD80 PROTEIN AND ANTICANCER DRUG

Technical Field

The present invention relates to a pharmaceutical composition for treating cancer, comprising, as active ingredients, a fusion protein comprising an IL-2 protein and a CD80 protein, and an anticancer agent.

Background Art

Interleukin 2 (IL-2), also called T-cell growth factor (TCGF), is a globular glycoprotein that plays a central role in lymphocyte production, survival, and homeostasis. IL-2 has a protein size of 15.5 kDa to 16 kDa and consists of 133 amino acids. IL-2 mediates various immune actions by binding to an IL-2 receptor composed of three distinct subunits.

In addition, IL-2 is synthesized mainly by activated T cells, in particular by CD4⁺ helper T cells. IL-2 stimulates proliferation and differentiation of T cells, and induces production of cytotoxic T lymphocytes (CTLs) and differentiation of peripheral blood lymphocytes into cytotoxic cells and lymphokine-activated killer cells (LAK cells).

Meanwhile, CD80, also known as B7-1, is a member of the B7 family of membrane-bound proteins that are involved in immune regulation by binding to its ligand by way of delivering costimulatory responses and coinhibitory responses. CD80 is a transmembrane protein expressed on the surface of T cells, B cells, dendritic cells, and monocytes. CD80 is known to bind CD28, CTLA-4 (CD152), and PD-L1 (programmed cell death ligand 1). CD80, CD86, CTLA-4, and CD28 are involved in a costimulatory-coinhibitory system. For example, they regulate activity of T cells and are involved in proliferation, differentiation, and survival thereof.

In addition, recently, immune checkpoint inhibitors such as Keytruda[®] are in the spotlight. Immune checkpoint inhibitors are anticancer agents that help to attack cancer cells by activating the body's immune system. Until now, cancer therapy has focused on killing rapidly dividing cells that are characteristic of cancer cells, so it has side effects by acting on rapidly proliferating cells among normal cells as well as cancer cells. However, it is known that immune anticancer agents affect cancer cells by utilizing the immune system of cancer patients, so there are few typical side effects exhibited by existing anticancer agents. Anti-PD-1 antibodies, such as Keytruda, bind to a specific receptor (PD-1) on T cells and block the pathway by which cancer

cells avoid the surveillance system of active T cells, thereby exhibiting anticancer effect through immune reactivation that allows T cells in the human body to attack cancer cells (KR 10-2018-0030580 A).

Detailed Description of Invention

Technical Problem

The present inventors have studied to develop IL-2 which is safe and effective. As a result, the present inventors have confirmed that a novel fusion protein, which comprises an IL-2 protein and a CD80 protein in one molecule, and an anticancer agent exhibit excellent anticancer effect, thereby completing the present invention.

Solution to Problem

In order to achieve the above object, in an aspect of the present invention, there is provided a pharmaceutical composition for treating cancer, comprising, as active ingredients, a fusion protein comprising an IL-2 protein and a CD80 protein, and an anticancer agent.

Effects of the Invention

A fusion protein comprising an IL-2 protein and a CD80 protein can not only activate immune cells owing to IL-2, but also effectively regulate Treg cells owing to CD80. In addition, it was confirmed that a synergistic effect appeared when administered in combination with an anticancer agent. Therefore, a pharmaceutical composition for treating cancer, comprising, as active ingredients, the fusion protein comprising an IL-2 protein and a CD80 protein, and an anticancer agent can be usefully employed for treatment of cancer disease.

Brief Description of Drawings

Fig. 1 illustrates a schematic diagram of an embodiment of a fusion protein dimer.

Fig. 2 illustrates a schematic diagram of a mechanism of action by which the fusion protein dimer exhibits in the lymph nodes.

Fig. 3 illustrates a schematic diagram of a mechanism of action by which the fusion protein dimer exhibits in the tumor microenvironment.

Fig. 4 illustrates a schematic view of the structure of the fusion protein. Here, each of GI101 and mGI101 is an embodiment of the fusion protein, and GI101C1, GI101C2, and mGI101C1 are comparative examples for comparison with activity of the fusion protein.

Fig. 5 illustrates various embodiments of the fusion protein. Human- and mouse-derived proteins may be combined to prepare a fusion protein. A CD80 protein and an IL-2 protein may be bound to each other via various linkers other than Fc.

Fig. 6 illustrates a result obtained by identifying the obtained fusion protein dimer (GI101) with SDS-PAGE.

Fig. 7 illustrates amounts of the fusion protein (GI101) measured by absorbance.

Fig. 8 illustrates a result obtained by analyzing the obtained fusion protein dimer (GI101) by size exclusion chromatography (SEC).

Fig. 9 illustrates a result obtained by identifying the obtained mGI101 fusion protein dimer with SDS-PAGE.

Fig. 10 illustrates a result obtained by identifying the obtained GI101C1 fusion protein dimer with SDS-PAGE.

Fig. 11 illustrates a result obtained by identifying the obtained GI101C2 fusion protein dimer with SDS-PAGE.

Fig. 12 illustrates a result obtained by identifying the obtained mGI101C1 fusion protein dimer with SDS-PAGE.

Fig. 13 illustrates a result obtained by identifying the obtained GI102-M45 fusion protein dimer with SDS-PAGE.

Fig. 14 illustrates a result obtained by identifying the obtained GI102-M61 fusion protein dimer with SDS-PAGE.

Fig. 15 illustrates a result obtained by identifying the obtained GI102-M72 fusion protein dimer with SDS-PAGE.

Fig. 16 illustrates binding affinity between hCTLA-4 and GI101.

Fig. 17 illustrates binding affinity between hPD-L1 and GI101.

Fig. 18 illustrates binding affinity between hPD-L1 and hPD-1.

Fig. 19 illustrates binding affinity between mCTLA-4 and mGI101.

Fig. 20 illustrates binding affinity between mPD-L1 and mGI101.

Fig. 21 illustrates a result obtained by identifying binding ability between GI101 (hCD80-Fc-hIL-2v) and CTLA-4. It was identified that GI101 (hCD80-Fc-hIL-2v) has high binding ability for CTLA-4.

Fig. 22 illustrates a result obtained by identifying binding affinity between GI101, and IL-2R α or IL-2R β .

Fig. 23 illustrates a result obtained by identifying binding affinity between GI101 and IL-2R α .

Fig. 24 illustrates a result obtained by identifying binding affinity between GI101 and IL-2R β .

Fig. 25 illustrates binding affinity between IL-2R α and GI102-M45.

Fig. 26 illustrates binding affinity between IL-2R α and GI102-M61.

Fig. 27 illustrates binding affinity between IL-2R α and GI102-M72.

Fig. 28 illustrates binding affinity between IL-2R β and GI102-M45.

Fig. 29 illustrates binding affinity between IL-2R β and GI102-M61.

Fig. 30 illustrates binding affinity between IL-2R β and GI102-M72.

Figs. 31 and 32 illustrate results obtained by measuring amounts of IFN- γ secreted from cells when the cells are treated and incubated with GI101, GI101C1, GI101C2, or IL-2 at respective concentrations.

Fig. 33 illustrates results obtained by identifying effects of GI101, GI101C1, GI101C2, and IL-2 (Proleukin) on proliferation of CD8 $^{+}$ T cells.

Fig. 34 illustrates a schematic diagram of a mechanism by which GI101 acts on effector T cells.

Fig. 35 illustrates results obtained by identifying effects of GI101 and GI102 on proliferation of CD8 $^{+}$ T cells and CD4 $^{+}$ T cells. Here, Fig. 35A illustrates proportions of CD8 $^{+}$ T cells and CD4 $^{+}$ T cells, Fig. 35B illustrates proliferation capacity of CD8 $^{+}$ T cells, and Fig. 35C illustrates a proportion of CD4 $^{+}$ /FoxP3 $^{+}$ Treg cells.

Figs. 36 and 37 illustrate results obtained by identifying effects of GI101 and GI101w on proliferation of CD8 $^{+}$ T cells and NK cells.

Figs. 38 and 39 illustrate results obtained by identifying an effect of GI101 on effector T cells.

Fig. 40 illustrates a result obtained by identifying effects of mGI101 and mGI102-M61 on mouse immune cells.

Figs. 41 and 42 illustrate results obtained by identifying a T cell activity inhibitory effect of GI101 on cancer cells expressing PD-L1 and CTLA-4.

Fig. 43 illustrates a result obtained by identifying a tumor inhibitory effect of mGI101, depending on its dose, in mice transplanted with mouse-derived colorectal cancer cells (CT26).

Fig. 44 illustrates results obtained by analyzing survival rate of mice, depending on the administration of mGI101, in mice transplanted with mouse-derived colorectal cancer cells (CT26).

Fig. 45 illustrates a result obtained by identifying a tumor inhibitory effect of GI101 in mice transplanted with mouse-derived colorectal cancer cells (CT26).

Fig. 46 illustrates results obtained by subjecting mice transplanted with mouse-derived colorectal cancer cells (CT26) to treatment with hIgG4, an anti-PD-1 antibody, or GI101, and then analyzing, with FACS, CD8 $^{+}$ T cells, IFN- γ T cells, CD4 $^{+}$ T cells, and Treg cells in cancer tissues.

Fig. 47 graphically illustrates results obtained by subjecting mice transplanted with mouse-derived colorectal cancer cells (CT26) to treatment with hIgG4, an anti-PD-1 antibody, or GI101, and then analyzing, with FACS, CD8 $^{+}$ T cells, IFN- γ T cells, CD4 $^{+}$ T cells, and Treg cells in cancer tissues.

Fig. 48 illustrates results obtained by subjecting mice transplanted with mouse-derived

colorectal cancer cells (CT26) to treatment with hIgG4, an anti-PD-1 antibody, or GI101, and then analyzing, with FACS, macrophages in cancer tissues.

Fig. 49 graphically illustrates results obtained by subjecting mice transplanted with mouse-derived colorectal cancer cells (CT26) to treatment with hIgG4, an anti-PD-1 antibody, or GI101, and then analyzing, with FACS, macrophages in cancer tissues.

Fig. 50 illustrates results obtained by subjecting mice transplanted with mouse-derived colorectal cancer cells (CT26) to treatment with hIgG4, an anti-PD-1 antibody, or GI101, and then analyzing, with FACS, dendritic cells in cancer tissues.

Fig. 51 graphically illustrates results obtained by subjecting mice transplanted with mouse-derived colorectal cancer cells (CT26) to treatment with hIgG4, an anti-PD-1 antibody, or GI101, and then analyzing, with FACS, dendritic cells in cancer tissues.

Fig. 52 illustrates a result obtained by identifying a tumor inhibitory effect of GI101 in mice transplanted with mouse-derived lung cancer cells (LL/2).

Fig. 53 graphically illustrates results obtained by subjecting mice transplanted with mouse-derived lung cancer cells (LL/2) to treatment with hIgG4, an anti-PD-1 antibody, or GI101, and then analyzing, with FACS, CD8⁺ T cells, IFN- γ T cells, CD4⁺ T cells, and Treg cells in cancer tissues.

Fig. 54 graphically illustrates results obtained by subjecting mice transplanted with mouse-derived lung cancer cells (LL/2) to treatment with hIgG4, an anti-PD-1 antibody, or GI101, and then analyzing, with FACS, macrophages in cancer tissues.

Fig. 55 graphically illustrates results obtained by subjecting mice transplanted with mouse-derived lung cancer cells (LL/2) to treatment with hIgG4, an anti-PD-1 antibody, or GI101, and then analyzing, with FACS, dendritic cells in cancer tissues.

Fig. 56 illustrates a result obtained by identifying a tumor inhibitory effect of mGI102-M61 in mice transplanted with mouse-derived colorectal cancer cells (CT26).

Fig. 57 illustrates results obtained by analyzing survival rate of mice, depending on the administration of mGI102-M61, in mice transplanted with mouse-derived colorectal cancer cells (CT26).

Fig. 58 illustrates a result obtained by identifying a tumor inhibitory effect of mGI101 in mice transplanted with mouse-derived colorectal cancer cells (CT26).

Fig. 59 illustrates tumor inhibition rate of mGI101 in mice transplanted with mouse-derived colorectal cancer cells (CT26).

Fig. 60 illustrates a graph showing tumor growth when a combination of GI101 and Keytruda is used in mice transplanted with human-derived breast cancer cells (MDA-MB-231). The groups having received each of GI101 and Keytruda exhibited tumor growth inhibition as compared with the control (hIgG4). The group having received a combination of GI101 and

Keytruda exhibited tumor growth inhibition as compared with the control. The group having received a combination of GI-101 and Keytruda exhibited tumor growth inhibition as compared with the group having received each of GI101 and Keytruda.

Fig. 61 illustrates a tumor growth inhibition rate when a combination of GI-101 and Keytruda is used in mice transplanted with human-derived breast cancer cells (MDA-MB-231). The groups having received IgG4 exhibited a tumor growth inhibition rate of 30% or more in 2 mice, 50% or more in 1 mouse, and 80% or more in 1 mouse. The group having received GI101 exhibited a tumor growth inhibition rate of 30% or more in 5 mice, 50% or more in 5 mice, and 80% or more in 2 mice. The group having received Keytruda exhibited a tumor growth inhibition rate of 30% or more in 7 mice, 50% or more in 5 mice, and 80% or more in 3 mice. The group having received a combination of GI101 and Keytruda exhibited a tumor growth inhibition rate of 30% or more in 8 mice, 50% or more in 8 mice, and 80% or more in 6 mice.

Fig. 62 illustrates the degree of tumor growth of individual experimental animals of each treatment group when a combination of GI101 and Keytruda is used in mice transplanted with human-derived breast cancer cells (MDA-MB-231).

Fig. 63 illustrates the degree of tumor growth of individual experimental animals of the group having received hIgG4 in mice transplanted with human-derived breast cancer cells (MDA-MB-231).

Fig. 64 illustrates the degree of tumor growth of individual experimental animals of the group having received GI101 in mice transplanted with human-derived breast cancer cells (MDA-MB-231).

Fig. 65 illustrates the degree of tumor growth of individual experimental animals of the group having received Keytruda in mice transplanted with human-derived breast cancer cells (MDA-MB-231).

Fig. 66 illustrates the degree of tumor growth of individual experimental animals of the group having received a combination of GI101 and Keytruda in mice transplanted with human-derived breast cancer cells (MDA-MB-231).

Fig. 67 illustrates a graph of tumor growth when mGI101 and an anti-PD-1 antibody are administered in combination in mice transplanted with rodent-derived colorectal cancer cells (MC38).

Fig. 68 illustrates a tumor growth inhibition rate when mGI101 and an anti-PD-1 antibody are administered in combination in mice transplanted with rodent-derived colorectal cancer cells (MC38).

Fig. 69 illustrates the degree of tumor growth of individual experimental animals of each treatment group when mGI101 and an anti-PD-1 antibody are administered in combination in mice transplanted with rodent-derived colorectal cancer cells (MC38).

Fig. 70 illustrates the degree of tumor growth of individual experimental animals of the group having received hIgG4 in mice transplanted with rodent-derived colorectal cancer cells (MC38).

Fig. 71 illustrates the degree of tumor growth of individual experimental animals of the group having received mGI101 in mice transplanted with rodent-derived colorectal cancer cells (MC38).

Fig. 72 illustrates the degree of tumor growth of individual experimental animals of the group having received an anti-PD-1 antibody in mice transplanted with rodent-derived colorectal cancer cells (MC38).

Fig. 73 illustrates the degree of tumor growth of individual experimental animals of the group having received a combination of mGI101 and an anti-PD-1 antibody in mice transplanted with rodent-derived colorectal cancer cells (MC38).

Fig. 74 illustrates the degree of tumor growth of individual experimental animals after reinjecting rodent-derived colorectal cancer cells into an experimental animal showing a complete remission, among the group having received a combination of mGI101 and an anti-PD-1 antibody in mice transplanted with rodent-derived colorectal cancer cells (MC38).

Fig. 75 illustrates a graph of tumor growth when mGI101 and an anti-PD-L1 antibody are administered in combination in mice transplanted with rodent-derived colorectal cancer cells (CT26).

Fig. 76 illustrates a graph of tumor growth when mGI101 and an anti-TIGIT antibody are administered in combination in mice transplanted with rodent-derived colorectal cancer cells (CT26).

Fig. 77 illustrates a graph of tumor growth when mGI101 and Galunisertib, a TGF- β R inhibitor, are administered in combination in mice transplanted with rodent-derived colorectal cancer cells (CT26).

Fig. 78 illustrates a tumor growth inhibition rate when mGI101 and Galunisertib, a TGF- β R inhibitor, are administered in combination in mice transplanted with rodent-derived colorectal cancer cells (CT26).

Fig. 79 illustrates the degree of tumor growth of individual experimental animals when mGI101, Galunisertib, a TGF- β R inhibitor, and a combination thereof are administered in mice transplanted with rodent-derived colorectal cancer cells (CT26).

Fig. 80 illustrates a graph of tumor growth when mGI101 and Axitinib, a VEGFR inhibitor, are administered in combination in mice transplanted with rodent-derived colorectal cancer cells (CT26).

Fig. 81 illustrates a tumor growth inhibition rate when mGI101 and Axitinib, a VEGFR inhibitor, are administered in combination in mice transplanted with rodent-derived colorectal

cancer cells (CT26).

Fig. 82 illustrates the degree of tumor growth of individual experimental animals when mGI101 and Axitinib, a VEGFR inhibitor, are administered in combination in mice transplanted with rodent-derived colorectal cancer cells (CT26).

Fig. 83 illustrates a graph of tumor growth when mGI101 and Axitinib, a VEGFR inhibitor, are administered in combination in mice transplanted with rodent-derived lung cancer cells (LL/2).

Fig. 84 illustrates a tumor growth inhibition rate when mGI101 and Axitinib, a VEGFR inhibitor, are administered in combination in mice transplanted with rodent-derived lung cancer cells (LL/2).

Fig. 85 illustrates the degree of tumor growth of individual experimental animals when mGI101 and Axitinib, a VEGFR inhibitor, are administered in combination in mice transplanted with rodent-derived lung cancer cells (LL/2).

Fig. 86 illustrates a graph of tumor growth when mGI101 and Lenvatinib, a VEGFR inhibitor, are administered in combination in mice transplanted with rodent-derived colorectal cancer cells (CT26).

Fig. 87 illustrates a tumor growth inhibition rate when mGI101 and Lenvatinib, a VEGFR inhibitor, are administered in combination in mice transplanted with rodent-derived colorectal cancer cells (CT26).

Fig. 88 illustrates the degree of tumor growth of individual experimental animals when mGI101 and Lenvatinib, a VEGFR inhibitor, are administered in combination in mice transplanted with rodent-derived colorectal cancer cells (CT26).

Fig. 89 illustrates a graph of tumor growth when mGI101 and Lenvatinib, a VEGFR inhibitor, are administered in combination in mice transplanted with rodent-derived renal cancer cells (Renca).

Fig. 90 illustrates a tumor growth inhibition rate when mGI101 and Lenvatinib, a VEGFR inhibitor, are administered in combination in mice transplanted with rodent-derived renal cancer cells (Renca).

Fig. 91 illustrates the degree of tumor growth of individual experimental animals when mGI101 and Lenvatinib, a VEGFR inhibitor, are administered in combination in mice transplanted with rodent-derived renal cancer cells (Renca).

Fig. 92 illustrates the effect of killing cancer cell line when mGI101, Cetuximab as an EGFR inhibitor, and a combination thereof are administered in human-derived colorectal cancer cell line (HCT116).

Fig. 93 illustrates a graph of tumor growth when mGI101 and Olaparib, a PARP inhibitor, are administered in combination in mice transplanted with rodent-derived breast cancer cells (4T1).

Fig. 94 illustrates a tumor growth inhibition rate when mGI101 and Olaparib, a PARP

inhibitor, are administered in combination in mice transplanted with rodent-derived breast cancer cells (4T1).

Fig. 95 illustrates the degree of tumor growth of individual experimental animals when mGI101 and Olaparib, a PARP inhibitor, are administered in combination in mice transplanted with rodent-derived breast cancer cells (4T1).

Fig. 96 is a schematic diagram of an experimental schedule for the administration of mGI101 and Guadecitabine, a DNA methyltransferase inhibitor, in combination in mice transplanted with rodent-derived colorectal cancer cells (CT26).

Fig. 97 illustrates a graph of tumor growth when mGI101 (0.6 mpk) and Guadecitabine, a DNA methyltransferase inhibitor, are administered in combination in mice transplanted with rodent-derived colorectal cancer cells (CT26).

Fig. 98 illustrates a tumor growth inhibition rate when mGI101 (0.6 mpk) and Guadecitabine, a DNA methyltransferase inhibitor, are administered in combination in mice transplanted with rodent-derived colorectal cancer cells (CT26).

Fig. 99 illustrates the degree of tumor growth of individual experimental animals when mGI101 (0.6 mpk) and Guadecitabine, a DNA methyltransferase inhibitor, are administered in combination in mice transplanted with rodent-derived colorectal cancer cells (CT26).

Fig. 100 illustrates a graph of tumor growth when mGI101 (3 mpk) and Guadecitabine, a DNA methyltransferase inhibitor, are administered in combination in mice transplanted with rodent-derived colorectal cancer cells (CT26).

Fig. 101 illustrates a tumor growth inhibition rate when mGI101 (3 mpk) and Guadecitabine, a DNA methyltransferase inhibitor, are administered in combination in mice transplanted with rodent-derived colorectal cancer cells (CT26).

Fig. 102 illustrates the degree of tumor growth of individual experimental animals when mGI101 (3 mpk) and Guadecitabine, a DNA methyltransferase inhibitor, are administered in combination in mice transplanted with rodent-derived colorectal cancer cells (CT26).

Fig. 103 illustrates a graph of tumor growth when mGI101, Docetaxel, and an anti-PD-L1 antibody are administered in combination in mice transplanted with rodent-derived breast cancer cells (4T1).

Fig. 104 illustrates a tumor growth inhibition rate when mGI101, Docetaxel, and an anti-PD-L1 antibody are administered in combination in mice transplanted with rodent-derived breast cancer cells (4T1).

Fig. 105 illustrates the degree of tumor growth of individual experimental animals when mGI101, Docetaxel, and an anti-PD-L1 antibody are administered in combination in mice transplanted with rodent-derived breast cancer cells (4T1).

Fig. 106 is a schematic diagram of an experimental schedule for the administration of

mGI101 and Paclitaxel in combination in mice transplanted with rodent-derived breast cancer cells (EMT6).

Fig. 107 illustrates a graph of tumor growth when mGI101 and Paclitaxel are administered in combination in mice transplanted with rodent-derived breast cancer cells (EMT6).

Fig. 108 illustrates a tumor growth inhibition rate when mGI101 and Paclitaxel are administered in combination in mice transplanted with rodent-derived breast cancer cells (EMT6).

Fig. 109 illustrates the degree of tumor growth of individual experimental animals when mGI101 and Paclitaxel are administered in combination in mice transplanted with rodent-derived breast cancer cells (EMT6).

Fig. 110 is an experimental schedule for identifying an anticancer effect after mGI101, Cisplatin, Pemetrexed, and an anti-PD-1 antibody are administered in combination in mice transplanted with rodent-derived lung cancer cells (TC1). In addition, a schematic diagram of an experimental schedule for identifying an effect of maintenance therapy by mGI101 is also shown in the above figure.

Fig. 111 illustrates a graph of tumor growth when mGI101, Cisplatin, Pemetrexed, and an anti-PD-1 antibody are administered in combination and maintenance therapy is performed in mice transplanted with rodent-derived lung cancer cells (TC1).

Fig. 112 illustrates the degree of tumor growth of individual experimental animals when mGI101, Cisplatin, Pemetrexed, and an anti-PD-1 antibody are administered in combination and maintenance therapy is performed in mice transplanted with rodent-derived lung cancer cells (TC1).

Figs. 113 to 117 illustrate the degree of tumor growth of individual experimental animals for each experimental group when mGI101, Cisplatin, Pemetrexed, and an anti-PD-1 antibody are administered in combination and maintenance therapy is performed in mice transplanted with rodent-derived lung cancer cells (TC1).

Fig. 118 illustrates a survival rate of mice after mGI101, Cisplatin, Pemetrexed, and an anti-PD-1 antibody are administered in combination and maintenance therapy is performed in mice transplanted with rodent-derived lung cancer cells (TC1).

Fig. 119 illustrates a graph of tumor growth when GI101 and Trastuzumab are administered in combination in mice transplanted with human-derived breast cancer cells (BT-474).

Fig. 120 illustrates a tumor growth inhibition rate when GI101 and Trastuzumab are administered in combination in mice transplanted with human-derived breast cancer cells (BT-474).

Fig. 121 illustrates the degree of tumor growth of individual experimental animals when GI101 and Trastuzumab are administered in combination in mice transplanted with human-derived

breast cancer cells (BT-474).

Fig. 122 illustrates the effect of killing cancer cell line depending on the concentrations of GI101 when GI101, Pertuzumab, a Her2 inhibitor, and a combination thereof are administered in human colorectal cancer cell line (HCT116).

Fig. 123 illustrates a graph of tumor growth when mGI101 and Abemaciclib, a CDK4/6 inhibitor, are administered in combination in mice transplanted with rodent-derived breast cancer cells (4T1).

Fig. 124 illustrates a tumor growth inhibition rate when mGI101 and Abemaciclib, a CDK4/6 inhibitor, are administered in combination in mice transplanted with rodent-derived breast cancer cells (4T1).

Fig. 125 illustrates the degree of tumor growth of individual experimental animals when mGI101 and Abemaciclib, a CDK4/6 inhibitor, are administered in combination in mice transplanted with rodent-derived breast cancer cells (4T1).

Fig. 126 illustrates the effect of killing cancer cell line when GI101, Ribociclib, a CDK4/6 inhibitor, and a combination thereof are administered in human-derived breast cancer cell line cells (MDA-MB-231).

Fig. 127 illustrates a schematic diagram of an experimental schedule for the administration of mGI101 and DMXAA, a STING agonist, in combination in mice transplanted with rodent-derived colorectal cancer cells (MC38).

Fig. 128 illustrates a survival rate of mice after mGI101 and DMXAA, a STING agonist, are administered in combination in mice transplanted with rodent-derived colorectal cancer cells (MC38).

Fig. 129 illustrates the degree of tumor growth of individual experimental animals after mGI101 and DMXAA, a STING agonist, are administered in combination in mice transplanted with rodent-derived colorectal cancer cells (MC38).

Figs. 130 to 133 illustrate the degree of tumor growth of individual experimental animals for each experimental group after mGI101 and DMXAA, a STING agonist, are administered in combination in mice transplanted with rodent-derived colorectal cancer cells (MC38).

Best Mode for Carrying out the Invention

Combination therapy of fusion protein

In an aspect of the present invention, there is provided a pharmaceutical composition for preventing or treating cancer, comprising, as active ingredients, a fusion protein dimer comprising a CD80 protein or a fragment thereof, and an IL-2 protein or a variant thereof; and an anticancer agent.

Since the fusion protein dimer comprising an IL-2 protein and a CD80 protein increases

the immune activity in the body, it may be used in combination with various anticancer treatment methods that have been conventionally used. Specifically, the conventional treatment method that can be used in combination may be selected from the group consisting of an anticancer chemotherapeutic agent for chemotherapy, a target anticancer agent, an anticancer virus, an antibody therapeutic agent, a cell therapeutic agent, an immune checkpoint inhibitor, and a combination thereof.

As used herein, the term "anticancer chemotherapeutic agent" is also referred to as an antineoplastic agent or a cytotoxic agent. It is a generic term for drugs that exhibit anticancer activity mainly by acting directly on DNA to block DNA replication, transcription and translation processes, or by interfering with the synthesis of nucleic acid precursors in the metabolic pathway, and by inhibiting cell division. The antineoplastic agent exhibits cytotoxicity by acting not only on tumor cells but also on normal cells. The anticancer chemotherapeutic agent may be used in maintenance therapy. In addition, as used herein, the term "maintenance therapy" refers to treatment of cancer with drugs after initial anticancer treatment, and refers to a treatment method performed to prevent or delay recurrence of cancer.

Specifically, an anticancer chemotherapeutic agent may be any one selected from the group consisting of an alkylating agent, a microtubule inhibitor, an anti-metabolite, and a topoisomerase inhibitor. The alkylating agent may be any one selected from the group consisting of Mechlorethamine, Cyclophosphamide, Ifosfamide, Melphalan, Chlorambucil, Thiotepe, Altretamine, Procarbazine, Busulfan, Streptozocin, Carmustine, Lomustine, Dacarbazine, Cisplatin, Carboplatin, and Oxaliplatin. The microtubule inhibitor may be any one selected from the group consisting of Docetaxel, Velban, Oncovin, and Navelbine. The anti-metabolite may be any one selected from the group consisting of Fluorouracil, Capecitabine, Cytarabine, Gemcitabine, Fludarabine, Methotrexate, Pemetrexed, and Mercaptopurine. The topoisomerase inhibitor may be any one selected from the group consisting of Hycamtin, Camptosar, Vepesid, Paclitaxel, Blenoxane, Adriamycin, and Cerubidine.

As used herein, the term "target anticancer agent" is a therapeutic agent that specifically kills cancer cells by blocking signals involved in the growth and development of cancer by targeting specific proteins or specific genetic changes that are frequently present only in cancer cells. It is classified into monoclonal antibodies that react outside the cell, and small molecule substances that act inside the cell. Monoclonal antibodies are anticancer agents that block cancer cell induction signals transmitted to the outside of cells, and act on initiation signals related to proliferation, death and the like; and small molecule substances act on complex signal transduction occurring inside the cells.

Specifically, proteins to be targeted may be EGFR, VEGFR, CD20, CD38, RAK-L, BTK, Bcr-abl, PDGFR/FGFR family, MEK/RAF, HER2/Neu, Ubiquitin, JAK, ALK, PARP,

TGF β RI, Proteasome, Bcl-2, C-Met, VR1, VR2, VR3, c-kit, AXL, RET, Braf, DNMT, CDK4/6, STING, and the like.

The target anticancer agent may be any one selected from the group consisting of Cetuximab, Trastuzumab, Pertuzumab, Axitinib, Lenvatinib, Bevacizumab, Ramucirumab, Aflibercept, Rituximab, Obinutuzumab, Daratumumab, Denosumab, Ibrutinib, Dasatinib, Nilotinib, Imatinib, Bosutinib, Galunisertib, Vactosertib, Nintedanib, Sunitinib, Sorafenib, Cabozantinib, Regorafenib, Masitinib, Semaxanib, Tivozanib, Vandetanib, Pazopanib, Trametinib, Dabrafenib, Trastuzumab, Afatinib, Lapatinib, Neratinib, Lenalidomide, Ixazomib, Ruxolitinib, Lestaurtinib, Pacritinib, Cobimethinib, Selumetinib, Trametinib, Binimetinib, Alectinib, Crizotinib, Venetoclax, Crizotinib, Cabozantinib, Bemcentinib, Gilteritinib, Selpercatinib, Pralsetinib, Vemurafenib, Olaparib, Talazoparib, Niraparib, Rucaparib, Azacitidine, Decitabine, Guadecitabine, Abemaciclib, Ribociclib, Palbociclib, CDNs, SB11285, and DMXAA.

As used herein, the term "epidermal growth factor receptor (EGFR)" is a cell membrane receptor that regulates cell growth, division, survival, and death. In various cancers, the expression of EGFR is increased in tumor tissues. It is known that tumor tissues with the increased EGFR are invasive, metastatic, and highly resistant to anticancer agents. The EGFR inhibitor may be a substance that inhibits the EGFR. In an embodiment, it may be Cetuximab, Trastuzumab, Pertuzumab, Gefitinib, Elotinib, or Panitumumab.

As used herein, the term "vascular endothelial growth factor receptor (VEGFR)" is a cell membrane receptor of a vascular endothelial growth factor that induces angiogenesis, and a VEGFR inhibitor inhibits the angiogenesis to suppress tumor growth and metastasis. In an embodiment, the VEGFR inhibitor may be Axitinib, Lenvatinib, Bevacizumab, Ramucirumab, or Aflibercept.

As used herein, the term "CD20 (B lymphocyte antigen CD20)" is a protein expressed on the surface of B cells and is used as a target protein for the treatment of B cell lymphoma. The CD20 target inhibitor may be Rituximab or Obinutuzumab.

As used herein, the term "CD38 (cluster of differentiation 38)" is a protein that regulates cell proliferation and death while acting as a signal transduction receptor in immune cells, and an inhibitor targeting it may be Daratumumab.

As used herein, the term "RANK-L (Receptor activator of nuclear factor kappa-B ligand)" is a RANK receptor expressed on the surface of osteoclasts, and when it is activated by binding to its ligand, it acts to cause bone destruction. The RANK-L inhibitor is mainly used for cancer patients suffering from bone metastasis or osteoporosis, and it may be specifically Denosumab.

As used herein, the term "BTK (Bruton's tyrosine kinase)" is an enzyme involved in the proliferation of B cells and may develop into hematologic malignancy when overexpressed. In an embodiment, the BTK target inhibitor may be Ibrutinib.

As used herein, the term "Bcr-abl" is a fusion protein that is highly expressed in chronic myelogenous leukemia patients, and is known to induce abnormal proliferation of blood cells. Specifically, the inhibitor of the protein may be Dasatinib, Nilotinib, Imatinib, or Bosutinib.

As used herein, the term "tumor growth factor β receptor (TGF β R)" is a cell membrane receptor of a tumor growth factor, and regulates the growth, migration, differentiation, death and the like of epithelial cells and hematopoietic cells. The TGF β R target inhibitor includes, but is not limited to, Galunisertib, Vactosertib or the like.

As used herein, the term "PDGFR (platelet derived growth factor receptor)" is a cell membrane receptor of PDGF that is frequently expressed in cancer cells, and is known to regulate cancer growth, metastasis, and drug resistance by participating in angiogenesis. FGFR (Fibroblast growth factor receptor) is a receptor of fibroblast growth factor (FGF), and regulates various biological processes including cell growth, differentiation, migration, and the like. The FGFR gene is easily mutated, and these variants are commonly observed in breast cancer, uterine cancer, ovarian cancer, cervical cancer, and the like. The Inhibitor targeting PDGFR or FGFR may be Nintedanib, Sunitinib, Sorafenib, Cabozantinib, Lenvatinib, Regorafenib, Masitinib, Semaxanib, Tivozanib, Vandetanib, Axitinib, or Pazopanib.

As used herein, the term "MEK/RAF" is an intracellular signaling mediator involved in cell proliferation, cell cycle regulation, cell survival, angiogenesis, cell migration, and the like, and is overactivated in cancer cells. The inhibitor targeting MEK/RAF may be Trametinib or Dabrafenib.

As used herein, the term "HER-2/neu (human epidermal growth factor receptor 2)" regulates cell proliferation through activation of PI3K/AkT. It is known that it is overexpressed in metastatic breast cancer, and ovarian cancer and the like, and induces resistance against anticancer agents. The Her2/neu target anticancer agent may be Trastuzumab, Afatinib, Lapatinib, or Neratinib.

As used herein, the term "ubiquitin" maintains cell homeostasis by binding to other proteins and inducing proteolysis (ubiquitin-proteasome system, UPS) by proteasome, which is a proteolytic enzyme. Abnormal expression or activity of the UPS is observed in various tumors, and its inhibitor exhibits anticancer activity. Specifically, the inhibitor targeting ubiquitin or proteasome may be Lenalidomide or Ixazomib.

As used herein, the term "JAK (Janus kinase)" is an upstream protein of STAT, which is a transcription factor that regulates cell proliferation, cell survival, cell migration, and immune response. A JAK inhibitor is known to decrease cell proliferation and induce cell death by inhibiting the activity of STAT. The JAK target inhibitor may be Ruxolitinib, Lestaurtinib, or Pacritinib.

As used herein, the term "MAP2K (Mitogen-activated protein kinase kinase)" is an

intracellular signaling mediator involved in cell proliferation, cell cycle regulation, cell survival, angiogenesis, cell migration and the like by phosphorylating MAPK, and it is overactivated in cancer cells. The MAP2K target inhibitor may be Cobimetinib, Selumetinib, Trametinib, or Binimetinib.

As used herein, the term "ALK (Anaplastic lymphoma kinase)" is a signaling mediator that promotes cell proliferation, cell migration and angiogenesis and inhibits cell death; and it is overactivated in various cancer tissues. The ALK target inhibitor may be Alectinib or Crizotinib.

As used herein, the term "Bcl-2" is a protein that inhibits cell death, and it is overexpressed or overactivated in various cancer tissues. The inhibitor targeting Bcl-2 may be Venetoclax.

As used herein, the term "C-Met" is a receptor of hepatocyte growth factor (HGF), and activates signal transduction related to cell growth, formation, motility, survival, angiogenesis and the like. The C-Met target anticancer agent may be Crizotinib or Cabozantinib.

As used herein, the term "VR (vanilloid receptor)" is also known as TRPV (Transient receptor potential vanilloid), and exists in the form of VR1, VR2, VR3, VR4, VR5, and VR6. VR is known to regulate proliferation, death, migration, infiltration and angiogenesis of cancer cells at each stage in the process of cancer progression.

As used herein, the term "c-kit" is also known as CD117, and induces signal transduction that activates cell survival, proliferation and differentiation. c-kit is a proto-oncogene, and overexpression or mutation of its gene is related to the onset of cancer.

As used herein, the term "AXL (tyrosine-protein kinase receptor UFO)" is a tyrosine kinase receptor present on the cell surface, and mediates signal transduction involved in cell proliferation and survival. It is known to be involved in anticancer agent resistance in anticancer treatment. In an embodiment, the AXL target anticancer agent may be Bemcentinib or Gilteritinib.

As used herein, the term "RET (REarranged during transfection)" is a receptor that mediates signals involved in cell proliferation, cell death, and survival; and mutations in RET are known to be involved in cancer development. The RET target inhibitor may be Selpercatinib or Pralsetinib, but is not limited thereto.

As used herein, the term "Braf" is a MAPK signaling mediator involved in cell proliferation, cell cycle regulation, cell survival, angiogenesis, cell migration, and the like, and genetic mutations are observed in cancer cells. The inhibitor targeting Braf may be Vemurafenib.

As used herein, the term "PARP (Poly[ADP-ribose]polymerase)" is a protein that recognizes damaged DNA in the nucleus and is activated, and then activates a DNA repair-related protein. The PARP target inhibitor suppresses proliferation of cancer cells by inhibiting DNA repair of cancer cells. In an embodiment, the PARP target inhibitor may be Olaparib, Talazoparib, Niraparib, or Rucaparib.

As used herein, the term "DNA methyltransferase (DNMT)" is an enzyme that transfers a methyl group to DNA, and expression of a gene is inhibited through the above process. The DNMT target inhibitor exhibits anticancer activity by inhibiting hypermethylation of the cancer suppressor gene and inducing normal expression of the cancer suppressor gene. In an embodiment, the DNMT target inhibitor may be Azacitidine, Decitabine, or Guadecitabine.

As used herein, the term "CDK (cyclin dependent kinase) 4/6" is a protein that regulates the cell cycle and promotes cell growth, and is overactivated in the development and progression stages of various malignant tumors. The CDK4/6 target inhibitor exhibits anticancer activity by inhibiting cell cycle of cancer cells, inhibiting cell proliferation, and inducing cell death. The CDK4/6 target inhibitor may be Abemaciclib or Palbociclib.

As used herein, the term "STING (Stimulator of Interferon Genes)" is an *in vivo* sensor that recognizes DNA fragments derived from cancer cells, and activates immune cells in the body such as dendritic cells by stimulating interferon genes. The STING agonist exhibits an immune enhancing effect and a cancer angiogenesis inhibitory effect. For example, the STING agonist may be CDNs, SB11285, DMXAA, or the like.

As used herein, the term "anticancer virus therapeutic agent" is a therapeutic agent that kills cancer by inserting a specific gene targeting cancer cells into a virus that is capable of proliferation and has infectivity. The anticancer virus therapeutic agent may be Talimogenem or Laherparepvec.

As used herein, the term "antibody therapeutic agent" is a therapeutic agent that exhibits anticancer effect by using an antibody that recognizes a specific protein of cancer cells as an antigen. The antibody therapeutic agent may be Trastuzumab, Emtansine, Emtansine, Rituximab, Ibritumomab, Tositumomab, Brentuximab, Ofatumumab, Obinutuzumab, Necitumumab, Bevacizumab, Ramucirumab, Nivolumab, Pembrolizumab, Atezolizumab, Durvalumab, Ipilimumab, or the like.

As used herein, the term "immune cell therapeutic agent" is a therapeutic agent that exhibits anticancer effect by activating an immune response in the body using immune cells such as dendritic cells, natural killer cells, and T cells. The immune cell therapeutic agent is used after extracting and potentiating immune cells in the body or genetically engineering them to be reinjected into the body. The representative immune cell therapeutic agent includes T cell receptor-modified T cells (TCR-T), chimeric antigen receptor-modified T cells (CAR-T), and the like. Specifically, it may be Tisagenlecleucel or Axicabtagene Ciloleucel, but is not limited thereto.

As used herein, the term "immune checkpoint inhibitor" is a substance that inhibits the activity of an immune checkpoint protein that inhibits differentiation, proliferation, and activity of immune cells, and it is known to eliminate cancer cells by preventing them from exerting the

function of evading the immune system. The immune checkpoint inhibitor may be any one selected from the group consisting of an anti-CTLA-4 antibody, an anti-PD-1 antibody, an anti-PD-L1 antibody, an anti-PD-L2 antibody, an anti-B7-H4 antibody, an anti-HVEM antibody, an anti-TIM3 antibody, an anti-GAL9 antibody, an anti-LAG3 antibody, an anti-VISTA antibody, an anti-KIR antibody, an anti-BTLA antibody, and an anti-TIGIT antibody. In an embodiment, the immune checkpoint inhibitor may be Ipilimumab, Pembrolizumab, Nivolumab, Cemiplimab, Atezolizumab, Avelumab, Duralumab and the like, but is not limited thereto.

As used herein, the term "ADC (antibody drug conjugate)" is a therapeutic agent that chemically binds an antibody and a cytotoxic drug to exhibit high anticancer effect through target delivery. It may be Gemtuzumab-Ozogamicin, Brentuximab-Vedotin, Trastuzumab-Emtansine, Inotuzumab-Ozogamicin, Eribulin-Mesylate, and the like.

The fusion protein dimer comprising an IL-2 protein and a CD80 protein may be used in combination with an anticancer vaccine or the like.

In addition, an anticancer agent may be used not only in combination with the anticancer agent described above, but also in combination with an anticancer vaccine or the like.

Preferably, the anticancer agent may be any one selected from the group consisting of Cisplatin, Oxaliplatin, ALTIMA, Axitinib (VR1,2,3, PDGFR, c-kit), Galunisertib (TGF β RI), Lenvatinib (VR1,2,3), Ramucirumab (VR2), Cabozatinib (c-Met, VR2, AXL, RET), Olaparib (PARP), Guadecitabine (DNMT), Docetaxel, Paclitaxel, Pemetrexed, Vemurafenib (Braf), Abemaciclib (CDK4/6), Cetuximab (EGFR), Durvalumab (PD-L1), Trastuzumab (Her2), DMXAA, NK cell, T cell, and Keytruda (PD-1).

In addition, the anticancer agent may include one or more anticancer agents. Specifically, the fusion protein dimer may be used commonly together with two anticancer agents. As an example, it may be an anticancer chemotherapeutic agent and a target anticancer agent; an anticancer chemotherapeutic agent and an anticancer virus; a target anticancer agent and an antibody therapeutic agent; an anticancer chemotherapeutic agent and a cell therapeutic agent; and an anticancer chemotherapeutic agent and an immune checkpoint inhibitor. In addition, it may be a target anticancer agent and an anticancer virus; a target anticancer agent and an antibody therapeutic agent; a target anticancer agent and a cell therapeutic agent; a target anticancer agent and an immune checkpoint inhibitor. In addition, it may be an anticancer virus and an antibody therapeutic agent; an anticancer virus and a cell therapeutic agent; and an anticancer virus and an immune checkpoint inhibitor. In addition, it may be an antibody therapeutic agent and a cell therapeutic agent; and an antibody therapeutic agent and an immune checkpoint inhibitor.

In addition, the fusion protein dimer may be used together with three anticancer agents. In addition to the two anticancer agents, a different anticancer agent may be further included and used.

In an embodiment, the anticancer agent may be an anticancer chemotherapeutic agent and a target anticancer agent; an anticancer chemotherapeutic agent and an immune checkpoint inhibitor; or an anticancer chemotherapeutic agent, a target anticancer agent and an immune checkpoint inhibitor.

Use of fusion protein dimer in anticancer maintenance therapy

In another aspect of the present invention, there is provided a composition for anticancer maintenance therapy, comprising, as an active ingredient, a fusion protein dimer comprising a CD80 protein or a fragment thereof and an IL-2 protein or a variant thereof.

As described above, "maintenance therapy" refers to treating cancer after initial anticancer treatment. In particular, it is a treatment method that increases the effect of cancer treatment by preventing or delaying the recurrence of cancer.

Here, it may further include at least one anticancer agent for maintenance therapy. Here, the anticancer agent is as described above.

Kit comprising fusion protein dimer

In another aspect of the present invention, there is provided a kit for preventing or treating cancer, comprising, as active ingredients, a fusion protein dimer comprising a CD80 protein or a fragment thereof and an IL-2 protein or a variant thereof, and an anticancer agent.

In another aspect of the present invention, there is provided a kit for anticancer maintenance therapy, comprising, as active ingredients, a fusion protein dimer comprising a CD80 protein or a fragment thereof and an IL-2 protein or a variant thereof, and an anticancer agent.

Fusion protein comprising IL-2 protein and CD80 protein

As used herein, the term "IL-2" or "interleukin-2", unless otherwise stated, refers to any wild-type IL-2 obtained from any vertebrate source, including mammals, for example, primates (such as humans) and rodents (such as mice and rats). IL-2 may be obtained from animal cells, and also includes one obtained from recombinant cells capable of producing IL-2. In addition, IL-2 may be wild-type IL-2 or a variant thereof.

In the present specification, IL-2 or a variant thereof may be collectively expressed by the term "IL-2 protein" or "IL-2 polypeptide." IL-2, an IL-2 protein, an IL-2 polypeptide, and an IL-2 variant specifically bind to, for example, an IL-2 receptor. This specific binding may be identified by methods known to those skilled in the art.

An embodiment of IL-2 may have the amino acid sequence of SEQ ID NO: 35 or SEQ ID NO: 36. Here, IL-2 may also be in a mature form. Specifically, the mature IL-2 may not contain a signal sequence, and may have the amino acid sequence of SEQ ID NO: 10. Here, IL-2 may be used under a concept encompassing a fragment of wild-type IL-2 in which a portion of N-terminus or C-terminus of the wild-type IL-2 is truncated.

In addition, the fragment of IL-2 may be in a form in which 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11,

12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 contiguous amino acids are truncated from N-terminus of a protein having the amino acid sequence of SEQ ID NO: 35 or SEQ ID NO: 36. In addition, the fragment of IL-2 may be in a form in which 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 contiguous amino acids are truncated from C-terminus of a protein having the amino acid sequence of SEQ ID NO: 35 or SEQ ID NO: 36.

As used herein, the term “IL-2 variant” refers to a form in which a portion of amino acids in the full-length IL-2 or the above-described fragment of IL-2 is substituted. That is, an IL-2 variant may have an amino acid sequence different from wild-type IL-2 or a fragment thereof. However, an IL-2 variant may have activity equivalent or similar to the wild-type IL-2. Here, “IL-2 activity” may, for example, refer to specific binding to an IL-2 receptor, which specific binding can be measured by methods known to those skilled in the art.

Specifically, an IL-2 variant may be obtained by substitution of a portion of amino acids in the wild-type IL-2. An embodiment of the IL-2 variant obtained by amino acid substitution may be obtained by substitution of at least one of the 38th, 42nd, 45th, 61st, and 72nd amino acids in the amino acid sequence of SEQ ID NO: 10.

Specifically, the IL-2 variant may be obtained by substitution of at least one of the 38th, 42nd, 45th, 61st, or 72nd amino acid in the amino acid sequence of SEQ ID NO: 10 with another amino acid. In addition, when IL-2 is in a form in which a portion of N-terminus in the amino acid sequence of SEQ ID NO: 35 is truncated, the amino acid at a position complementarily corresponding to that in the amino acid sequence of SEQ ID NO: 10 may be substituted with another amino acid. For example, when IL-2 has the amino acid sequence of SEQ ID NO: 35, its IL-2 variant may be obtained by substitution of at least one of 58th, 62nd, 65th, 81st, or 92nd amino acid in the amino acid sequence of SEQ ID NO: 35 with another amino acid. These amino acid residues correspond to the 38th, 42nd, 45th, 61st, and 72nd amino acid residues in the amino acid sequence of SEQ ID NO: 10, respectively. According to an embodiment, one, two, three, four, five, six, seven, eight, nine, or ten amino acids may be substituted as long as such IL-2 variant maintains IL-2 activity. According to another embodiment, one to five amino acids may be substituted.

In an embodiment, an IL-2 variant may be in a form in which two amino acids are substituted. Specifically, the IL-2 variant may be obtained by substitution of the 38th and 42nd amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of the 38th and 45th amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of the 38th and 61st amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of the 38th and 72nd amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the

IL-2 variant may be obtained by substitution of the 42nd and 45th amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of the 42nd and 61st amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of the 42nd and 72nd amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of the 45th and 61st amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of the 45th and 72nd amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of the 61st and 72nd amino acids in the amino acid sequence of SEQ ID NO: 10.

Furthermore, an IL-2 variant may be in a form in which three amino acids are substituted. Specifically, the IL-2 variant may be obtained by substitution of the 38th, 42nd, and 45th amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of the 38th, 42nd, and 61st amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of the 38th, 42nd, and 72nd amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of the 38th, 45th, and 61st amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of the 38th, 45th, and 72nd amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of the 38th, 61st, and 72nd amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of the 42nd, 45th, and 61st amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of the 42nd, 45th, and 72nd amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of the 45th, 61st, and 72nd amino acids in the amino acid sequence of SEQ ID NO: 10.

In addition, an IL-2 variant may be in a form in which four amino acids are substituted. Specifically, the IL-2 variant may be obtained by substitution of the 38th, 42nd, 45th, and 61st amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of the 38th, 42nd, 45th, and 72nd amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of the 38th, 45th, 61st, and 72nd amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of the 38th, 42nd, 61st, and 72nd amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of 42nd, 45th, 61st,

and 72nd amino acids in the amino acid sequence of SEQ ID NO: 10.

Furthermore, an IL-2 variant may be in a form in which five amino acids are substituted. Specifically, the IL-2 variant may be obtained by substitution of each of the 38th, 42nd, 45th, 61st, and 72nd amino acids in the amino acid sequence of SEQ ID NO: 10 with another amino acid.

Here, the “another amino acid” introduced by the substitution may be any one selected from the group consisting of alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. However, regarding amino acid substitution for the IL-2 variant, in the amino acid sequence of SEQ ID NO: 10, the 38th amino acid cannot be substituted with arginine, the 42nd amino acid cannot be substituted with phenylalanine, the 45th amino acid cannot be substituted with tyrosine, the 61st amino acid cannot be substituted with glutamic acid, and the 72nd amino acid cannot be substituted with leucine.

Regarding amino acid substitution for an IL-2 variant, in the amino acid sequence of SEQ ID NO: 10, the 38th amino acid, arginine, may be substituted with an amino acid other than arginine. Preferably, regarding amino acid substitution for an IL-2 variant, in the amino acid sequence of SEQ ID NO: 10, the 38th amino acid, arginine, may be substituted with alanine (R38A).

Regarding amino acid substitution for an IL-2 variant, in the amino acid sequence of SEQ ID NO: 10, the 42nd amino acid, phenylalanine, may be substituted with an amino acid other than phenylalanine. Preferably, regarding amino acid substitution for an IL-2 variant, in the amino acid sequence of SEQ ID NO: 10, the 42nd amino acid, phenylalanine, may be substituted with alanine (F42A).

Regarding amino acid substitution for an IL-2 variant, in the amino acid sequence of SEQ ID NO: 10, the 45th amino acid, tyrosine, may be substituted with an amino acid other than tyrosine. Preferably, regarding amino acid substitution for an IL-2 variant, in the amino acid sequence of SEQ ID NO: 10, the 45th amino acid, tyrosine, may be substituted with alanine (Y45A).

Regarding amino acid substitution for an IL-2 variant, in the amino acid sequence of SEQ ID NO: 10, the 61st amino acid, glutamic acid, may be substituted with an amino acid other than glutamic acid. Preferably, regarding amino acid substitution for an IL-2 variant, in the amino acid sequence of SEQ ID NO: 10, the 61st amino acid, glutamic acid, may be substituted with arginine (E61R).

Regarding amino acid substitution for an IL-2 variant, in the amino acid sequence of SEQ ID NO: 10, the 72nd amino acid, leucine, may be substituted with an amino acid other than leucine. Preferably, regarding amino acid substitution for an IL-2 variant, in the amino acid sequence of SEQ ID NO: 10, the 72nd amino acid, leucine, may be substituted with glycine (L72G).

Specifically, an IL-2 variant may be obtained by at least one substitution selected from the group consisting of R38A, F42A, Y45A, E61R, and L72G, in the amino acid sequence of SEQ

ID NO: 10.

Specifically, an IL-2 variant may be obtained by amino acid substitutions at two, three, four, or five positions among the positions selected from the group consisting of R38A, F42A, Y45A, E61R, and L72G.

In addition, an IL-2 variant may be in a form in which two amino acids are substituted. Specifically, an IL-2 variant may be obtained by the substitutions, R38A and F42A. In addition, in an embodiment, an IL-2 variant may be obtained by the substitutions, R38A and Y45A. In addition, in an embodiment, an IL-2 variant may be obtained by the substitutions, R38A and E61R. In addition, in an embodiment, an IL-2 variant may be obtained by the substitutions, R38A and L72G. In addition, in an embodiment, an IL-2 variant may be obtained by the substitutions, F42A and Y45A. In addition, in an embodiment, an IL-2 variant may be obtained by the substitutions, F42A and E61R. In addition, in an embodiment, an IL-2 variant may be obtained by the substitutions, F42A and L72G. In addition, in an embodiment, an IL-2 variant may be obtained by the substitutions, E61R and L72G.

Furthermore, an IL-2 variant may be in a form in which three amino acids are substituted. Specifically, an IL-2 variant may be obtained by the substitutions, R38A, F42A, and Y45A. In addition, in an embodiment, an IL-2 variant may be obtained by the substitutions, R38A, F42A, and E61R. In addition, in an embodiment, an IL-2 variant may be obtained by the substitutions, R38A, F42A, and L72G. In addition, in an embodiment, an IL-2 variant may be obtained by the substitutions, R38A, Y45A, and E61R. In addition, in an embodiment, an IL-2 variant may be obtained by the substitutions, R38A, Y45A, and L72G. In addition, in an embodiment, an IL-2 variant may be obtained by the substitutions, F42A, Y45A, and E61R. In addition, in an embodiment, an IL-2 variant may be obtained by the substitutions, F42A, Y45A, and L72G. In addition, in an embodiment, an IL-2 variant may be obtained by the substitutions, F42A, E61R, and L72G. In addition, in an embodiment, an IL-2 variant may be obtained by the substitutions, Y45A, E61R, and L72G.

In addition, an IL-2 variant may be in a form in which four amino acids are substituted. Specifically, an IL-2 variant may be obtained by the substitutions, R38A, F42A, Y45A, and E61R. In addition, in an embodiment, an IL-2 variant may be obtained by the substitutions, R38A, F42A, Y45A, and L72G. In addition, in an embodiment, an IL-2 variant may be obtained by the substitutions, R38A, F42A, E61R, and L72G. In addition, in an embodiment, an IL-2 variant may be obtained by the substitutions, R38A, Y45A, E61R, and L72G. In addition, in an embodiment, an IL-2 variant may be obtained by the substitutions, F42A, Y45A, E61R, and L72G.

Furthermore, an IL-2 variant may be obtained by the substitutions, R38A, F42A, Y45A, E61R, and L72G.

Preferably, an embodiment of the IL-2 variant may contain which are any one selected

from the following substitution combinations (a) to (d) in the amino acid sequence of SEQ ID NO: 10:

- (a) R38A/F42A
- (b) R38A/F42A/Y45A
- (c) R38A/F42A/E61R
- (d) R38A/F42A/L72G

Here, when IL-2 has the amino acid sequence of SEQ ID NO: 35, an amino acid substitution may be present at a position complementarily corresponding to that in the amino acid sequence of SEQ ID NO: 10. In addition, even when IL-2 is a fragment of the amino acid sequence of SEQ ID NO: 35, an amino acid substitution may be present at a position complementarily corresponding to that in the amino acid sequence of SEQ ID NO: 10.

Specifically, an IL-2 variant may have the amino acid sequence of SEQ ID NO: 6, 22, 23, or 24.

In addition, an IL-2 variant may be characterized by having low *in vivo* toxicity. Here, the low *in vivo* toxicity may be a side effect caused by binding of IL-2 to the IL-2 receptor alpha chain (IL-2R α). Various IL-2 variants have been developed to ameliorate the side effect caused by binding of IL-2 to IL-2R α , and such IL-2 variants may be those disclosed in US Patent No. 5,229,109 and Korean Patent No. 1667096. In particular, IL-2 variants described in the present application have low binding ability for the IL-2 receptor alpha chain (IL-2R α) and thus have lower *in vivo* toxicity than the wild-type IL-2.

As used herein, the term “CD80”, also called “B7-1”, is a membrane protein present in dendritic cells, activated B cells, and monocytes. CD80 provides co-stimulatory signals essential for activation and survival of T cells. CD80 is known as a ligand for the two different proteins, CD28 and CTLA-4, present on the surface of T cells. CD80 is composed of 288 amino acids, and may specifically have the amino acid sequence of SEQ ID NO: 11. In addition, as used herein, the term “CD80 protein” refers to the full-length CD80 or a CD80 fragment.

As used herein, the term “CD80 fragment” refers to a cleaved form of CD80. In addition, the CD80 fragment may be an extracellular domain of CD80. An embodiment of the CD80 fragment may be obtained by elimination of the 1st to 34th amino acids from N-terminus which are a signal sequence of CD80. Specifically, an embodiment of the CD80 fragment may be a protein composed of the 35th to 288th amino acids in SEQ ID NO: 11. In addition, an embodiment of the CD80 fragment may be a protein composed of the 35th to 242nd amino acids in SEQ ID NO: 11. In addition, an embodiment of the CD80 fragment may be a protein composed of the 35th to 232nd amino acids in SEQ ID NO: 11. In addition, an embodiment of the CD80 fragment may be a protein composed of the 35th to 139th amino acids in SEQ ID NO: 11. In addition, an embodiment of the CD80 fragment may be a protein composed of the 142nd to 242nd amino acids in SEQ ID

NO: 11. In an embodiment, a CD80 fragment may have the amino acid sequence of SEQ ID NO: 2.

In addition, the IL-2 protein and the CD80 protein may be attached to each other via a linker or a carrier. Specifically, the IL-2 or a variant thereof and the CD80 (B7-1) or a fragment thereof may be attached to each other via a linker or a carrier. In the present description, the linker and the carrier may be used interchangeably.

The linker links two proteins. An embodiment of the linker may include 1 to 50 amino acids, albumin or a fragment thereof, an Fc domain of an immunoglobulin, or the like. Here, the Fc domain of immunoglobulin refers to a protein that contains heavy chain constant region 2 (CH2) and heavy chain constant region 3 (CH3) of an immunoglobulin, and does not contain heavy and light chain variable regions and light chain constant region 1 (CH1) of an immunoglobulin. The immunoglobulin may be IgG, IgA, IgE, IgD, or IgM, and may preferably be IgG4. Here, Fc domain of wild-type immunoglobulin G4 may have the amino acid sequence of SEQ ID NO: 4.

In addition, the Fc domain of an immunoglobulin may be an Fc domain variant as well as wild-type Fc domain. In addition, as used herein, the term “Fc domain variant” may refer to a form which is different from the wild-type Fc domain in terms of glycosylation pattern, has a high glycosylation as compared with the wild-type Fc domain, or has a low glycosylation as compared with the wild-type Fc domain, or a deglycosylated form. In addition, an aglycosylated Fc domain is included therein. The Fc domain or a variant thereof may be adapted to have an adjusted number of sialic acids, fucosylations, or glycosylations, through culture conditions or genetic manipulation of a host.

In addition, glycosylation of the Fc domain of an immunoglobulin may be modified by conventional methods such as chemical methods, enzymatic methods, and genetic engineering methods using microorganisms. In addition, the Fc domain variant may be in a mixed form of respective Fc regions of immunoglobulins, IgG, IgA, IgE, IgD, and IgM. In addition, the Fc domain variant may be in a form in which some amino acids of the Fc domain are substituted with other amino acids. An embodiment of the Fc domain variant may have the amino acid sequence of SEQ ID NO: 12.

The fusion protein may have a structure in which, using an Fc domain as a linker (or carrier), a CD80 protein and an IL-2 protein, or an IL-2 protein and a CD80 protein are linked to N-terminus and C-terminus of the linker or carrier, respectively. Linkage between N-terminus or C-terminus of the Fc domain and CD-80 or IL-2 may optionally be achieved by a linker peptide.

Specifically, a fusion protein may consist of the following structural formula (I) or (II):

N'-X-[linker (1)]_n-Fc domain-[linker (2)]_m-Y-C' (I)

N'-Y-[linker (1)]_n-Fc domain-[linker (2)]_m-X-C' (II)

Here, in the structural formulas (I) and (II),

N' is the N-terminus of the fusion protein,
C' is the C-terminus of the fusion protein,
X is a CD80 protein,
Y is an IL-2 protein,
the linkers (1) and (2) are peptide linkers, and
n and m are each independently 0 or 1.

Preferably, the fusion protein may consist of the structural formula (I). The IL-2 protein is as described above. In addition, the CD80 protein is as described above. According to an embodiment, the IL-2 protein may be an IL-2 variant with one to five amino acid substitutions as compared with the wild-type IL-2. The CD80 protein may be a fragment obtained by truncation of up to about 34 contiguous amino acid residues from the N-terminus or C-terminus of the wild-type CD80. Alternatively, the CD protein may be an extracellular immunoglobulin-like domain having the activity of binding to the T cell surface receptors CTLA-4 and CD28.

Specifically, the fusion protein may have the amino acid sequence of SEQ ID NO: 9, 26, 28, or 30. According to another embodiment, the fusion protein includes a polypeptide having a sequence identity of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% to the amino acid sequence of SEQ ID NO: 9, 26, 28, or 30. Here, the identity is, for example, percent homology, and may be determined through homology comparison software such as BlastN software of the National Center of Biotechnology Information (NCBI).

The peptide linker (1) may be included between the CD80 protein and the Fc domain. The peptide linker (1) may consist of 5 to 80 contiguous amino acids, 20 to 60 contiguous amino acids, 25 to 50 contiguous amino acids, or 30 to 40 contiguous amino acids. In an embodiment, the peptide linker (1) may consist of 30 amino acids. In addition, the peptide linker (1) may contain at least one cysteine. Specifically, the peptide linker (1) may contain one, two, or three cysteines. In addition, the peptide linker (1) may be derived from the hinge of an immunoglobulin. In an embodiment, the peptide linker (1) may be a peptide linker consisting of the amino acid sequence of SEQ ID NO: 3.

The peptide linker (2) may consist of 1 to 50 contiguous amino acids, 3 to 30 contiguous amino acids, or 5 to 15 contiguous amino acids. In an embodiment, the peptide linker (2) may be (G4S)_n (where n is an integer of 1 to 10). Here, in (G4S)_n, n may be 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10. In an embodiment, the peptide linker (2) may be a peptide linker consisting of the amino acid sequence of SEQ ID NO: 5.

In another aspect of the present invention, there is provided a dimer obtained by binding of two fusion proteins, each of which comprises an IL-2 protein and a CD80 protein. The fusion protein comprising IL-2 or a variant thereof and CD80 or a fragment thereof is as described above.

Here, the binding between the fusion proteins constituting the dimer may be achieved by,

but is not limited to, a disulfide bond formed by cysteines present in the linker. The fusion proteins constituting the dimer may be the same or different fusion proteins from each other. Preferably, the dimer may be a homodimer. An embodiment of the fusion protein constituting the dimer may be a protein having the amino acid sequence of SEQ ID NO: 9.

Pharmaceutical use

The pharmaceutical composition for treating or preventing cancer of the present invention, the composition comprising, as an active ingredient, a fusion protein comprising an IL-2 protein and a CD80 protein, and an anticancer agent may enhance efficacy for treating and/or preventing cancer.

The fusion protein comprising an IL-2 protein and a CD80 protein, or the fusion protein dimer where the two fusion proteins are attached is as described above.

The cancer may be selected from the group consisting of gastric cancer, liver cancer, lung cancer, colorectal cancer, breast cancer, prostate cancer, ovarian cancer, pancreatic cancer, cervical cancer, thyroid cancer, laryngeal cancer, acute myeloid leukemia, brain tumor, neuroblastoma, retinoblastoma, head and neck cancer, salivary gland cancer, and lymphoma.

A preferred dose of the pharmaceutical composition varies depending on the patient's condition and body weight, severity of disease, form of drug, route and duration of administration and may be appropriately selected by those skilled in the art. In the pharmaceutical composition for treating or preventing cancer of the present invention, the active ingredient may be contained in any amount (effective amount) depending on application, dosage form, blending purpose, and the like, as long as the active ingredient can exhibit anticancer activity. A conventional effective amount thereof will be determined within a range of 0.001 wt% to 20.0 wt% by weight, based on the total weight of the composition. Here, the term “effective amount” refers to an amount of an active ingredient capable of inducing an anticancer effect. Such an effective amount can be experimentally determined within the scope of common knowledge of those skilled in the art.

As used herein, the term “treatment” may be used to mean both therapeutic and prophylactic treatment. Here, prophylaxis may be used to mean that a pathological condition or disease of an individual is alleviated or mitigated. In an embodiment, the term “treatment” includes both application or any form of administration for treating a disease in a mammal, including a human. In addition, the term includes inhibiting or slowing down a disease or disease progression; and includes meanings of restoring or repairing impaired or lost function so that a disease is partially or completely alleviated; stimulating inefficient processes; or alleviating a serious disease.

As used herein, the term “efficacy” refers to capacity that can be determined by one or parameters, for example, survival or disease-free survival over a certain period of time such as one year, five years, or ten years. In addition, the parameter may include inhibition of size of at least

one tumor in an individual.

Pharmacokinetic parameters such as bioavailability and underlying parameters such as clearance rate may also affect efficacy. Thus, “enhanced efficacy” (for example, improvement in efficacy) may be due to enhanced pharmacokinetic parameters and improved efficacy, which may be measured by comparing clearance rate and tumor growth in test animals or human subjects, or by comparing parameters such as survival, recurrence, or disease-free survival.

As used herein, the term “therapeutically effective amount” or “pharmaceutically effective amount” refers to an amount of a compound or composition effective to prevent or treat the disease in question, which is sufficient to treat the disease at a reasonable benefit/risk ratio applicable to medical treatment and does not cause adverse effects. A level of the effective amount may be determined depending on factors including the patient's health condition, type and severity of disease, activity of drug, the patient's sensitivity to drug, mode of administration, time of administration, route of administration and excretion rate, duration of treatment, formulation or simultaneously used drugs, and other factors well known in the medical field. In an embodiment, the therapeutically effective amount means an amount of drug effective to treat cancer.

Here, the pharmaceutical composition may further comprise a pharmaceutically acceptable carrier. The pharmaceutically acceptable carrier may be any carrier as long as the carrier is a non-toxic substance suitable for delivery to a patient. Distilled water, alcohol, fat, wax, and inert solid may be contained as the carrier. A pharmaceutically acceptable adjuvant (buffer, dispersant) may also be contained in the pharmaceutical composition.

Specifically, by including a pharmaceutically acceptable carrier in addition to the active ingredient, the pharmaceutical composition may be prepared into a parenteral formulation depending on its route of administration using conventional methods known in the art. Here, the term “pharmaceutically acceptable” means that the carrier does not have more toxicity than the subject to be applied (prescribed) can adapt while not inhibiting activity of the active ingredient.

When the pharmaceutical composition is prepared into a parenteral formulation, it may be made into preparations in the form of injections, transdermal patches, nasal inhalants, or suppositories with suitable carriers according to methods known in the art. In a case of being made into injections, sterile water, ethanol, polyol such as glycerol or propylene glycol, or a mixture thereof may be used as a suitable carrier; and an isotonic solution, such as Ringer's solution, phosphate buffered saline (PBS) containing triethanol amine or sterile water for injection, and 5% dextrose, or the like may preferably be used. Formulation of pharmaceutical compositions is known in the art, and reference may specifically be made to Remington's Pharmaceutical Sciences (19th ed., 1995) and the like. This document is considered part of the present description.

A preferred dose of the pharmaceutical composition may range from 0.01 $\mu\text{g/kg}$ to 10 g/kg, or 0.01 mg/kg to 1 g/kg, per day, depending on the patient's condition, body weight, sex, age,

severity of the patient, and route of administration. The dose may be administered once a day or may be divided into several times a day. Such a dose should not be construed as limiting the scope of the present invention in any aspect.

Subjects to which the pharmaceutical composition can be applied (prescribed) are mammals and humans, with humans being particularly preferred. In addition to the active ingredient, the pharmaceutical composition of the present application may further contain any compound or natural extract, which has already been validated for safety and is known to have anticancer activity or a therapeutic effect on an infectious disease, so as to boost or reinforce anticancer activity.

Use of composition comprising fusion protein dimer and anticancer agent

In another aspect of the present invention, there is provided a use of a composition for combination administration comprising a fusion protein dimer comprising a CD80 protein or a fragment thereof and an IL-2 protein or a variant thereof, and an anticancer agent for the treatment of cancer disease.

In another aspect of the present invention, there is provided a use of a composition for combination administration comprising a fusion protein dimer comprising an IL-2 protein and a CD80 protein, and an anticancer agent for enhancing the therapeutic effect of cancer disease.

In another aspect of the present invention, there is provided a use of a fusion protein dimer comprising a CD80 protein or a fragment thereof and an IL-2 protein or a variant thereof for maintenance therapy. Here, an anticancer agent may be further included.

In another aspect of the present invention, there is provided a method for treating cancer disease and/or a method for enhancing therapeutic effect, comprising a step of administering, to a subject, a fusion protein comprising an IL-2 protein and a CD80 protein or a fusion protein dimer in which the two fusion proteins are bound to each other, and an anticancer agent.

The subject may be a subject suffering from cancer. In addition, the subject may be a mammal, preferably a human. The fusion protein comprising an IL-2 protein and a CD80 protein or the fusion protein dimer in which the two fusion proteins are bound to each other is as described above.

Route of administration, dose, and frequency of administration of the fusion protein or the fusion protein dimer may vary depending on the patient's condition and the presence or absence of side effects, and thus the fusion protein or the fusion protein dimer may be administered to a subject in various ways and amounts. The optimal administration method, dose, and frequency of administration may be selected in an appropriate range by those skilled in the art. In addition, the fusion protein or the fusion protein dimer may be administered in combination with other drugs (for example, the above described anticancer agent) or physiologically active substances whose therapeutic effect is known with respect to a disease to be treated, or may be formulated in the

form of combination preparations with other drugs.

Due to IL-2 activity, the fusion protein in an embodiment of the present invention can activate immune cells such as natural killer cells. Thus, the fusion protein can be effectively used for cancer disease. In particular, it was identified that as compared with the wild type, an IL-2 variant with two to five amino acid substitutions, in particular, an IL-2 variant that contains amino acid substitutions at two, three, four, or five positions among the positions selected from the group consisting of R38A, F42A, Y45A, E61R, and L72G in the amino acid sequence of SEQ ID NO: 10, has low binding ability for the IL-2 receptor alpha chain and thus exhibits improved characteristics with respect to pharmacological side effects of conventional IL-2. Thus, such an IL-2 variant, when used alone or in the form of a fusion protein, can decrease incidence of vascular (or capillary) leakage syndrome (VLS), which is a conventionally known problem of IL-2.

Pharmaceutical composition comprising, as active ingredients, IL-2 protein or variant thereof, CD80 protein or variant thereof, and anticancer agent

In another aspect of the present invention, there is provided a composition for treating cancer, comprising, as active ingredients, IL-2 or a variant thereof, a CD80 protein or a variant thereof, and an anticancer agent.

Here, the IL-2 or variant thereof is as described above. In addition, the IL-2 or variant thereof may further include an immunoglobulin Fc region. Here, the IL-2 or variant thereof may bind to the N-terminus or C-terminus of the Fc region. In an embodiment, the IL-2 or variant thereof may bind to the C-terminus of the Fc region. In addition, as described above, the variant of IL-2 may be a form in which two amino acids are substituted or a form in which three amino acids are substituted. Here, the IL-2 or variant thereof may be directly bound to the Fc region, but may be bound through a peptide linker. Here, the peptide linker may be any one of the linkers described above.

In addition, the CD80 protein or variant thereof is as described above. In addition, the CD80 or variant thereof may further include an immunoglobulin Fc region. Here, the CD80 or variant thereof may bind to the N-terminus or C-terminus of the Fc region. Here, the CD80 may be in the form of a fragment, and may be a fragment of CD80 including a V domain. In an embodiment, the CD80 or variant may bind to the N-terminus of the Fc region. In addition, the variant of CD80 may be a variant in various forms as long as its activity is maintained.

In addition, the anticancer agent may be any one selected from various types of the anticancer agents described above.

Mode for Carrying out the Invention

Hereinafter, the present invention will be described in more detail by way of the following examples. However, the following examples are only for illustrating the present invention, and the scope of the present invention is not limited thereto.

I. Preparation of fusion protein

Preparation Example 1. Preparation of hCD80-Fc-IL-2 variant (2M): GI101

In order to produce a fusion protein comprising a human CD80 fragment, an Fc domain, and an IL-2 variant, a polynucleotide was synthesized through the Invitrogen GeneArt Gene Synthesis service of ThermoFisher Scientific. Specifically, the polynucleotide contains a nucleotide sequence (SEQ ID NO: 8) which encodes a fusion protein that contains a signal peptide (SEQ ID NO: 1), a CD80 fragment (SEQ ID NO: 2), an Ig hinge (SEQ ID NO: 3), an Fc domain (SEQ ID NO: 4), a linker (SEQ ID NO: 5), and an IL-2 variant (2M) (R38A, F42A) (SEQ ID NO: 6) having two amino acid substitutions, in this order, from the N-terminus. The polynucleotide was inserted into pcDNA3_4 vector. In addition, the vector was introduced into CHO cells (Expi-CHO™) to express the fusion protein of SEQ ID NO: 9. After the vector was introduced, culture was performed for 7 days in an environment of 37°C, 125 rpm, and 8% CO₂ concentration. Then, the culture was harvested and the fusion protein was purified therefrom. The purified fusion protein was designated "GI101".

Purification was carried out using chromatography containing MabSelect SuRe protein A resin. The fusion protein was bound thereto under a condition of 25 mM Tris, 25 mM NaCl, pH 7.4. Then, elution was performed with 100 mM NaCl and 100 mM acetic acid at pH 3. 20% 1 M Tris-HCl at pH 9 was placed in a collection tube, and then the fusion protein was collected. For the collected fusion protein, the buffer was exchanged through dialysis with PBS buffer for 16 hours.

Thereafter, absorbance at 280 nm wavelength was measured, over time, with size exclusion chromatography using a TSKgel G3000SWXL column (TOSOH Bioscience), to obtain a highly concentrated fusion protein. Here, the isolated and purified fusion protein was subjected to SDS-PAGE under reduced (R) or non-reduced (NR) condition, and stained with Coomassie Blue to check its purity (Fig. 6). It was identified that the fusion protein was contained at a concentration of 2.78 mg/ml when detected with NanoDrop (Fig. 7). In addition, the results obtained by analysis using size exclusion chromatography are provided in Fig. 8.

Preparation Example 2. Preparation of mCD80-Fc-IL-2 variant (2M): mGI101

In order to produce a fusion protein comprising a mouse CD80, an Fc domain, and an IL-2 variant, a polynucleotide was synthesized through the Invitrogen GeneArt Gene Synthesis service of ThermoFisher Scientific. Specifically, the polynucleotide contains a nucleotide sequence (SEQ ID NO: 14) which encodes a fusion protein that contains a signal peptide (SEQ ID NO: 1), a mCD80 (SEQ ID NO: 13), an Ig hinge (SEQ ID NO: 3), an Fc domain (SEQ ID NO: 4), a linker (SEQ ID NO: 5), and an IL-2 variant (2M) (R38A, F42A) (SEQ ID NO: 6) with two amino acid substitutions, in this order, from the N-terminus. The polynucleotide was inserted into pcDNA3_4 vector. In addition, the vector was introduced into CHO cells (Expi-CHO™) to

express the fusion protein of SEQ ID NO: 15. After the vector was introduced, culture was performed for 7 days in an environment of 37°C, 125 rpm, and 8% CO₂ concentration. Then, the culture was harvested and the fusion protein was purified therefrom. The purified fusion protein was designated "mGI101".

The purification and collection of the fusion protein were carried out in the same manner as in Preparation Example 1. The isolated and purified fusion protein was subjected to SDS-PAGE under reduced (R) or non-reduced (NR) condition and stained with Coomassie Blue to check its purity (Fig. 9). It was found that the fusion protein was contained at a concentration of 1.95 mg/ml when detected by absorbance at 280 nm using NanoDrop.

Preparation Example 3. Preparation of hCD80-Fc: GI101C1

In order to produce a fusion protein comprising a human CD80 fragment and an Fc domain, a polynucleotide was synthesized through the Invitrogen GeneArt Gene Synthesis service of ThermoFisher Scientific. Specifically, the polynucleotide contains a nucleotide sequence (SEQ ID NO: 16) which encodes a fusion protein that contains a signal peptide (SEQ ID NO: 1), a CD80 fragment (SEQ ID NO: 2), an Ig hinge (SEQ ID NO: 3), and an Fc domain (SEQ ID NO: 4). The polynucleotide was inserted into pcDNA3_4 vector. In addition, the vector was introduced into CHO cells (Expi-CHO™) to express the fusion protein of SEQ ID NO: 17. After the vector was introduced, culture was performed for 7 days in an environment of 37°C, 125 rpm, and 8% CO₂ concentration. Then, the culture was harvested and the fusion protein was purified therefrom. The purified fusion protein was designated "GI101C1".

The purification and collection of the fusion protein were carried out in the same manner as in Preparation Example 1. The isolated and purified fusion protein was subjected to SDS-PAGE under reduced (R) or non-reduced (NR) condition and stained with Coomassie Blue to check its purity (Fig. 10). It was observed that the fusion protein was contained at a concentration of 3.61 mg/ml when detected by absorbance at 280 nm using NanoDrop.

Preparation Example 4. Preparation of Fc-IL-2 variant (2M): GI101C2

In order to produce a fusion protein comprising an Fc domain and an IL-2 variant, a polynucleotide was synthesized through the Invitrogen GeneArt Gene Synthesis service of ThermoFisher Scientific. Specifically, the polynucleotide contains a nucleotide sequence (SEQ ID NO: 18) which encodes a fusion protein that contains a signal peptide (SEQ ID NO: 1), an Fc domain (SEQ ID NO: 4), a linker (SEQ ID NO: 5), and an IL-2 variant (2M) (R38A, F42A) (SEQ ID NO: 6) with two amino acid substitutions, in this order, from the N-terminus. The polynucleotide was inserted into pcDNA3_4 vector. In addition, the vector was introduced into CHO cells (Expi-CHO™) to express the fusion protein of SEQ ID NO: 19. After the vector was introduced, culture was performed for 7 days in an environment of 37°C, 125 rpm, and 8% CO₂ concentration. Then, the culture was harvested and the fusion protein was purified therefrom.

The purified fusion protein was designated "GI101C2".

The purification and collection of the fusion protein were carried out in the same manner as in Preparation Example 1. The isolated and purified fusion protein was subjected to SDS-PAGE under reduced (R) or non-reduced (NR) condition and stained with Coomassie Blue to check its purity (Fig. 11). It was found that the fusion protein was contained at a concentration of 4.79 mg/ml when detected by absorbance at 280 nm using NanoDrop.

Preparation Example 5. Preparation of mCD80-Fc: mGI101C1

In order to produce a fusion protein comprising a mouse CD80 and an Fc domain, a polynucleotide was synthesized through the Invitrogen GeneArt Gene Synthesis service of ThermoFisher Scientific. Specifically, the polynucleotide contains a nucleotide sequence (SEQ ID NO: 20) which encodes a fusion protein that contains a signal peptide (SEQ ID NO: 1), a mouse CD80 (SEQ ID NO: 13), an Ig hinge (SEQ ID NO: 3), and an Fc domain (SEQ ID NO: 4), in this order, from the N-terminus. The polynucleotide was inserted into pcDNA3_4 vector. In addition, the vector was introduced into CHO cells (Expi-CHO™) to express the fusion protein of SEQ ID NO: 21. After the vector was introduced, culture was performed for 7 days in an environment of 37°C, 125 rpm, and 8% CO₂ concentration. Then, the culture was harvested and the fusion protein was purified therefrom. The purified fusion protein was designated "mGI101C1".

The purification and collection of the fusion protein were carried out in the same manner as in Preparation Example 1. The isolated and purified fusion protein was subjected to SDS-PAGE under reduced (R) or non-reduced (NR) condition and stained with Coomassie Blue to check its purity (Fig. 12). It was observed that the fusion protein was contained at a concentration of 2.49 mg/ml when detected by absorbance at 280 nm using NanoDrop.

The fusion proteins prepared in Preparation Examples 1 to 5 are summarized in Table 1 below.

[Table 1]

Item	N-terminus	Linker	C-terminus
Preparation Example 1 (GI101)	hCD80 fragment	Fc domain	hIL-2m
Preparation Example 2 (mGI101)	mCD80 fragment	Fc domain	hIL-2m
Preparation Example 3 (GI101C1)	CD80 fragment	Fc domain	-
Preparation Example 4 (GI101C2)	-	Fc domain	IL-2m

Preparation Example 5 (mGI101C1)	mCD80 fragment	Fc domain	-
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Preparation Example 6. Preparation of CD80-Fc-IL-2: GI101w

In order to produce a fusion protein comprising a human CD80 fragment, an Fc domain, and a human IL-2, a polynucleotide was synthesized through the Invitrogen GeneArt Gene Synthesis service of ThermoFisher Scientific. Specifically, the polynucleotide contains a nucleotide sequence (SEQ ID NO: 31) which encodes a fusion protein that contains a signal peptide (SEQ ID NO: 1), a CD80 fragment (SEQ ID NO: 2), an Ig hinge (SEQ ID NO: 3), an Fc domain (SEQ ID NO: 4), a linker (SEQ ID NO: 5), and mature human IL-2 (SEQ ID NO: 10), in this order, from the N-terminus. The polynucleotide was inserted into pcDNA3_4 vector. In addition, the vector was introduced into CHO cells (Expi-CHO™) to express the fusion protein of SEQ ID NO: 32. After the vector was introduced, culture was performed for 7 days in an environment of 37°C, 125 rpm, and 8% CO₂ concentration. Then, the culture was harvested and the fusion protein was purified therefrom. The purified fusion protein was designated "GI101w". The purification and collection of the fusion protein were carried out in the same manner as in Preparation Example 1.

Preparation Example 7. Preparation of hCD80-Fc-IL-2 variant (3M): GI102-M45

In order to produce a fusion protein comprising a human CD80 fragment, an Fc domain, and an IL-2 variant (3M) (R38A, F42A, and Y45A) (GI102-M45) with three amino acid substitutions, a polynucleotide was synthesized through the Invitrogen GeneArt Gene Synthesis service of ThermoFisher Scientific. Specifically, the polynucleotide contains a nucleotide sequence (SEQ ID NO: 25) which encodes a fusion protein that contains a signal peptide (SEQ ID NO: 1), a CD80 fragment (SEQ ID NO: 2), an Ig hinge (SEQ ID NO: 3), an Fc domain (SEQ ID NO: 4), a linker (SEQ ID NO: 5), and an IL-2 variant (SEQ ID NO: 22), in this order, from the N-terminus. The polynucleotide was inserted into pcDNA3_4 vector. In addition, the vector was introduced into CHO cells (Expi-CHO™) to express the fusion protein of SEQ ID NO: 26. After the vector was introduced, culture was performed for 7 days in an environment of 37°C, 125 rpm, and 8% CO₂ concentration. Then, the culture was harvested and the fusion protein was purified therefrom. The purified fusion protein was designated "GI102-M45".

The purification and collection of the fusion protein were carried out in the same manner as in Preparation Example 1. The isolated and purified fusion protein was subjected to SDS-PAGE under reduced (R) or non-reduced (NR) condition and stained with Coomassie Blue to check its purity (Fig. 13).

Preparation Example 8. Preparation of hCD80-Fc-IL-2 variant (3M): GI102-M61

In order to produce a fusion protein comprising a human CD80 fragment, an Fc domain, and an IL-2 variant (3M) (R38A, F42A, and E61R) (GI102-M61) with three amino acid

substitutions, a polynucleotide was synthesized through the Invitrogen GeneArt Gene Synthesis service of ThermoFisher Scientific. Specifically, the polynucleotide contains a nucleotide sequence (SEQ ID NO: 27) which encodes a fusion protein that contains a signal peptide (SEQ ID NO: 1), a CD80 fragment (SEQ ID NO: 2), an Ig hinge (SEQ ID NO: 3), an Fc domain (SEQ ID NO: 4), a linker (SEQ ID NO: 5), and an IL-2 variant (SEQ ID NO: 23), in this order, from the N-terminus. The polynucleotide was inserted into pcDNA3_4 vector. In addition, the vector was introduced into CHO cells (Expi-CHO™) to express the fusion protein of SEQ ID NO: 28. After the vector was introduced, culture was performed for 7 days in an environment of 37°C, 125 rpm, and 8% CO₂ concentration. Then, the culture was harvested and the fusion protein was purified therefrom. The purified fusion protein was designated "GI102-M61".

The purification and collection of the fusion protein were carried out in the same manner as in Preparation Example 1. The isolated and purified fusion protein was subjected to SDS-PAGE under reduced (R) or non-reduced (NR) condition and stained with Coomassie Blue to check its purity (Fig. 14).

Preparation Example 9. Preparation of hCD80-Fc-IL-3M: GI102-M72

In order to produce a fusion protein comprising a human CD80 fragment, an Fc domain, and an IL-2 variant (3M) (R38A, F42A, and L72G) (GI102-M72) with three amino acid substitutions, a polynucleotide was synthesized through the Invitrogen GeneArt Gene Synthesis service of ThermoFisher Scientific. Specifically, the polynucleotide contains a nucleotide sequence (SEQ ID NO: 29) which encodes a fusion protein that contains a signal peptide (SEQ ID NO: 1), a CD80 fragment (SEQ ID NO: 2), an Ig hinge (SEQ ID NO: 3), an Fc domain (SEQ ID NO: 4), a linker (SEQ ID NO: 5), and an IL-2 variant (SEQ ID NO: 24), in this order, from the N-terminus. The polynucleotide was inserted into pcDNA3_4 vector. In addition, the vector was introduced into CHO cells (Expi-CHO™) to express the fusion protein of SEQ ID NO: 30. After the vector was introduced, culture was performed for 7 days in an environment of 37°C, 125 rpm, and 8% CO₂ concentration. Then, the culture was harvested and the fusion protein was purified therefrom. The purified fusion protein was designated "GI102-M72".

The purification and collection of the fusion protein were carried out in the same manner as in Preparation Example 1. The isolated and purified fusion protein was subjected to SDS-PAGE under reduced (R) or non-reduced (NR) condition and stained with Coomassie Blue to check its purity (Fig. 15).

Preparation Example 10. Preparation of mCD80-Fc-IL-3M: mGI102-M61

In order to produce a fusion protein comprising a mouse CD80 fragment, an Fc domain, and an IL-2 variant (3M) (R38A, F42A, and E61R) (GI102-M61) with three amino acid substitutions, a polynucleotide was synthesized through the Invitrogen GeneArt Gene Synthesis service of ThermoFisher Scientific. Specifically, the polynucleotide contains a nucleotide

sequence (SEQ ID NO: 33) which encodes a fusion protein that contains a signal peptide (SEQ ID NO: 1), a mCD80 fragment (SEQ ID NO: 13), an Ig hinge (SEQ ID NO: 3), an Fc domain (SEQ ID NO: 4), a linker (SEQ ID NO: 5), and an IL-2 variant (SEQ ID NO: 23), in this order, from the N-terminus. The polynucleotide was inserted into pcDNA3_4 vector. In addition, the vector was introduced into CHO cells (Expi-CHO™) to express the fusion protein of SEQ ID NO: 34. After the vector was introduced, culture was performed for 7 days in an environment of 37°C, 125 rpm, and 8% CO₂ concentration. Then, the culture was harvested and the fusion protein was purified therefrom. The purified fusion protein was designated "mGI102-M61".

The purification and collection of the fusion protein were carried out in the same manner as in Preparation Example 1.

II. Identification of binding affinity between fusion protein and its ligand

In order to identify the binding affinity between the fusion protein and its ligand, the binding affinity was measured using Octet RED 384.

Experimental Example 1. Identification of binding affinity between hCTLA-4 and GI101

AR2G biosensor (Amine Reactive 2nd gen, ForteBio, Cat: 18-5092) was previously hydrated with 200 µl of distilled water in a 96-well microplate (GreinerBio-one, Cat: 655209). A ligand (CTLA-4, Human CTLA-4/CD152, His tag, Sino Biological, Cat: 11159-H08H) to be attached to the AR2G biosensor was diluted with 10 mM acetate buffer (pH 5, AR2G reagent Kit, ForteBio, Cat: 18-5095) to a concentration of 5 µg/ml. In addition, GI101 to be attached to the ligand was diluted with 1X AR2G kinetic buffer (AR2G reagent Kit, ForteBio, Cat: 18-5095) to a concentration of 1,000 nM, 500 nM, 250 nM, 125 nM, or 62.5 nM. Activation buffer was prepared by mixing 20 mM EDC and 10 mM s-NHS (AR2G reagent Kit, ForteBio, Cat: 18-5095) in distilled water. 80 µl of each reagent was placed in a 384-well microplate (GreinerBio-one, Cat: 781209) and the program was set up.

As a result, the binding affinity between hCTLA-4 and GI101 was measured as illustrated in Fig. 16.

Experimental Example 2. Identification of binding affinity between hPD-L1/GI101 and hPD-L1/PD-1

Ni-NTA (Nickel charged Tris-NTA, Ni-NTA Biosensors, ForteBio, 18-5101) was previously hydrated with 200 µl of 1X Ni-NTA kinetic buffer (10X Kinetics buffer, ForteBio, 18-1042) in a 96-well microplate. A ligand (Human PD-L1/B7-H1 protein, His-tag, Sino biological, Cat: 10084-H08H) to be attached to the Ni-NTA Biosensors was diluted with 1X Ni-NTA kinetic buffer to a concentration of 5 µg/ml. GI101 to be attached to the ligand was diluted with 1X Ni-NTA kinetic buffer to 1,000 nM, 500 nM, 250 nM, 125 nM, or 62.5 nM. In addition, human PD-

1/PDCD1 (Human PD-1/PDCD1, Fc Tag, Sino Biological, Cat: 10377-H02H) to be attached to the ligand was diluted with 1X Ni-NTA kinetic buffer to a concentration of 2,000 nM, 1,000 nM, 500 nM, 250 nM, or 125 nM. Then, 80 µl of each reagent was placed in a 384-well microplate and the program was set up.

As a result, the binding affinity between hPD-L1 and GI101 was measured as illustrated in Fig. 17. In addition, the binding affinity between hPD-L1 and hPD-1 was measured as illustrated in Fig. 18.

Experimental Example 3. Identification of binding affinity between mCTLA-4 and mGI101

The binding affinity between mCTLA-4 and mGI101 was identified in the same manner as in Experimental Example 1. Here, the equipment used is as follows: Biosensor: AR2G, Ligand: mCTLA-4 (Recombinant Mouse CTLA-4 Fc chimera, R&D Systems, Cat: 434-CT-200), Analyte: mGI101 (500 nM, 250 nM, 125 nM, 62.5 nM, 31.3 nM).

As a result, the binding affinity between mCTLA-4 and mGI101 was measured as illustrated in Fig. 19.

Experimental Example 4. Identification of binding affinity between mPD-L1 and mGI101

The binding affinity between mPD-L1 and mGI101 was identified in the same manner as in Experimental Example 1. Here, the equipment used is as follows. Biosensor: AR2G, Ligand: mPD-L1 (Recombinant Mouse B7-H1/PD-L1 Fc chimera, R&D Systems, Cat: 434-CT-200), Analyte: mGI101 (500 nM, 250 nM, 125 nM, 62.5 nM, 31.3 nM).

As a result, the binding affinity between mPD-L1 and mGI101 was measured as illustrated in Fig. 20.

Experimental Example 5. Identification of binding ability of GI-101 (hCD80-Fc-hIL-2v) to CTLA-4

Binding kinetics measurements were performed using the Octet RED 384 instrument (ForteBio, Pall Life Science) with agitation at 30°C and 1,000 rpm. The binding ability for CTLA-4 was measured using the Amine Reactive 2 generation (AR2G) biosensor chip, and the binding ability for PD-L1 was measured using the Nickel charged Tris-NTA (Ni-NTA) biosensor chip. The AR2G biosensor chip was activated with a combination of 400 mM EDC and 100 mM sulfo-NHS. Then, Human CTLA-4-His Tag (Sino Biological, Cat: 11159-H08H) was diluted with 10 mM acetate buffer (pH 5) to 5 µg/ml, and loaded on the AR2G biosensor chip for 300 seconds and fixed.

Then, binding of CTLA-4 to GI-101 (hCD80-Fc-hIL-2v), GI-101C1 (hCD80-Fc), Ipilimumab (Bristol-Myers Squibb), and GI-101C2 (Fc-hIL-2v) at various concentrations was measured for 300 seconds and dissociation thereof was also measured for 300 seconds. Binding

kinetics analysis was performed using Octet Data Analysis HT software ver. 10 provided by Pall Corporation. The results are illustrated in Fig. 21.

Experimental Example 6. Identification of binding affinity between IL-2R α or IL-2R β , and GI101

The binding ability for IL-2R α was measured using the AR2G biosensor, and the binding ability for IL-2R β was measured using the Ni-NTA biosensors (Nickel charged Tris-NTA, Ni-NTA Biosensors, ForteBio, 18-5101).

A ligand (IL-2R α -His Tag, Acro, Cat: ILA-H52H9) to be attached to the AR2G biosensor was diluted with 10 mM acetate buffer (pH 5, AR2G reagent Kit, ForteBio, Cat: 18-5095) to a concentration of 5 μ g/ml. The AR2G biosensor was activated with a buffer prepared by mixing 400 mM EDC and 100 mM sulfo-NHS, and then the diluted ligand was loaded on the AR2G biosensor for 300 seconds and fixed.

Meanwhile, a ligand (IL-2R β -His Tag, Acro, Cat: CD2-H5221) to be attached to the Ni-NTA biosensor was diluted with 1X Ni-NTA kinetic buffer to a concentration of 5 μ g/ml. The diluted ligand was loaded on the Ni-NTA biosensor for 600 seconds and fixed.

Thereafter, GI101, GI101w, or Proleukin (Novartis, hIL-2), at various concentrations, to be attached to the ligand was loaded thereon for 300 seconds. Then, binding thereof was measured and dissociation thereof was also measured for 300 seconds. Binding kinetics analysis was performed using Octet Data Analysis HT software ver. 10 provided by Pall Corporation. The results are illustrated in Figs. 22 to 24.

As a result, it was identified that GI101 has low binding ability for the IL-2 receptor IL-2R α and high binding ability for IL-2R β , as compared with GI101w and Proleukin.

Experimental Example 7. Measurement of binding affinity between fusion protein and ligand

In order to identify the binding affinity between the fusion protein and its ligand, the binding affinity was measured using Octet RED 384.

Experimental Example 7.1. Identification of binding affinity between IL-2 alpha receptor, and GI101-M45, GI101-M61, or GI101-M72

AR2G biosensor (Amine Reactive 2nd gen, ForteBio, Cat: 18-5092) was previously hydrated with 200 μ l of distilled water (DW) in a 96-well microplate (GreinerBio-one, Cat: 655209). A ligand (Human IL-2 R alpha protein, His Tag, Acro, ILA-H52H9) to be attached to the biosensor was diluted with 10 mM acetate buffer (pH 5) (AR2G reagent Kit, ForteBio, Cat: 18-5095) to a concentration of 5 μ g/ml. An analyte (GI101-M45, GI101-M61, GI101-M72) to be attached to the ligand was diluted with 1X AR2G kinetic buffer (AR2G reagent Kit, ForteBio, Cat: 18-5095) to 500 nM, 250 nM, 125 nM, and 62.5 nM, respectively. Activation buffer was prepared by mixing 20 mM EDC and 10 mM s-NHS (AR2G reagent Kit, ForteBio, Cat: 18-5095)

in DW. 80 µl of each reagent was placed in a 384-well microplate (GreinerBio-one, Cat: 781209) and the program was set up.

As a result, the binding affinity between IL-2 alpha receptor and GI101-M45 is illustrated in Fig. 25. In addition, the binding affinity between IL-2 alpha receptor and GI101-M61 is illustrated in Fig. 26, and the binding affinity between IL-2 alpha receptor and GI101-M72 is illustrated in Fig. 27.

Experimental Example 7.2. Identification of binding affinity of GI102-M45, GI102-M61, and GI102-M72 to IL-2Rβ

Ni-NTA Biosensors were previously hydrated with 200 µl of 1X Ni-NTA kinetic buffer (10X Kinetics buffer, ForteBio, 18-1042) in a 96-well microplate. A ligand (Human IL-2 R beta protein, His-Tag, Acro, CD2-H5221) to be attached to the biosensor was diluted with 1X Ni-NTA kinetic buffer to a concentration of 2 µg/ml. GI102-M45, GI102-M61, or GI102-M72 to be attached to the ligand was diluted with 1X Ni-NTA kinetic buffer to a concentration of 500 nM, 250 nM, 125 nM, or 62.5 nM. 80 µl of each reagent was placed in a 384-well microplate and the program was set up.

As a result, the binding affinity between IL-2Rβ and GI102-M45 was measured as illustrated in Fig. 28, and the binding affinity between IL-2Rβ and GI102-M61 was measured as illustrated in Fig. 29. In addition, the binding affinity between IL-2Rβ and GI102-M72 was measured as illustrated in Fig. 30.

III. Identification of immune activity of fusion protein

Experimental Example 8. Identification of IFN-γ production caused by fusion protein

Experimental Example 8.1. Culture of CFSE-labeled PBMCs

Peripheral blood mononuclear cells (PBMCs) isolated from a human were labeled with carboxyfluorescein succinimidyl ester (CFSE) by being reacted with 1 µM CellTrace CFSE dye at 37°C for 20 minutes. CFSE not bound to the cells was removed by being reacted for 5 minutes with a culture medium having a 5-fold volume of the staining reaction solution and then by being centrifuged at 1,300 rpm for 5 minutes. The CFSE-labeled PBMCs were resuspended in the culture medium (RPMI1640 medium containing 10% fetal bovine serum (FBS), 10 mM HEPES, 100 U/ml penicillin/streptomycin, 1 mM sodium pyruvate, 55 µM 2-mercaptoethanol, 1 mM non-essential amino acid, and 2 mM L-glutamine), and then added to a 96-well microplate at 1×10^5 cells per well. Treatment with 5 µg/ml of PHA (Lectin from Phaseolus Vulgaris, red kidney bean, Sigma-Aldrich, St. Louis, MO, USA, cat No. L1668-5MG), and GI101, GI101C1, GI101C2, or IL-2 (Aldesleukin; human recombinant IL-2, Novartis) was performed and incubation was performed in a 5% CO₂ incubator at 37°C for 6 days.

Here, the treatment with GI101, GI101C1, GI101C2, and IL-2 was performed at a concentration of 1 nM, 10 nM, or 100 nM. The cells were analyzed by FACS, and human IFN- γ present in the culture medium was measured using an ELISA kit (Biolegend, San Diego, CA, USA, cat No.430103).

Experimental Example 8.2. FACS analysis

The cell pellets obtained by removing the supernatant were washed with FACS buffer (3% fetal bovine serum, 10 mM EDTA, 1 M HEPES, 100 unit/ml penicillin, streptomycin, 1 mM sodium pyruvate), and then reacted with Fc blocker (Biolegend, cat NO. 422302) at 4°C for 5 minutes. Then, treatment with APC anti-CD3 Ab (Biolegend, cat NO. 300412) and PE anti-CD8a Ab (Biolegend, cat NO. 300908) was performed and reaction was allowed to proceed at 4°C for 20 minutes. Then, the resultant was washed with FACS buffer. The cell pellets were resuspended in FACS buffer and then analyzed using BD LSR Fortessa (BD biosciences, San Diego, CA, USA) and FlowJo Software.

Experimental Example 8.3. Human IFN- γ ELISA

The amount of human IFN- γ secreted into the supernatant of each sample in which the cells had been cultured was measured using a human IFN- γ ELISA kit (Biolegend, cat No.430103). Briefly, anti-human-IFN- γ antibodies were added to an ELISA plate, and reaction was allowed to proceed overnight at 4°C so that these antibodies were coated thereon. Then, blocking was performed at room temperature for 1 hour with a PBS solution to which 1% BSA had been added. Washing with a washing buffer (0.05% Tween-20 in PBS) was performed, and then a standard solution and each sample were properly diluted and added thereto. Then, reaction was allowed to proceed at room temperature for 2 hours.

After the reaction was completed, the plate was washed and secondary antibodies (detection antibodies) were added thereto. Reaction was allowed to proceed at room temperature for 1 hour. Washing with a washing buffer was performed, and then an Avidin-HRP solution was added thereto. Reaction was allowed to proceed at room temperature for 30 minutes. A substrate solution was added thereto and color development reaction was induced in the dark at room temperature for 20 minutes. Finally, H₂SO₄ was added thereto to stop the color development reaction, and the absorbance at 450 nm was measured with Epoch Microplate Spectrophotometer (BioTek instruments, Winooski, VT, USA), and the concentration was calculated.

As a result, it was found that cells treated with GI101 exhibited a remarkable increase in IFN- γ secretion, as compared with cells treated with GI101C1, GI101C2, or IL-2 (Figs. 31 and 32).

Experimental Example 9. Identification of effect of GI101 on proliferation of CD8+ T cells

Peripheral blood mononuclear cells (PBMCs) isolated from a human were labeled with CFSE by being reacted with 1 μ M CellTrace CFSE dye at 37°C for 20 minutes. CFSE not bound to the cells was removed by being reacted for 5 minutes with a culture medium having a 5-fold volume of the staining reaction solution and then by being centrifuged at 1,300 rpm for 5 minutes. The CFSE-labeled PBMCs were resuspended in the culture medium (RPMI1640 medium containing 10% fetal bovine serum, 10 mM HEPES, 100 U/ml penicillin/streptomycin, 1 mM sodium pyruvate, 55 μ M 2-mercaptoethanol, 1 mM non-essential amino acid, and 2 mM L-glutamine), and then added to a 96-well microplate at 1×10^5 cells per well.

Thereafter, treatment with 1 μ g/ml of anti-CD3 ϵ antibody (Biolegend cat No. L1668-5MG), and GI101, GI101C1, GI101C2, or Proleukin (Novartis) was performed and incubation was performed in a 5% CO₂ incubator at 37°C for 6 days. Here, the cells were treated with GI101, GI101C1, GI101C2, and IL-2 at a concentration of 100 nM. The incubated cells were examined for their degree of proliferation by measuring, with FACS analysis using APC-TCR $\alpha\beta$ and PE-CD8 α antibodies, a proportion of CD8⁺ T cells that had not been labeled with CFSE.

As a result, it was found that GI101 activated proliferation of CD8⁺ T cells *in vitro* to a similar extent to the wild-type IL-2 Proleukin (Figs. 33 and 34).

Experimental Example 10. Identification of effect of GI101 and GI102 on proliferation of CD8⁺ T cells

Human PBMCs were purchased from Allcells (Lot#3014928, USA). 1M CellTrace CFSE dye was used, which was reacted with the human PBMCs under a light-blocking condition at room temperature for 20 minutes. The cells were labeled with CFSE by being reacted with 1 μ M CellTrace CFSE dye at 37°C for 20 minutes. CFSE not bound to the cells was removed by being reacted for 5 minutes with a culture medium having a 5-fold volume of the staining reaction solution and then by being centrifuged at 1,300 rpm for 5 minutes. The CFSE-labeled PBMCs were resuspended in the culture medium (RPMI1640 medium containing 10% fetal bovine serum, 10 mM HEPES, 100 U/ml penicillin/streptomycin, 1 mM sodium pyruvate, 55 μ M 2-mercaptoethanol, 1 mM non-essential amino acid, and 2 mM L-glutamine), and then added to a 96-well microplate at 1×10^5 cells per well.

Thereafter, the CFSE-labeled PBMCs were subjected to treatment with 1 μ g/ml of anti-CD3 ϵ antibody (OKT3, eBioscience, USA), and GI101, GI101C1, GI101C2, or Proleukin (Novartis), and incubation was performed in a 5% CO₂ incubator at 37°C for 7 days. Here, the cells were subjected to treatment with GI101, GI101C1, GI101C2, and IL-2 at a concentration of 10 μ M.

The incubated cells were examined for their degree of proliferation by measuring, with FACS analysis using anti-human CD4-PE antibody (BioLegend, USA), anti-human CD8-PE/Cy7 antibody (BioLegend, USA), and anti-human FoxP3-APC antibody (BioLegend, USA), a

proportion of CD8⁺ T cells that had not been labeled with CFSE.

As a result, the GI101, GI102_M61, GI101C2, and Proleukin treatment groups exhibited a significant increase in proportion of CD8⁺ T cells, as compared with the control (no stimulus), the anti-CD3 antibody alone treatment group, and the GI101C1 treatment group. In addition, as compared with the negative control (no stimulus) and the anti-CD3 antibody alone treatment group, the GI101, GI101C2, and Proleukin treatment groups exhibited a significant increase in proliferation of CD4⁺/FoxP3⁺ Treg cells, whereas the GI102 and GI101C1 treatment groups did not exhibit a significant increase in proliferation of CD4⁺/FoxP3⁺ Treg cells (Fig. 35).

Experimental Example 11. Identification of effect of GI101 or GI101w on proliferation of CD8⁺ T cells and NK cells

7-week-old C57BL/6 mice purchased from Orient Bio (Korea) were divided into 3 groups, each group including 3 mice, and PBS, GI101, or GI101w was injected intraperitoneally thereinto. Here, GI101 and GI101w were respectively prepared to be at 40.5 µg in 200 µl of PBS, and injected intraperitoneally thereinto. Five days after the injection, the spleens were removed from the mice of each group. The cells were isolated therefrom, and the total number of cells was measured using a hemacytometer. Splenocytes were examined for proportions of CD8⁺ T cells and NK cells therein, with FACS analysis using staining with APC-CD3ε antibody (Biolegend; 145-2C11), PE-NK1.1 antibody (Biolegend; PK136), and Pacific blue-CD8α antibody (BD; 53-6.7). As such, the numbers of CD8⁺ T cells and NK cells present in the spleen were calculated.

As a result, it was identified that GI101 activated proliferation of CD8⁺ T cells and NK cells *in vivo* as compared with GI101w (Figs. 36 and 37).

Experimental Example 12. Identification of effect of GI101 on function of T cells

An experiment was performed using a CTLA-4 blockade bioassay kit (Promega cat No. JA4005). The experiment is briefly described as follows. CTLA-4 effector cells kept in liquid nitrogen were thawed in a 37°C constant temperature water bath for 3 minutes, and 0.8 ml of CTLA-4 effector cells were mixed well with 3.2 ml of pre-warmed assay buffer (90% RPMI + 10% fetal bovine serum). Then, the mixture was added to a 96-well white cell culture plate (SPL, cat No. 30196) at 25 µl per well. Then, 25 µl of GI101 at various concentrations was added thereto. For a negative control, 25 µl of assay buffer was added thereto. Then, the 96-well white cell culture plate was covered and placed at room temperature until aAPC/Raji cells were prepared.

aAPC/Raji cells kept in liquid nitrogen were thawed in a 37°C constant temperature water bath for 3 minutes, and 0.8 ml of aAPC/Raji cells were mixed well with 3.2 ml of pre-warmed assay buffer. Then, 25 µl of the mixture was added to the plate at per well, and reaction was allowed to proceed in a 5% CO₂ incubator at 37°C for 16 hours. After the reaction was completed, the resultant was allowed to stand at room temperature for 15 minutes, and then the Bio-Glo reagent was added thereto while taking care to avoid bubbles. The Bio-Glo reagent was also

added to three of the outermost wells and the wells were used as blanks to correct the background signal. Reaction was allowed to proceed at room temperature for 10 minutes, and then luminescence was measured with Cytation 3 (BioTek instruments, Winooski, VT, USA). Final data analysis was performed by calculating RLU (GI101-background)/RLU (no treatment-background).

As a result, it was found that GI101 binds to CTLA-4 expressed on effector T cells, and activates the function of T cells rather than inhibiting the same (Figs. 38 and 39).

Experimental Example 13. Identification of effect of mGI101 and mGI102 on immune cells

7-week-old C57BL/6 mice purchased from Orient Bio (Korea) were divided into 3 groups, each group including 3 mice, and PBS, 3 mg/kg, 6 mg/kg, or 12 mg/kg of GI101, or 3 mg/kg, 6 mg/kg, or 12 mg/kg of mGI102 (mGI102-M61) was administered intravenously thereinto. On days 1, 3, 5, 7, and 14 after the injection, the spleen tissues were removed from the mice of each group. Thereafter, for the spleen tissue, the numbers of effector CD8⁺ T cells, NK cells, and Treg cells were calculated with FACS analysis using respective antibodies, and proportions of effector CD8⁺ T cells and NK cells with respect to Treg cells were respectively calculated. The information on the antibodies used in each cell analysis is as follows:

Effector CD8⁺ T cell: PB anti-mouse CD3 ϵ antibody (Biolegend, # 155612; KT3.1.1), FITC anti-mouse CD8 α antibody (BD, # 553031, 53-6.7), PE/Cy7 anti-mouse CD44 antibody (Biolegend, # 103030; IM7), APC anti-mouse CD122 antibody (Biolegend, # 123214; TM- β 1)

NK cell: PB anti-mouse CD3 ϵ antibody (Biolegend, # 155612; KT3.1.1), PE anti-mouse NK-1.1 (Biolegend, # 108708; PK136)

Treg cell: FITC anti-mouse CD3 antibody (Biolegend, # 100204; 17A2), PB anti-mouse CD4 antibody (Biolegend, # 100531; RM4-5), PE anti-mouse CD25 antibody (Biolegend, # 102008; PC61), APC anti-mouse Foxp3 antibody (Invitrogen, # FJK-16s, 17-5773-82).

As a result, the group having received mGI101 or mGI102 (mGI102-M61) exhibited a significant increase in numbers of CD8⁺ T cells and NK cells at the time points from the 3rd to 14th days after administration, as compared with the PBS administration group. In addition, it was found that the group having received mGI102 exhibited a significant increase in proportions of activated CD8⁺ T cells/Treg cells and NK cells/Treg cells at the time points from the 3rd to 7th days after administration, as compared with the PBS administration group (Fig. 40).

IV. Identification of anticancer effect of fusion protein

Experimental Example 14. Identification of effect of GI101 on T cell activity inhibition by cancer cells expressing PD-L1 and CTLA-4

NCI-H292 cancer cell line expressing PD-L1 and CTLA-4 was cultured for 3 hours in a

culture medium containing 10 µg/ml Mitomycin C (Sigma), and then Mitomycin C was removed by washing with the culture medium. Thereafter, 5×10^4 cells of the Mitomycin C-treated NCI-H292 cancer cell line were incubated with 1×10^5 cells of human PBMCs in a 96-well microplate. Here, treatment with 5 µg/ml of PHA (Sigma) was performed for T cell activity. In addition, GI101C1 and GI101 at a concentration of 50 nM were reacted with IgG1-Fc (Biolegend) or abatacept (= Orencia; Bristol-Myers Squibb) at a concentration of 50 nM for 30 minutes at 4°C, and then the resultant was used to treat the NCI-H292 cancer cells. After 3 days, the supernatant of the cell culture was collected and the amount of IFN-γ was quantified using an ELISA kit (Biolegend).

As a positive control, human PBMCs stimulated with PHA in the absence of the Mitomycin C-treated NCI-H292 cancer cell line were used; and as a negative control, human PBMCs stimulated with PHA in the presence of the Mitomycin C-treated NCI-H292 cancer cell line were used. An experimental method using the IFN-γ ELISA kit was carried out in the same manner as in Experimental Example 9.3.

As a result, GI101 effectively activated the immune response that had been inhibited by the cancer cell line overexpressing PD-L1. In addition, it was identified that GI101 inhibited signaling of CTLA-4 expressed on effector T cells (Figs. 41 and 42).

Experimental Example 15. Identification of anticancer effect of mGI101 in mice transplanted with mouse-derived colorectal cancer cells

BALB/c mice (female, 7-week-old) purchased from Orient Bio were subjected to an acclimation period of 7 days. Then, 5×10^6 cells of CT-26 cancer cell line (ATCC, USA) were mixed with 0.05 ml of MATRIGEL matrix phenol red-free (BD), and allotransplantation of the mixture was performed by subcutaneous administration at 0.1 ml in the right dorsal region of the mice. A certain period of time after the cancer cell transplantation, the tumor volume was measured and subjects that reached about 28 mm³ were selected, and then the selected mice were grouped evenly based on tumor size and body weight, each group including 10 mice. Thereafter, using a disposable syringe (31G, 1 ml), hIgG4 was administered at a dose of 6 mg/kg to a negative control. For experimental groups, mGI101 at a dose of 3 mg/kg, 6 mg/kg, or 12 mg/kg was administered intravenously thereto. A total of three administrations were given once every three days after the first administration. The tumor size was measured daily.

As a result, it was found that the experimental group having received mGI101 at a dose of 6 mg/kg and 12 mg/kg exhibited significant tumor growth inhibition at some measurement time points and at the end of the test, as compared with the negative control (Fig. 43). In addition, as a result of measuring a survival rate, it was found that the experimental group having received mGI101 at a dose of 6 mg/kg exhibited significant improvement at some measurement time points and at the end of the test, as compared with the negative control (Fig. 44).

Experimental Example 16. Identification of anticancer effect of GI101 in mice transplanted with mouse-derived colorectal cancer cells

Experimental Example 16.1. Identification of tumor inhibitory effect

BALB/c mice (female, 7-week-old) purchased from Orient Bio were subjected to an acclimation period of 7 days. Then, 5×10^6 cells of CT-26 cancer cell line (ATCC, USA) were suspended in 0.1 ml PBS, and allotransplantation of the suspension was performed by subcutaneous administration at 0.1 ml in the right dorsal region of the mice. A certain period of time after the cancer cell transplantation, the tumor volume was measured and subjects that reached about 50 mm³ to 200 mm³ were selected, and then the selected mice were grouped evenly based on tumor size and body weight, each group including 10 mice. Thereafter, using a disposable syringe (31G, 1 mL), no drug was administered to a negative control, and an anti-PD-1 antibody at a dose of 5 mg/kg, or an anti-PD-1 antibody at a dose of 5 mg/kg and an anti-CTLA-4 antibody at a dose of 5 mg/kg were administered intravenously to positive controls. For experimental groups, GI101 at a dose of 0.1 mg/kg or 1 mg/kg was administered intravenously thereto. A total of three administrations were given once every three days after the first administration. The tumor size was measured daily.

As a result, in the mice transplanted with CT-26 cancer cell line, all groups having received anti-PD-1 antibody; anti-PD-1 antibody and anti-CTLA-4 antibody; or GI101 at a dose of 0.1 mg/kg or 1 mg/kg exhibited significant tumor growth inhibition, as compared with the negative control. In particular, the experimental group having received GI101 at a dose of 0.1 mg/kg exhibited a significant tumor inhibitory effect, as compared with the group having received an anti-PD-1 antibody (* $p < 0.05$) (Fig. 45).

Experimental Example 16.2. Immune cell analysis in cancer tissue

The mice of each group in Experimental Example 16.1 were sacrificed when the tumor volume reached an average of 200 mm³, and cancer tissues were collected. Thereafter, the cancer tissues were separated to a single-cell level to analyze immune cells therein, and then FACS analysis was performed on immune cells in the cancer tissues using the following antibodies: Anti-mouse-CD3 (Biolegend, Cat. No. 100320), Anti-mouse-CD4 (Biolegend, Cat. No. 100526), Anti-mouse-CD8 (Biolegend, Cat. No. 100750), Anti-mouse-FoxP3 (eBioscience, Cat. No. 12-5773-82), Anti-mouse-CD25 (Biolegend, Cat. No. 102049), Anti-mouse-CD44 (eBioscience, Cat. No. 61-0441-82), Anti-mouse-PD-1 (Biolegend, Cat. No. 135218), Anti-mouse-IFN-gamma (Biolegend, Cat. No. 505832), Anti-mouse-CD49b (Biolegend, Cat. No. 108906), Anti-mouse-H2 (Invitrogen, Cat. No. A15443), Anti-mouse-CD11c (Biolegend, Cat. No. 117343), Anti-mouse-CD80 (eBioscience, Cat. No. 47-4801-82), Anti-mouse-CD86 (Biolegend, Cat. No. 104729), Anti-mouse-F4/80 (eBioscience, Cat. No. 47-4801-82), and Anti-mouse-CD206 (eBioscience, Cat. No. 17-2061-80).

As a result, the experimental group having received GI101 at a dose of 0.1 mg/kg exhibited a significant increase in CD8⁺ T cells, as compared with the positive control having received anti-PD-1 antibody alone at a dose of 5 mg/kg (* $p < 0.05$, Figs. 46 and 47). Furthermore, all experimental groups having received GI101 exhibited a significantly increased level of expression of IFN- γ in T cells, as compared with the negative control (* $p < 0.05$, Figs. 46 and 47). In addition, the experimental group having received GI101 at a dose of 0.1 mg/kg exhibited an increase in M1 macrophages as compared with the negative control and the positive control having received anti-PD-1 antibody alone (Figs. 48 and 49). In addition, all experimental groups having received GI101 exhibited an increased level of CD86 expression in macrophages and dendritic cells (* $p < 0.05$, Figs. 48 to 51).

Experimental Example 17. Identification of anticancer effect of GI101 in mice transplanted with mouse-derived lung cancer cells

Experimental Example 17.1. Identification of tumor inhibitory effect

C57BL/6 mice (female, 7-week-old) purchased from Orient Bio were subjected to an acclimation period of 7 days. Then, 5×10^6 cells of LL/2 cancer cell line (ATCC, USA) were suspended in 0.1 ml PBS, and allotransplantation of the suspension was performed by subcutaneous administration at 0.1 ml in the right dorsal region of the mice. A certain period of time after the cancer cell transplantation, the tumor volume was measured and subjects that reached about 50 mm³ to 200 mm³ were selected, and then the selected mice were grouped evenly based on tumor size and body weight, each group including 10 mice. Thereafter, using a disposable syringe (31G, 1 mL), no drug was administered to a negative control, and an anti-PD-1 antibody at a dose of 5 mg/kg, or an anti-PD-1 antibody at a dose of 5 mg/kg and an anti-CTLA-4 antibody at a dose of 5 mg/kg were administered intravenously to positive controls. For experimental groups, GI101 at a dose of 0.1 mg/kg or 1 mg/kg was administered intravenously thereto. A total of three administrations were given once every three days after the first administration. The tumor size was measured daily.

As a result, all experimental groups exhibited a significant tumor inhibitory effect, as compared with the negative control (* $p < 0.05$) (Fig. 52).

Experimental Example 17.2. Immune cell analysis in cancer tissue

The mice of each group in Experimental Example 17.1. were sacrificed when the tumor volume reached an average of 200 mm³, and cancer tissues were collected. Thereafter, FACS analysis was performed in the same manner as Experimental Example 16.2. to analyze immune cells in the cancer tissues.

As a result, the experimental group having received GI101 at a dose of 0.1 mg/kg exhibited a significant increase in CD8⁺ T cells, as compared with the positive control having received anti-PD-1 antibody alone (* $p < 0.05$, Fig. 59). Furthermore, all experimental groups having received

GI101 exhibited a significantly increased level of expression of IFN- γ , as compared with the negative control (* $p < 0.05$, Fig. 59). In addition, all experimental groups having received GI101 exhibited an increased level of CD86 expression in macrophages and dendritic cells (* $p < 0.05$, Figs. 53 to 55).

Experimental Example 18. Identification of anticancer effect of mGI102-M61 in mice transplanted with mouse-derived colorectal cancer cells

BALB/c mice (female, 7-week-old) purchased from Orient Bio were subjected to an acclimation period of 7 days. Then, 5×10^6 cells of CT26 cancer cell line (ATCC, USA) were mixed with 0.05 ml of MATRIGEL matrix phenol red-free (BD), and allotransplantation of the mixture was performed by subcutaneous administration at 0.1 ml in the right dorsal region of the mice. A certain period of time after the cancer cell transplantation, the tumor volume was measured and subjects that reached about 28 mm³ were selected, and then the selected mice were grouped evenly based on tumor size and body weight, each group including 10 mice. Thereafter, using a disposable syringe (31G, 1 mL), hIgG4 was administered at a dose of 6 mg/kg to a negative control. For experimental groups, mGI102-M61 at a dose of 3 mg/kg, 6 mg/kg, or 12 mg/kg was administered intravenously thereto. A total of three administrations were given once every three days after the first administration. The tumor size was measured daily.

As a result, it was identified that the experimental group having received mGI102-M61 at a dose of 12 mg/kg exhibited significant tumor growth inhibition at some measurement time points and at the end of the test, as compared with the negative control (Fig. 56). In addition, as a result of measuring a survival rate, it was identified that the experimental group having received mGI102-M61 at a dose of 12 mg/kg exhibited significant improvement at some measurement time points and at the end of the test, as compared with the negative control (Fig. 57).

Experimental Example 19. Identification of anticancer effect of mGI101 in mice transplanted with mouse-derived colorectal cancer cells

BALB/c mice (female, 7-week-old) purchased from Orient Bio (Korea) were subjected to an acclimation period of 7 days. Then, 5×10^6 cells of CT-26 cancer cell line (ATCC, USA) were mixed with 0.05 ml of MATRIGEL matrix phenol red-free (BD), and allotransplantation of the mixture was performed by subcutaneous administration at 0.1 ml in the right dorsal region of the mice. A certain period of time after the cancer cell transplantation, the tumor volume was measured and subjects that reached about 200 mm³ to 250 mm³ were selected, and then the selected mice were grouped evenly based on tumor size and body weight, each group including 10 mice.

Thereafter, using a disposable syringe (31G, 1 mL), hIgG4 was administered at a dose of 4 mg/kg to a negative control. For experimental groups, mGI101 at a dose of 1 mg/kg, 4 mg/kg, or 6 mg/kg was administered intravenously thereto. Additionally, groups having received mCD80 at 4.9 mg/kg or Fc-IL-2v (GI101C2) at 2.8 mg/kg were set as controls. In addition, a

group having simultaneously received mCD80 at 4.9 mg/kg and Fc-IL-2v (GI101C2) at 2.8 mg/kg was set as a control.

In tumor volume measurement, it was identified that the group having received mGI101 at a dose of 6 mg/kg exhibited significant inhibition at some measurement time points and at the end of the test, as compared with the negative control. An excellent tumor growth inhibition rate was observed as compared with the group having received a combination of mCD80 and Fc-IL-2v (GI101C2) (Figs. 58 and 59).

In conclusion, in the tumor growth-inhibitory efficacy test on BALB/c mice allotransplanted with CT-26, a BALB/c mouse-derived colorectal cancer cell line, it was demonstrated that the test substance mGI101 had tumor inhibitory efficacy under this test condition as compared with respective mCD80 and IL-2v single formulations; and it was identified that mGI101 exhibited excellent anticancer efficacy as compared with the group having received a combination of mCD80 and IL-2v (Figs. 58 and 59). In particular, the group having received mGI101 at a dose of 6 mg/kg exhibited significant inhibition of tumor size, as compared with the negative control and the group having received a combination of mCD80 and Fc-IL2v (GI101C2).

V. Identification of anticancer effect according to administration of combination of fusion protein dimer and immune checkpoint inhibitor

Experimental Example 20. Identification of anticancer effect by administration of combination of GI101 and anti-PD-1 antibody in mice transplanted with human-derived breast cancer cells

This test was to evaluate the tumor growth inhibitory effect after intraperitoneal administration of GI101 as a test substance alone or in combination with Keytruda (Pembrolizumab, MSD), which is an anti-PD-1 antibody, as a positive control substance in a tumor model xenotransplanted with MDA-MB-231 cells, which are human-derived breast cancer cells, using a humanized mouse model prepared by xenotransplanting human PBMCs into NSG^{b2m} mice.

The stock solution of the test substance, negative control substance, and positive control substance described in Table 2 was diluted by adding excipients according to each dose.

[Table 2]

-	Test substance	Positive control substance	Negative control substance	Excipient
Substance name	GI101	Keytruda	hIgG4	PBS
Appearance	clear liquid	clear liquid	clear liquid	clear

				liquid
Component	Fc fusion protein	anti-PD-1 antibody	-	-
pH	7.5	-	-	-
Storage condition	refrigerated storage (4°C)	refrigerated storage (4°C)	refrigerated storage (4°C)	refrigerated storage (4°C)
Precautions for handling	keep refrigerated until administration, and prepare and use on the day of administration	keep refrigerated until administration, and prepare and use on the day of administration	keep refrigerated until administration, and prepare and use on the day of administration	-

Human-derived breast cancer cells, MDA-MB-231 (*Homo sapiens*, human mammary gland/breast; derived from metastatic site: pleural effusion), were purchased from the Korea cell line bank (Korea) and used for the test. The cell culture medium has a composition as shown in the table below. Fetal bovine serum (FBS, 16000-044, Thermofisher scientific, USA), penicillin-streptomycin; 10,000 units/ml penicillin and 10,000 µg/ml streptomycin (15140122, Thermofisher scientific, USA); and RPMI1640 (A1049101, Thermofisher scientific, USA) per 100 ml were mixed and used.

[Table 3]

Name	Composition (ml)
FBS	10
Penicillin-Streptomycin	1
RPMI1640	89
Total volume	100

The cells to be used for the test were thawed, placed in a cell culture flask, and cultured in a 37°C, 5% CO₂ incubator (MCO-170M, Panasonic, Japan). The cells were suspended using Trypsin-EDTA (Cat. 25200-072, Thermofisher scientific, USA). The suspended cells were collected by centrifugation (125×g, 5 minutes) using a centrifuge, transferred to a new medium and a new flask, and passage cultured. The cultured cells were put to a centrifugation tube on the day of cell line transplantation and then collected. Thereafter, centrifugation (125×g, 5 minutes) was performed to discard the supernatant, and a cell suspension (5×10^6 cells/0.05 ml) was prepared with PBS (Cat. LB 001-04, Welgene, KOREA) and stored on ice until inoculation. 8-week-old female NSG^{b2m} (NOD.Cg-B2m^{tm1Unc}Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ) mice were purchased from Joongang Bio (Korea) and used for the test. The body weight was measured the next day after the end of the quarantine and acclimation period, and then the human-derived PBMC cell

suspension (5×10^6 cells/0.2 ml) prepared for healthy animals was filled into a disposable syringe and administered to the caudal vein of the animals. General symptoms were observed once a day after cell transplantation.

MATRIGEL matrix phenol red-free (0.05 ml, 356237, BD, USA) was added to the prepared MDA-MB-231 cell suspension (5×10^6 cells/0.05 ml) to prepared the solution, and the solution was filled into a disposable syringe, and transplantation of the solution was performed by subcutaneous administration at 0.1 ml/head in the right dorsal region of the animals transplanted with human PBMCs. General symptoms were observed once a day during the engraftment and growth period after cell line transplantation.

A certain period of time after the cell transplantation, the tumor volume was measured for animals with no abnormalities in the health condition of the animals, and 32 subjects were selected so that the average of each group reached 40 to 80 mm³. The selected animals were grouped into a total of 4 groups as evenly as possible based on tumor volume and body weight, each group including 8 animals.

As shown in Table 4, the test groups were configured. The test substance was administered to the animals using a disposable syringe (31G, 1 ml), and the administration frequency was 2 times/week, a total of 4 administrations were performed.

[Table 4]

Group		Dosage amount (mg/kg)	Dosage volume (ml/kg)	Number of animals
G1	hIgG4	6	10	8
G2	GI101	6	10	8
G3	Keytruda	5	10	8
G7	GI101 + Keytruda	6 + 5	10	8

General symptoms such as appearance, behavior, and excrement were observed once a day during the observation period, and deceased animals were identified. Body weight was measured on the day of cell line transplantation, twice a week, and on the day of animal sacrifice. The major axis (maximum length, L) and minor axis (perpendicular width, W) of the tumor were measured using a caliper (Digital caliper, mitutoyo, Japan) three times a week during the observation period, and the tumor volume (TV) was calculated by substituting them into the following equations.

[Equation 1]

$$TV (mm^3) = (W^2 \times L)/2$$

[Equation 2]

$$\%TGI (\text{Tumor Growth Inhibition}) = (1 - (T_i - T_0) / (V_i - V_0)) \times 100$$

The tumor volume before administration of each subject was set as the value measured at the time of grouping.

As a result of administering the drugs shown in Table 4 on days 21, 25, 28, and 31 after tumor transplantation, respectively, the group having received each of GI101 and Keytruda exhibited tumor growth inhibition as compared with the control (hIgG4). The group having received a combination of GI101 and Keytruda exhibited tumor growth inhibition as compared with the control. The group having received a combination of GI101 and Keytruda exhibited tumor growth inhibition as compared with the groups having received each of GI101 and Keytruda (Fig. 60).

As a result of calculating the tumor growth inhibition rate at the end of the experiment (after tumor transplantation, day 42) as compared with on day 1 of drug treatment (after tumor transplantation, day 21), the group having received hIgG4 exhibited the tumor growth inhibition rate of 30% or more in 2 mice, 50% or more in 1 mouse, and 80% or more in 1 mouse. In addition, the group having received GI101 exhibited the tumor growth inhibition rate of 30% or more in 5 mice, 50% or more in 5 mice, and 80% or more in 2 mice, and the group having received Keytruda exhibited the tumor growth inhibition rate of 30% or more in 7 mice, 50% or more in 5 mice, and 80% or more in 3 mice. In addition, the group having received a combination of GI101 and Keytruda exhibited the tumor growth inhibition rate of 30% or more in 8 mice, 50% or more in 8 mice, and 80% or more in 6 mice (Fig. 61).

In addition, the degree of tumor growth of individual experimental animals of each treatment group when a combination of GI101 and Keytruda is used in mice transplanted with human-derived breast cancer cells is shown in Figs. 62 to 66.

Experimental Example 21. Identification of anticancer effect by administration of combination of mGI101 and anti-PD-1 antibody in mice transplanted with mouse-derived colorectal cancer cells

This test was to evaluate the tumor growth inhibitory effect after intraperitoneal administration of mGI101 as a test substance alone or in combination with an anti-PD-1 antibody as a positive control substance in a tumor model allotransplanted with MC38 into C57BL/6 mice.

Rodent-derived colorectal cancer cells, MC38, were purchased from Kerafast (USA) and used for the test. MC38 cells were cultured in RPMI1640 medium (Gibco) containing 10% fetal bovine serum (Gibco) and 1% antibiotic/antifungal agent (Gibco). The cultured cells were harvested using trypsin and then suspended in PBS. In order to establish an allotransplanted tumor model, 1×10^6 MC38 cells were subcutaneously injected into the right flank of C57BL/6 female mice (7-week-old).

The mice were randomly assigned based on tumor volume (30 mm^3), each group including 5 mice. The tumor grafts were identified about day 2 after cell inoculation. As shown in Table

5, the test groups were configured and the test substances were administered.

[Table 5]

Experimental group		Route of administration, dosing cycle	Dosage amount	Number of animal
G1	Vehicle control (hIgG4)	<i>i.p.</i> BIW x 16 days	10 mg/kg	5
G2	mGI101	<i>i.p.</i> day 1, 5, 9	6 mg/kg	6
G3	Anti-PD-1 antibody (cloneRMP1-14, InVivoMab)	<i>i.p.</i> BIW x 16 days	5 mg/kg	5
G4	mGI101 + anti-PD-1 antibody	<i>i.p.</i> day 1, 5, 9 (mGI101)	0.6 mg/kg	5
		<i>i.p.</i> BIW x 16 days (anti-PD-1 antibody)	5 mg/kg	

Clinical symptoms such as a disease and a behavioral change were observed once a day during the test period, and deceased animals were identified. At the end of the test period, the animals were sacrificed. The size of the MC38 solid cancer was measured using a tumor 3D scanner (TM900, Peria, Belgium). For each experimental group, the average loss and percentage change of body weight and the average tumor growth inhibition were calculated. The anti-tumor efficacy was evaluated as compared with the vehicle control. All statistical calculations were performed using Prism 8.0 (Graph Pad Software Inc, USA). The comparison of tumor volume measurements was made through one-way analysis of variance (at the end of this test) followed by Bonferroni's multiple comparison test. A p value of less than 0.05 was considered significant.

All test animals maintained a healthy state without signs of pathological abnormalities after administration of mGI101 alone and with combination in an anti-PD-1 antibody. The results of combination therapy using mGI101 and/or an anti-PD-1 antibody against the MC38 tumor are shown in Fig. 67. The anticancer effect was observed in the group having received the drug as compared with the control, and the difference in tumor size was noticeable during the test period of 16 days. The MC38 tumor is known as a model responsive to an anti-PD-1 antibody in previous literature, and the anticancer effect was also observed in the group having received the anti-PD-1 antibody of this test ($p > 0.01$). The anticancer effect was also shown in the group having received mGI101 (6 mpk) alone as well as the group having received the anti-PD-1 antibody ($p > 0.01$). The group having received a combination of mGI101 (0.6 mpk) + anti-PD-1 (5 mpk) exhibited a remarkably excellent anticancer effect ($p > 0.0001$).

Individual tumor sizes for each test group are shown in Figs. 69 to 73. According to the results of individual tumor sizes, slight tumor regression was observed in some animals of the group having received the anti-PD-1 antibody. The group having received mGI101 (6 mpk) alone exhibited a more excellent tumor growth inhibitory effect as compared with the group having

received the anti-PD-1 antibody. The tumor size was maintained at the same size from day 5 to day 7, but was regrown after day 7. The group having received the combination (GI101 (0.6 mpk) + an anti-PD-1 antibody (5 mpk)) exhibited a remarkably excellent tumor growth inhibition. In particular, two mice of the group having received the combination showed a complete remission (no tumor).

MC38 cells were reinjected into the left flank of two mice of the group having received the combination that showed a complete remission (the site opposite to the first injection site of cancer cells). These mice maintained an anti-PD-1 antibody administration (5 mpk, BIW) until day 32 (Fig. 74). A tumor with a small size ($> 30 \text{ mm}^3$) was observed in one of the two mice, but the tumor size did not grow any more until day 35 (Fig. 69). In another mouse, no tumor was observed after the tumor was reinjected (Figs. 69 and 74).

In conclusion, as a result of testing the anti-tumor efficacy of mGI101 alone and in combination with an anti-PD-1 antibody in the MC38 allotransplanted tumor model, the most excellent anti-tumor efficacy was shown in the group having received the combination (GI101 (0.6 mpk) + anti-PD-1 (5 mpk)). Two of the experimental animals in the group having received the combination showed a complete remission, and the complete remission mice reinjected with MC38 showed a cancer resistance effect (Table 6).

[Table 6]

Days After treatment	CR mouse	
	No.1 Tumor Vol. (mm^3)	No.2 Tumor Vol. (mm^3)
D1	78.2	23.6
D2	84.5	13.5
D3	36.3	0
D4	0	0
D5	0	0
D6	0	0
D7	0	0
D8	0	0
D9	0	0
D10	0	0
D11	0	0
D12	0	0
D13	0	0
D14	0	0

D15	0	0
D16	0	0
D17	0	0
D18	0	0
D19	0	0
D20	0	0
D21	0	0
D22	0	0
D23	0	0
D24	0	0
*D25	0	0
D26	0	0
D27	0	0
D28	0	0
D29	0	0
D30	0	0
D31	0	0
D32	12.8	0

Experimental Example 22. Identification of anticancer effect by administration of combination of mGI101 and anti-PD-L1 antibody in mice transplanted with mouse-derived colorectal cancer cells

This test was to evaluate the tumor growth inhibitory effect after administration of mGI101 as a test substance alone or in combination with an anti-PD-L1 antibody (BioXcell, Cat# BE0101) as a positive control substance in a tumor model allotransplanted with CT26 cells (murine colon carcinoma cells) into BALB/c mice.

CT26 cells were cultured in RPMI1640 medium (Gibco) containing 10% fetal bovine serum (Gibco) and 1% antibiotic/antifungal agent (Gibco). The cultured cells were harvested using trypsin and then suspended in PBS. In order to establish an allotransplanted tumor model, 5×10^5 CT26 cells were subcutaneously injected into the right flank of BALB/c female mice (7-week-old).

The mice were randomly assigned based on tumor volume (50 to 120 mm³), each group including 4 mice. The tumor grafts were identified about day 2 after cell inoculation. As shown in Table 7, the test groups were configured and the test substances were administered.

[Table 7]

Experimental group	Route of administration,	Dosage	Number of
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		dosing cycle	amount	animals
G1	Vehicle control (PBS)	<i>i.p.</i> BIW x 9 days	-	4
G2	mGI101	<i>i.v.</i> QW x 9 days	3 mg/kg	4
G3	Anti-PD-L1 antibody (BioXcell, Cat# BE0101)	<i>i.p.</i> BIW x 9 days	10 mg/kg	4
G4	mGI101 + anti-PD-L1 antibody	<i>i.v.</i> QW x 9 days (mGI101)	3 mg/kg	4
		<i>i.p.</i> BIW x 9 days (anti-PD-L1 antibody)	10 mg/kg	

Clinical symptoms such as a disease and a behavioral change were observed once a day during the test period, and deceased animals were identified. At the end of the test period, the animals were sacrificed. The size of the CT26 solid cancer was measured using a tumor 3D scanner (TM900, Peria, Belgium). For each experimental group, the average loss and percentage change of body weight and the average tumor growth inhibition were calculated. The anti-tumor efficacy was evaluated as compared with the vehicle control. All statistical calculations were performed using Prism 8.0 (Graph Pad Software Inc, USA). The comparison of tumor volume measurements was made through one-way analysis of variance (at the end of this test) followed by Bonferroni's multiple comparison test. A p value of less than 0.05 was considered significant.

As a result of testing the anti-tumor efficacy of mGI101 alone and in combination with an anti-PD-L1 antibody in the CT26 allotransplanted tumor model, the most excellent anti-tumor efficacy was shown in the group having received the combination (mGI101 (3 mpk) + anti-PD-L1 (10 mpk)) (Fig. 75).

Experimental Example 23. Identification of anticancer effect by administration of combination of mGI101 and anti-TIGIT antibody in mice transplanted with mouse-derived colorectal cancer cells

This test was to evaluate the tumor growth inhibitory effect after administration of mGI101 as a test substance alone or in combination with an anti-TIGIT antibody specifically binding to an extracellular domain (ECD) of TIGIT as a positive control substance in a tumor model allotransplanted with CT26 cells (murine colon carcinoma cells) into BALB/c mice.

CT26 cells were cultured in RPMI1640 medium (Gibco) containing 10% fetal bovine serum (Gibco) and 1% antibiotic/antifungal agent (Gibco). The cultured cells were harvested using trypsin and then suspended in PBS. In order to establish an allotransplanted tumor model, 5×10^5 CT26 cells were subcutaneously injected into the right flank of BALB/c female mice (7-week-old).

The mice were randomly assigned based on tumor volume (50 to 120 mm³), each group

including 5 mice. The tumor grafts were identified about day 2 after cell inoculation. As shown in Table 8, the test groups were configured and the test substances were administered.

[Table 8]

Experimental group		Route of administration, dosing cycle	Dsage amount	Number of animals
G1	Vehicle control (PBS)	<i>i.p.</i> BIW x 9 days	-	5
G2	mGI101	<i>i.v.</i> QW x 9 days	3 mg/kg	5
G3	Anti-TIGIT antibody	<i>i.p.</i> BIW x 9 days	20 mg/kg	5
G4	mGI101 + anti-TIGIT antibody	<i>i.v.</i> QW x 9 days (mGI101)	3 mg/kg	5
		<i>i.p.</i> BIW x 9 days (anti-TIGIT antibody)	20 mg/kg	

Clinical symptoms such as a disease and a behavioral change were observed once a day during the test period, and deceased animals were identified. At the end of the test period, the animals were sacrificed. The size of the CT26 solid cancer was measured using a tumor 3D scanner (TM900, Peria, Belgium). For each experimental group, the average loss and percentage change of body weight and the average tumor growth inhibition were calculated. The anti-tumor efficacy was evaluated as compared with the vehicle control. All statistical calculations were performed using Prism 8.0 (Graph Pad Software Inc, USA). The comparison of tumor volume measurements was made through one-way analysis of variance (at the end of this test) followed by Bonferroni's multiple comparison test. A p value of less than 0.05 was considered significant.

As a result of testing the anti-tumor efficacy of mGI101 alone and in combination with an anti-TIGIT antibody in the CT26 allotransplanted tumor model, the most excellent anti-tumor efficacy was shown in the group having received the combination (mGI101 (3 mpk) + anti-TIGIT (20 mpk)) (Fig. 76). No anti-tumor effect was observed in the group having received the anti-TIGIT antibody alone as compared with the control, but the group having received a combination with mGI101 exhibited a remarkably excellent anti-tumor effect as compared with the group having received mGI101 alone.

VI. Identification of anticancer effect according to administration of combination of fusion protein dimer and TGF- β R inhibitor

Experimental Example 24. Identification of anticancer effect by administration of combination of mGI-101 and TGF- β R inhibitor (Galunisertib) in mice transplanted with mouse-derived colorectal cancer cells

This test was to evaluate the tumor growth inhibitory effect after administration of mGI-

101 as a test substance alone or in combination with Galunisertib as a positive control substance in a tumor model allotransplanted with CT26 (mouse colon carcinoma) cells into mice.

The stock solution of the test substance, negative control substance, and positive control substance described in Table 9 was diluted by adding excipients according to each dose.

[Table 9]

-	Test substance	Positive control substance	Negative control substance
Substance name	mGI-101	Galunisertib	hIgG4
Appearance	clear liquid	clear liquid	clear liquid
Component	Fc fusion protein	TGF- β inhibitor	-
Excipient	histidine buffer (20 mM), pH 7.0, poloxamer 188 0.07 w/w%, arginine-HCl 15 mg/mL, sucrose 150 mg/mL	1% CMC (carboxymethylcellulose)-Na	PBS
pH	7.5	-	-
Storage condition	refrigerated storage (4°C)	refrigerated storage (4°C)	refrigerated storage (4°C)
Precautions for handling	keep refrigerated until administration, and prepare and use on the day of administration	keep refrigerated until administration, and prepare and use on the day of administration	keep refrigerated until administration, and prepare and use on the day of administration

Mouse-derived colorectal cancer cells, CT26 (*Mus musculus*, Colon adenocarcinoma), were purchased from ATCC (USA) and used for the test. The cells to be used for the test were thawed, mixed with RPMI1640 (A1049101, Thermofisher scientific) medium containing 10% FBS (fetal bovine serum, Gibco, 10082-147), and then placed in a cell culture flask, and cultured in a 37°C, 5% CO₂ incubator. The cells were washed with PBS, and then the cells were isolated using Trypsin-EDTA (15090, Gibco), and centrifugation (125×g, 5 minutes) was performed to discard the supernatant, and then the cells were suspended in a new medium to obtain the cell suspension. The viability of the cells was identified, and then a cell line was prepared by diluting in a medium to a concentration of 5.0×10^6 cells/mL.

6-week-old male BALB/cAnHsd mice were purchased and used for the test. After the end of the acclimation period of 7 days, the cells were transplanted into healthy animals. The cell suspension (5×10^6 cells/mL) was dispensed, and filled into a disposable syringe, and transplantation of the suspension was performed by subcutaneous administration at 0.1 mL/head in the right dorsal region of the animals. General symptoms were observed once a day during the

engraftment and growth period after cell line transplantation.

After the inoculation of the cell line was performed, when the tumor size at the site to which the cell line was transplanted reached about 50 mm³, the tumor size of each group was distributed as uniformly as possible according to the tumor size.

As shown in Table 10, the test groups were configured. The test substance was administered orally or intraperitoneally, and administered for 3 weeks depending on the composition of the test group. In the case of oral administration, the animals were fixed with the cervical spine skin fixation method, and administered directly into the stomach using a sonde for oral administration. In the case of intraperitoneal administration, the animals were fixed with the cervical spine skin fixation method, and administered intraperitoneally using a syringe equipped with a 26 gauge needle. The administration rate was not to exceed 200 µl/min.

[Table 10]

Group	Sex	Administered substance	Frequency of administration	Route of administration	Dosage amount (mg/kg/day)	Dosage volume (mL/kg/day)
G1	M	Vehicle	2 times/week	<i>i.p.</i>	-	5
G2	M	mGI-101	2 times/week	<i>i.p.</i>	3	5
G3	M	Galunisertib	3 times/week	<i>p.o.</i>	75	10
G4	M	mGI-101	2 times/week	<i>i.p.</i>	3	5
		Galunisertib	3 times/week	<i>p.o.</i>	75	10

The type of general symptoms including death, the date of onset, and the severity of symptoms were observed once a day during the administration and observation period, and recorded for each subject. Body weight was measured on the day of grouping or on the day of start of administration of the test substance, and thereafter once a week.

The tumor size was measured three times a week for 3 weeks from the day of start of administration of the test substance. The major axis and minor axis of the tumor were measured using a caliper, and the tumor size (tumor volume, TV) was calculated using the following equations.

[Equation 1]

$$TV (mm^3) = (W^2 \times L)/2$$

The tumor growth inhibition rate was calculated using the tumor size measurement results. The tumor growth inhibition rate was calculated as follows.

[Equation 2]

$$\%TGI (\text{Tumor Growth Inhibition}) = (1 - (T_i - T_0) / (V_i - V_0)) \times 100$$

As a result of measuring the tumor size, the tumor size levels of G2 and G4 on day 4 after the start of administration of the test substance were statistically significantly lower than that of G1 ($p < 0.01$, $p < 0.001$), and the tumor size levels of G2 and G4 on day 7 after the start of

administration of the test substance were statistically significantly lower than that of G1 ($p < 0.05$ or $p < 0.01$).

From day 2 to day 7 after the start of administration of the test substance, the tumor size levels of G2 and G4 tended to be lower than that of G1. In addition, until day 7 after the start of administration of the test substance, the tumor size levels of G4 was lower than that of G2 or G3 (Figs. 77 and 79).

[Table 11]

Tumor size				Unit: mm ³
Day	Experimental group			
	G1	G2	G3	G4
0	61.97 ± 12.41	61.96 ± 11.81	61.98 ± 12.23	61.98 ± 12.16
2	210.34 ± 32.30	177.83 ± 78.22	187.20 ± 55.14	149.16 ± 43.02
4	363.47 ± 36.18	224.56 ± 105.31**	260.69 ± 78.70	187.35 ± 60.13***
7	488.92 ± 81.97	291.81 ± 155.23 (9)*	398.78 ± 146.30	232.95 ± 99.73**
N	10	10	10	10

The results were expressed as mean ± standard deviation. N: number of animals, G1: Vehicle control IP, G2: mGI-101 3 mg/kg IP, G3: Galunisertib 75 mg/kg PO, G4: mGI-101 3 mg/kg IP + Galunisertib 75 mg/kg PO

***/**/* A significant difference at $p < 0.001/p < 0.01/p < 0.05$ level compared to the G1

As a result of calculating the tumor growth inhibition rate, from day 4 to day 7 after the start of administration of the test substance, the tumor growth inhibition level of G2 was statistically significantly higher than that of G1. From day 2 to day 7 after the start of administration of the test substance, the tumor growth inhibition level of G4 was statistically significantly higher than that of G1. In addition, on day 7, the tumor growth inhibition level of G4 was statistically significantly higher than that of G3.

At the end of the experiment, mice with the tumor growth inhibition rate of 30%, 50%, or 80% or more are as shown in Fig. 78.

[Table 12]

Tumor growth inhibition				Unit: %
Day	Experimental group			
	G1	G2	G3	G4
2	0.00 ± 20.74	21.91 ± 53.41	15.60 ± 38.26	41.24 ± 25.98*
4	0.00 ± 10.50	46.07 ± 35.1**	34.09 ± 27.14	58.42 ± 17.88***
7	0.00 ± 19.21	46.48 ± 36.39 (9)**	21.12 ± 34.98	59.96 ± 21.45***,\$
N	10	10	10	10

The results were expressed as mean $\pm$ standard deviation. N: number of animals, G1: Vehicle control IP, G2: mGI-101 3 mg/kg IP, G3: Galunisertib 75 mg/kg PO, G4: mGI-101 3 mg/kg IP + Galunisertib 75 mg/kg PO

***/**/* A significant difference at $p < 0.001/p < 0.01/p < 0.05$ level compared to the G1

\$ A significant difference at $p < 0.05$ level compared to the G3

Experimental Example 25. Identification of anticancer effect by combination of mGI-101 and TGF- β R inhibitor (Vactosertib) in breast cancer cell line

This experiment was to evaluate the effect of killing cancer cells by treating MDA-MB-231 cells (human breast cancer cells) with the test substance GI-101 alone or in combination with the TGF-beta signal inhibitor Vactosertib substance in an *in vitro* environment.

MDA-MB-231 cells were purchased from the Korea cell line bank and cultured in RPMI1640 medium (Gibco) containing 10% FBS (Gibco) and 1% antibiotic/antifungal agent (Gibco). For use in cancer cell killing test, the cells were harvested using trypsin (Gibco), and then suspended in RPMI1640 medium, and then dead cells and debris were removed using Ficoll (GE Healthcare Life Sciences) solution. The cells suspended in RPMI1640 medium were carefully layered on ficoll solution. The cell layer with a low specific gravity formed by centrifuging at room temperature at $350 \times g$ for 20 minutes was collected with a pipette, washed with PBS (Gibco), and then centrifuged at room temperature at $350 \times g$ for 5 minutes. The separated cell layer was made into a suspension of 2×10^5 cells/mL with FBS-free RPMI1640 medium. The cancer cell suspension was stained at 37°C for 1 hour using CELLTRACKER™ Deep Red Dye (Thermo) in order to track proliferation or inhibition of the proliferation of cancer cells. After staining, it was centrifuged at 1300 rpm for 5 minutes, and then it was washed with FBS-free RPMI1640 medium, and then suspended in RPMI1640 medium containing 5% human AB serum (Sigma) to a concentration of 2×10^5 cells/mL. The cancer cell suspension was added to each well of a 96-well microplate (Corning) by 50 μl (1×10^4 cells), and then stabilized in an incubator (37°C , 5% CO_2) for 1 hour.

Human peripheral blood mononuclear cells (PBMCs) were used in order to identify the effect of killing cancer cells by GI-101. The human PBMCs were purchased from Zen-Bio, and the PBMCs stored frozen were placed in a 37°C water bath, and thawed as quickly as possible, and then transferred to RPMI1640 medium (Gibco) containing 10% FBS (Gibco) and 1% antibiotic/antifungal agent (Gibco), and centrifuged at 1300 rpm for 5 minutes. The separated cell layer was suspended in RPMI1640 medium, and then dead cells and debris were removed using Ficoll (GE Healthcare Life Sciences) solution in the same manner as the cancer cell line. The cells suspended in RPMI1640 medium were carefully layered on ficoll solution. The cell layer with a low specific gravity formed by centrifuging at room temperature at $350 \times g$ for 20 minutes was collected with a pipette, washed with PBS (Gibco), and then centrifuged at room

temperature at 350×g for 5 minutes. The separated cell layer was suspended in RPMI1640 medium containing 5% human AB serum (Sigma) to a concentration of 5×10^5 cells/mL. The PBMC suspension was dispensed 50 µl into each well of a 96-well microplate (Corning) in which cancer cell line has been dispensed, depending on the conditions.

In order to identify the effect of killing the cells, a CytoTox Green reagent (INCUCYTE™ CytoTox Green, Satorius) that binds to the DNA of cells to be killed was prepared in 1 µl per 1 mL of RPMI1640 medium containing 5% human AB serum (Sigma). The prepared medium was used for dilution of the test substance, and the effect of killing the cells could be quantitatively identified by staining the cells to be killed when the test substance was co-cultured with cancer cell lines and PBMCs.

Vactosertib power was dissolved in DMSO (Sigma) to a concentration of 48.4 mM, and diluted using RPMI1640 medium containing a CytoTox Green reagent, and then used in the experiment at a final concentration of 12.1 nM (50 µL) per well of a 96-well microplate.

GI-101 was diluted by 1/3 using RPMI1640 medium containing a CytoTox Green reagent, and then used in the experiment at final concentrations of 0.4 nM, 1.2 nM, 3.7 nM, 11.1 nM, 33.3 nM, and 100 nM by 50 µl per well of a 96-well microplate.

The prepared test substance was placed in each well of a 96-well microplate in which cancer cell lines and PBMCs were dispensed depending on the conditions, and cultured in an incubator (37°C, 5% CO₂) for 24 hours, and the proliferation or death of cancer cells was observed through the real-time cell imaging analysis equipment IncuCyte S3 (Satorius). The death of cancer cells was quantified by the integrated intensity of the cells stained in green with a CytoTox Green reagent.

As a result, it was identified that the group having received a combination of GI-101 and Vactosertib exhibited the excellent effect of killing cancer cells as compared with the group having received each drug alone.

VII. Identification of anticancer effect according to administration of combination of fusion protein dimer and VEGFR inhibitor

Experimental Example 26. Identification of anticancer effect by administration of combination of mGI-101 and VEGFR inhibitor (Axitinib)

This test was to evaluate the tumor growth inhibitory effect after administration of mGI-101 as a test substance alone or in combination with Axitinib as a positive control substance in a tumor model allotransplanted with CT26 cells (mouse colon carcinoma) or LL2 cells (mouse lung carcinoma) into mice.

The stock solution of the test substance, negative control substance, and positive control substance described in Table 13 was diluted by adding excipients according to each dose.

[Table 13]

-	Test substance	Positive control substance	Negative control substance
Substance name	mGI-101	Axitinib	hIgG4
Appearance	clear liquid	clear liquid	clear liquid
Component	Fc fusion protein	VEGFR inhibitor	-
Excipient	histidine buffer (20 mM), pH 7.0, poloxamer 188 0.07 w/w%, arginine-HCl 15 mg/mL, sucrose 150 mg/mL	0.5 % CMC (carboxymethylcellulose)	PBS
pH	7.5	-	-
Storage condition	refrigerated storage (4°C)	refrigerated storage (4°C)	refrigerated storage (4°C)
Precautions for handling	keep refrigerated until administration, and prepare and use on the day of administration	keep refrigerated until administration, and prepare and use on the day of administration	keep refrigerated until administration, and prepare and use on the day of administration

Mouse-derived colorectal cancer cells, CT26 (*Mus musculus*, Colon adenocarcinoma), and mouse-derived lung cancer cells, LL/2 (*Mus musculus*, Lung adenocarcinoma), were purchased from ATCC (USA) and used for the test. The cells to be used for the test were thawed, mixed with RPMI1640 (A1049101, Thermofisher scientific) medium containing 10% FBS (fetal bovine serum, Gibco, 10082-147), and then placed in a cell culture flask, and cultured in a 37°C, 5% CO₂ incubator. The cells were washed with PBS, and then the cells were isolated using Trypsin-EDTA (15090, Gibco), and centrifugation (125×g, 5 minutes) was performed to discard the supernatant, and then the cells were suspended in a new medium to obtain the cell suspension. The viability of the cells was identified, and then a cell line was prepared by diluting in a medium to a concentration of 5.0×10^6 cells/mL.

6-week-old male BALB/cAnHsd mice or C57BL/6NHsd mice were purchased and used for the test. After the end of the acclimation period of 7 days, the cells were transplanted into healthy animals. The cell suspension (5×10^6 cells/mL) was dispensed, and filled into a disposable syringe, and transplantation of the suspension was performed by subcutaneous administration at 0.1 mL/head in the right dorsal region of the animals. General symptoms were observed once a day during the engraftment and growth period after cell line transplantation.

After the inoculation of the cell line was performed, when the tumor size at the site to which the cell line was transplanted reached about 50 mm³, the tumor size of each group was

distributed as uniformly as possible according to the ranked tumor size.

As shown in Table 14, the test groups were configured. The test substance was administered orally or intraperitoneally, and administered for 3 weeks depending on the composition of the test group. In the case of oral administration, the animals were fixed with the cervical spine skin fixation method, and administered directly into the stomach using a sonde for oral administration. In the case of intraperitoneal administration, the animals were fixed with the cervical spine skin fixation method, and administered intraperitoneally using a syringe equipped with a 26 gauge needle. The administration rate was not to exceed 200 μ l/min.

[Table 14]

Group	Sex	Administered substance	Frequency of administration	Route of administration	Dosage amount (mg/kg/day)	Dosage volume (mL/kg/day)
G1	M	Vehicle	2 times/week	IP	3	5
G2	M	mGI-101	2 times/week	IP	3	5
G3	M	Axitinib	3 times/week	PO	30	10
G4	M	mGI-101	2 times/week	IP	3	5
		Axitinib	3 times/week	PO	30	10

The tumor size was measured in the same manner as described in Experimental Example 24.

Experimental Example 26.1. Mouse tumor model allotransplanted with colorectal cancer (CT26)

Syngeneic model was prepared by subcutaneously transplanting the CT26 cell line into Balb/c mice, and then the test substance was administered to evaluate the anticancer effect.

As a result of measuring the tumor size, the tumor size level of G4 on day 18 and day 21 after the start of administration of the test substance was statistically significantly lower than that of G1 and G2 ($p < 0.0001$, $p < 0.01$, or $p < 0.05$). The tumor size level of G4 on day 21 after the start of administration of the test substance was statistically significantly lower than that of G3 ($p < 0.01$). In addition, the tumor size level of G3 on day 21 after the start of administration of the test substance was statistically significantly lower than that of G1 and G2 ($p < 0.001$, $p < 0.01$) (Figs. 80 and 82).

[Table 15]

Tumor size					Unit: mm ³
Day	Experimental group				
	G1	G2	G3	G4	
0	59.15 $\pm$ 11.65	63.53 $\pm$ 11.56	61.67 $\pm$ 12.58	59.58 $\pm$ 11.14	
2	205.25 $\pm$ 31.92	148.98 $\pm$ 55.26	169.28 $\pm$ 32.19	127.90 $\pm$ 24.99	

5	354.24 ± 32.53	186.53 ± 76.61	261.76 ± 61.86	178.53 ± 84.43
7	468.75 ± 75.04	249.93 ± 149.08	354.05 ± 104.90	224.92 ± 158.21
9	573.13 ± 101.41	290.71 ± 165.17	442.98 ± 148.70	235.75 ± 158.35
12	789.72 ± 88.58	352.46 ± 195.64	529.85 ± 206.76	254.91 ± 176.31*
14	1206.38 ± 229.96	520.04 ± 307.47**	671.72 ± 303.14*	254.76 ± 159.31****
16	1693.18 ± 210.83	962.28 ± 634.90**	970.72 ± 475.99**	381.38 ± 235.37****, #, \$
19	2037.27 ± 341.20	1395.48 ± 982.61**	1129.12 ± 639.16****	465.02 ± 285.88****, ####, \$\$
21	3082.01 ± 462.37	1987.45 ± 1320.62****	1710.57 ± 1133.79****	880.10 ± 632.96****, ####, \$\$
N	8	8	8	8

The results were expressed as mean ± standard deviation. N: number of animals, G1: Vehicle control IP, G2: mGI-101 3 mg/kg IP, G3: Axitinib 30 mg/kg PO, G4: mGI-101 3 mg/kg IP + Axitinib 30 mg/kg PO

****/**/* A significant difference at p<0.0001/p<0.01/p<0.05 level compared to the Vehicle

####/# A significant difference at p<0.0001/p<0.05 level compared to the mGI-101

\$\$\$/\$/\$ A significant difference at p<0.001/p<0.01/p<0.05 level compared to the Axitinib

As a result of calculating the tumor growth inhibition rate, from day 2 to day 21 after the start of administration of the test substance, the tumor growth inhibition levels of G2 and G4 were statistically significantly higher than that of G1 (p<0.0001, p<0.001, p<0.01 or p<0.05), and from day 9 to day 14 after the start of administration of the test substance, the tumor growth inhibition level of G4 was statistically significantly higher than that of G3 (p<0.05). In addition, on day 19 after the start of administration of the test substance, the tumor growth inhibition level of G4 was statistically significantly higher than that of G2 (p<0.05).

At the end of the experiment, mice with the tumor growth inhibition rate of 30%, 50%, or 80% or more are as shown in Fig. 81.

[Table 16]

Tumor growth inhibition					Unit: %
Day	Experimental group				
	G1	G2	G3	G4	
2	0.00 ± 22.76	41.51 ± 36.11*	26.34 ± 20.47	53.24 ± 19.71***	
5	0.00 ± 10.69	58.32 ± 24.95****	32.19 ± 18.64	59.69 ± 29.99***	
7	0.00 ± 19.60	62.12 ± 38.89****	28.62 ± 24.35	59.63 ± 39.68***	
9	0.00 ± 20.29	62.87 ± 34.95****	25.81 ± 27.77	65.72 ± 31.54****, #, \$	

12	0.00 ± 11.98	66.48 ± 29.50****	35.92 ± 27.44	73.26 ± 24.71****,\$
14	0.00 ± 20.33	65.87 ± 29.12****	46.82 ± 25.79**	82.99 ± 14.19****,\$
16	0.00 ± 13.20	52.36 ± 41.33**	44.37 ± 28.66**	80.31 ± 14.77****
19	0.00 ± 17.31	41.48 ± 52.17*	46.04 ± 31.96**	79.50 ± 14.72****,#
21	0.00 ± 15.45	44.57 ± 46.55**	45.45 ± 37.30**	72.86 ± 21.07****
N	8	8	8	8

The results were expressed as mean ± standard deviation. N: number of animals, G1: Vehicle control IP, G2: mGI-101 3 mg/kg IP, G3: Axitinib 30 mg/kg PO, G4: mGI-101 3 mg/kg IP + Axitinib 30 mg/kg PO

****/****/****/ A significant difference at p<0.0001/p<0.001/p<0.01/p<0.05 level compared to the Vehicle

A significant difference at p<0.05 level compared to the mGI-101 3 mg/kg

\$ A significant difference at p<0.05 level compared to the mGI-101 3 mg/kg + Axitinib 30 mg/kg

Experimental Example 26.2. Mouse tumor model allotransplanted lung cancer (LL/2)

Syngeneic model was prepared by subcutaneously transplanting the LL/2 cell line into C57BL/6 mice, and then the test substance was administered to evaluate the anticancer effect.

As a result of measuring the tumor size, G3 and G4 maintained a lower tumor size level than that of G1 until the end of the test. In addition, the tumor size level of G4 during the entire test period was the lowest level among all test groups, and the tumor size level of G4 on day 19 after the start of administration of the test substance was statistically significantly lower than that of G1 (p<0.05) and G2 (p<0.01).

The tumor size level of G4 on day 21 after the start of administration of the test substance was statistically significantly lower than that of all groups of G1 (p<0.0001), G2 (p<0.0001), and G3 (p<0.01). In addition, the tumor size level of G3 on day 21 after the start of administration of the test substance was statistically significantly lower than that of G1 (p<0.01) and G2 (p<0.001) (Figs. 83 and 85).

[Table 17]

Tumor size				Unit: mm ³
Day	Experimental group			
	G1	G2	G3	G4
0	55.75 ± 12.29	55.70 ± 11.48	55.77 ± 11.25	55.73 ± 9.99
2	96.34 ± 25.87	99.18 ± 19.96	88.73 ± 21.26	98.80 ± 24.93
5	181.94 ± 40.25	179.58 ± 39.71	179.31 ± 52.62	179.21 ± 58.26
7	460.71 ± 126.65	409.37 ± 72.98	362.30 ± 97.92	341.77 ± 91.55

9	791.60 ± 247.83	749.00 ± 174.47	584.17 ± 172.94	544.37 ± 155.89
12	1141.58 ± 236.27	1066.72 ± 307.18	892.90 ± 256.88	841.86 ± 267.92
14	1637.35 ± 402.07	1608.00 ± 572.50	1292.27 ± 385.43	1242.66 ± 424.70
16	2219.74 ± 442.05	2200.03 ± 850.12	1775.26 ± 462.50 (9)	1693.31 ± 579.80
19	3044.87 ± 518.05	3223.87 ± 1191.11	2551.08 ± 695.31 (9)	2215.02 ± 667.76 ^{*,##}
21	5285.56 ± 1120.41	5431.08 ± 1673.60	4236.33 ± 1060.18 (9) ^{**,##}	3255.38 ± 819.52 ^{****,####,\$\$}
N	10	10	10	10

The results were expressed as mean ± standard deviation. N: number of animals, G1: Vehicle control IP, G2: mGI-101 3 mg/kg IP, G3: Axitinib 30 mg/kg PO, G4: mGI-101 3 mg/kg IP + Axitinib 30 mg/kg PO

^{****/*} A significant difference at p<0.0001/p<0.05 level compared to the Vehicle

^{####/##} A significant difference at p<0.0001/p<0.01 level compared to the mGI-101 3 mg/kg

^{\$\$} A significant difference at p<0.01 level compared to the mGI-101 3 mg/kg + Axitinib 30 mg/kg

As a result of calculating the tumor growth inhibition rate, the tumor growth inhibition level of G4 on day 19 and day 21 after the start of administration of the test substance was statistically significantly higher than that of G2 (p<0.01, p<0.05), and the tumor growth inhibition level of G4 on day 21 after the start of administration of the test substance was statistically significantly higher than that of G1 (p<0.05). The lowest tumor size level was observed in G4, and the tumor growth inhibition rate also tended to be highest.

At the end of the experiment, mice with the tumor growth inhibition rate of 30%, 50%, or 80% or more are as shown in Fig. 84.

[Table 18]

Tumor growth inhibition				Unit: %
Day	Experimental group			
	G1	G2	G3	G4
2	0.00 ± 46.91	-7.12 ± 34.31	18.79 ± 28.38	-6.12 ± 53.19
5	0.00 ± 26.04	1.83 ± 27.19	2.10 ± 36.27	2.15 ± 43.77
7	0.00 ± 29.89	12.67 ± 19.15	24.30 ± 23.63	29.36 ± 21.69 [*]
9	0.00 ± 33.79	5.78 ± 24.08	28.19 ± 23.46	33.59 ± 20.83
12	0.00 ± 21.78	6.89 ± 28.47	22.90 ± 23.42	27.60 ± 24.47
14	0.00 ± 25.38	1.85 ± 36.39	21.82 ± 24.12	24.95 ± 26.67
16	0.00 ± 20.30	0.91 ± 39.37	20.58 ± 21.15 (9)	24.33 ± 26.59

19	0.00 ± 17.10	-5.99 ± 39.91	16.55 ± 23.06 (9)	27.76 ± 22.20 [#]
21	0.00 ± 21.32	-2.78 ± 32.04	20.08 ± 20.18 (9)	38.82 ± 15.63 ^{*,##}
N	10	10	10	10

The results were expressed as mean ± standard deviation. N: number of animals, G1: Vehicle control IP, G2: mGI-101 3 mg/kg IP, G3: Axitinib 30 mg/kg PO, G4: mGI-101 3 mg/kg IP + Axitinib 30 mg/kg PO

* A significant difference at p<0.05 level compared to the Vehicle

A significant difference at p<0.01/p<0.05 level compared to the mGI-101 3 mg/kg

Experimental Example 27. Identification of anticancer effect by administration of combination of mGI-101 and VEGFR inhibitor (Lenvatinib)

Experimental Example 27.1. Identification of anticancer effect by administration of combination of mGI-101 and Lenvatinib in mice transplanted with mouse-derived colorectal cancer cells

This experiment was to evaluate the tumor growth inhibitory effect after administration of mGI-101 as a test substance alone or in combination with Lenvatinib substance in a tumor model allotransplanted with CT26 cells (murine colon carcinoma cells) into BALB/c mice.

CT26 cells were purchased from ATCC (USA) and cultured in RPMI1640 medium (Gibco) containing 10% FBS (Gibco) and 1% antibiotic/antifungal agent (Gibco). The cultured cells were harvested using trypsin (Gibco) and then suspended in PBS. In order to establish an allotransplanted tumor model, 1×10^6 CT26 cells were subcutaneously injected into the right flank of BALB/c female mice (7-week-old). General symptoms were observed once a day during the engraftment and growth period after cell line transplantation.

A certain period of time after the cell transplantation, the tumor volume was measured for animals with no abnormalities in the health condition of the animals, and the mice were assigned so that the average tumor volume of each group was less than 70-100 mm³, each group including 10 mice. As shown in Table 19, the test groups were configured and the test substances were administered.

In the case of Lenvatinib powder, the dose was calculated and weighed, and it was prepared to the dose concentration using a 0.5% methyl cellulose excipient. In order to minimize the loss of the test substance, the doses for 3 or 4 days were weighed, and prepared using the excipient on the day of administration and injected.

[Table 19]

Experimental group		Route of administration	Dosing cycle	Dosage amount	Number of animals
G1	Vehicle control (PBS)	i.v.	QW	- mg/kg	10

			(once/week)		
G2	mGI-101	i.v.	QW (once/week)	3 mg/kg	10
G3	Lenvatinib	p.o.	daily	3 mg/kg	10
G4	mGI-101 + Lenvatinib	i.v. + p.o.	mGI-101: QW (once/week), Lenvatinib: daily	3 mg/kg + 3 mg/kg	10

Clinical symptoms such as a disease and a behavioral change were observed once a day during the test period, and deceased animals were identified, and the mice were sacrificed when the tumor size reached a size of 4,000 mm³. The size of the CT26 solid cancer was measured twice a week during the observation period, and the major axis (maximum length, L) and minor axis (perpendicular width, W) of the tumor were measured using a caliper (Digital caliper, Mitutoyo, Japan), and the tumor volume (TV) and the tumor growth inhibition rate (TGI) were calculated by substituting them into the following equations.

[Equation 1]

$$TV (mm^3) = (W^2 \times L)/2$$

[Equation 2]

$$\%TGI (\text{Tumor Growth Inhibition}) = (1 - (T_i - T_0) / (V_i - V_0)) \times 100$$

The tumor volume before administration of each subject was set as the value measured at the time of grouping.

All statistical calculations were performed using Prism 8.0 (Graph Pad Software Inc, USA). The comparison of tumor volume measurements was made through two-way analysis of variance followed by Tukey's multiple comparison test. A p value of less than 0.05 was considered significant.

The results of tumor size upon administration of mGI-101 alone or in combination with Lenvatinib substance against the CT26 tumor are shown in Fig. 86. As a result of measuring the tumor size, as compared with the control, the statistically significant anticancer effect was observed in the group having received Lenvatinib alone and the group having received a combination of mGI-101 + Lenvatinib. The tumor size level of the group having received Lenvatinib alone on day 18 and day 21 after the start of administration of the test substance was statistically significantly lower than that of the control ($p < 0.5$, $p < 0.1$). The tumor size level of the group having received a combination of mGI-101 + Lenvatinib on day 16, day 18, and day 21 after the start of administration of the test substance was statistically significantly lower than that of the control ($p < 0.5$, $p < 0.001$, $p < 0.0001$), and was statistically significantly lower than that of the group having received mGI-101 alone on day 18 and day 21 ($p < 0.01$, $p < 0.0001$).

Individual tumor sizes for each test group are shown in Fig. 88. According to the results of individual tumor sizes, the group having received a combination of mGI-101 + Lenvatinib exhibited an excellent tumor growth inhibitory effect as compared with the group having received mGI-101 alone.

At the end of the experiment, mice with the tumor growth inhibition rate of 30%, 50%, or 80% or more are as shown in Fig. 87. The vehicle control exhibited a tumor growth inhibition rate of 30% or more in 4 mice, 50% or more in 3 mice, and 80% or more in 1 mouse. The group having received mGI-101 alone exhibited a tumor growth inhibition rate of 30% or more in 6 mice, 50% or more in 2 mice, and 80% or more in 1 mouse. The group having received Lenvatinib alone exhibited a tumor growth inhibition rate of 30% or more in 6 mice, 50% or more in 4 mice, and 80% or more in 1 mouse. The group having received a combination of mGI-101 + Lenvatinib exhibited a tumor growth inhibition rate of 30% or more in 10 mice, 50% or more in 6 mice, and 80% or more in no mouse.

Experimental Example 27.2. Identification of anticancer effect by administration of combination of mGI-101 and Lenvatinib in mice transplanted with mouse-derived renal cancer cell line

This experiment was to evaluate the tumor growth inhibitory effect after administration of mGI-101 as a test substance alone or in combination with Lenvatinib substance in a tumor model allotransplanted with Renca cells (mouse renal cancer cells) into BALB/c mice.

Renca cells were purchased from ATCC (USA) and cultured in RPMI1640 medium (Gibco) containing 10% FBS (Gibco) and 1% antibiotic/antifungal agent (Gibco). The cultured cells were harvested using trypsin (Gibco) and then suspended in PBS. In order to establish an allotransplanted tumor model, 5×10^6 Renca cells were subcutaneously injected into the back of BALB/c female mice (8-week-old). General symptoms were observed once a day during the engraftment and growth period after cell line transplantation.

A certain period of time after cell inoculation of the tumor grafts of the mice, the tumor volume was measured for animals with no abnormalities in the health condition of the animals, and the mice were randomly selected and assigned, each group including 10 mice. As shown in Table 20, the test groups were configured and the test substances were administered.

[Table 20]

Experimental group		Route of administration	Dosing cycle	Dosage amount	Number of animals
G1	Vehicle control (PBS)	i.p.	QW (once/week)	- mg/kg	10
G2	mGI-101	i.p.	BIW (2	3 mg/kg	10

			times/week)		
G3	Lenvatinib	p.o.	daily	3 mg/kg	10
G4	mGI-101 + Lenvatinib	i.p. + p.o.	mGI-101: BIW (2 times/week), Lenvatinib: daily	3 mg/kg + 3 mg/kg	10

The death of the mouse, the type of general symptoms, the date of onset, and the severity of symptoms were observed once a day during the test period, and recorded for each subject. The size of the Renca solid cancer was measured twice a week during the observation period, and the major axis (maximum length, L) and minor axis (perpendicular width, W) of the tumor were measured using a vernier caliper, and the tumor volume (TV) and the tumor growth inhibition rate (TGI) were calculated by substituting them into the following equations.

[Equation 1]

$$TV (mm^3) = (W^2 \times L)/2$$

[Equation 2]

$$\%TGI (\text{Tumor Growth Inhibition}) = (1 - (Ti - T0) / (Vi - V0)) \times 100$$

The tumor volume before administration of each subject was set as the value measured at the time of grouping, and the anti-tumor efficacy was evaluated as compared with the vehicle control.

All statistical calculations were performed using Prism 8.0 (Graph Pad Software Inc, USA). The comparison of tumor volume measurements was made through two-way analysis of variance followed by Tukey's multiple comparison test. A p value of less than 0.05 was considered significant.

The results of tumor size upon administration of mGI-101 alone or in combination with Lenvatinib substance against the Renca tumor are shown in Fig. 89. As a result of measuring the tumor size, the tumor size of the group having received a combination of mGI-101 (BIW) + Lenvatinib on day 15 after the start of administration of the test substance was statistically significantly lower than that of vehicle control and the group having received mGI-101 (BIW) alone ($p < 0.05$).

Individual tumor sizes for each test group are shown in Fig. 91. According to the results of individual tumor sizes, the group having received a combination of mGI-101 (BIW) + Lenvatinib exhibited an excellent tumor growth inhibitory effect.

Fig. 90 illustrates a tumor growth inhibition rate when mGI-101 and Lenvatinib are administered in combination in mice transplanted with Renca. The vehicle control exhibited a tumor growth inhibition rate of 30% or more in 4 mice, 50% or more in 3 mice, and 80% or more in 2 mice. The group having received mGI-101 alone once a week exhibited a tumor growth

inhibition rate of 30% or more in 5 mice, 50% or more in 2 mice, and 80% or more in 1 mouse. The group having received mGI-101 alone twice a week exhibited a tumor growth inhibition rate of 30% or more in 3 mice, 50% or more in 1 mouse, and 80% or more in no mouse. The group having received Lenvatinib alone exhibited a tumor growth inhibition rate of 30% or more in 4 mice, 50% or more in 2 mice, and 80% or more in no mouse. The group having received a combination of mGI-101 (BIW) + Lenvatinib exhibited a tumor growth inhibition rate of 30% or more in 8 mice, 50% or more in 6 mice, and 80% or more in 1 mouse.

VIII. Identification of anticancer effect according to administration of combination of fusion protein dimer and EGFR inhibitor

Experimental Example 28. Identification of anticancer effect of combination of mGI-101 and EGFR inhibitor (Cetuximab)

This experiment was to evaluate the effect of killing cancer cells by treating HCT116 cells (human colon cancer cells) with the test substance GI-101 alone or in combination with Cetuximab substance in an in vitro environment.

HCT116 cells were purchased from the Korea cell line bank and cultured in McCoy's 5A medium (ATCC) containing 10% FBS (Gibco) and 1% antibiotic/antifungal agent (Gibco). For use in cancer cell killing test, the cells were harvested using trypsin (Gibco), and then suspended in McCoy's 5A medium, and then dead cells and debris were removed using Ficoll (GE Healthcare Life Sciences) solution. The cells suspended in McCoy's 5A medium were carefully layered on ficoll solution. The cell layer with a low specific gravity formed by centrifuging at room temperature at $350\times g$ for 20 minutes was collected with a pipette, washed with PBS (Gibco), and then centrifuged at room temperature at $350\times g$ for 5 minutes. The separated cell layer was made into a suspension of 2×10^5 cells/mL with FBS-free RPMI1640 medium. The cancer cell suspension was stained at 37°C for 1 hour using CELLTRACKER™ Deep Red Dye (Thermo) in order to track proliferation of cancer cells or inhibition of the proliferation. After staining, it was centrifuged at 1300 rpm for 5 minutes, and then it was washed with FBS-free RPMI1640 medium, and then suspended in RPMI1640 medium containing 5% human AB serum (Sigma) to a concentration of 2×10^5 cells/mL. The cancer cell suspension was added to each well of a 96-well microplate (Corning) by 50 μL (1×10^4 cells), and then stabilized in an incubator (37°C , 5% CO_2) for 1 hour.

In order to identify the effect of killing cancer cells through antibody-dependent cellular cytotoxicity (ADCC) by the test substance, natural killer cells (NK cells) were isolated from human peripheral blood mononuclear cells (PBMCs) using a CD56+CD16+ NK cell isolation kit (Miltenyi Biotec) and used. In isolated NK cells, dead cells and debris were removed using Ficoll (GE Healthcare Life Sciences) solution in the same manner as the cancer cell line. The cells

suspended in RPMI1640 medium were carefully layered on ficoll solution. The cell layer with a low specific gravity formed by centrifuging at room temperature at 350×g for 20 minutes was collected with a pipette, washed with PBS (Gibco), and then centrifuged at room temperature at 350×g for 5 minutes. The separated cell layer was suspended in RPMI1640 medium containing 5% human AB serum (Sigma) to a concentration of 2×10^5 cells/mL. The PBMC suspension was dispensed 50 µl into each well of a 96-well microplate (Corning) in which cancer cell line has been dispensed, depending on the conditions.

In order to identify the effect of killing the cells, a CytoTox Green reagent (INCUCYTE™ CytoTox Green, Satorius) that binds to the DNA of cells to be killed was prepared in 1 µl per 1 mL of RPMI1640 medium containing 5% human AB serum (Sigma). The prepared medium was used for dilution of the test substance, and the effect of killing the cells could be quantitatively identified by staining the cells to be killed when the test substance was co-cultured with cancer cell lines and PBMCs.

Cetuximab was diluted using RPMI1640 medium containing a CytoTox Green reagent, and then used in the experiment at a final concentration of 68.6 nM (50 µl) per well of a 96-well microplate. GI-101 was diluted by 1/3 using RPMI1640 medium containing a CytoTox Green reagent, and then used in the experiment at a final concentration 100 nM by 50 µl per well of a 96-well microplate.

The prepared test substance was placed in each well of a 96-well microplate in which cancer cell lines and PBMCs were dispensed depending on the conditions, and cultured in an incubator (37°C, 5% CO₂) for 24 hours, and the proliferation or death of cancer cells was observed through the real-time cell imaging analysis equipment IncuCyte S3 (Satorius). The death of cancer cells was quantified by the integrated intensity of the cells stained in green with a CytoTox Green reagent.

Fig. 92 illustrates the degree of killing cancer cells measured when the cancer cells were treated with GI-101 at a concentration of 100 nM. All groups having received GI-101 alone, Cetuximab alone, and a combination of GI-101 + Cetuximab exhibited a high level of killing cancer cells, and the group having received a combination of GI-101 + Cetuximab exhibited the most excellent effect of killing cancer cells.

IX. Identification of anticancer effect according to administration of combination of fusion protein dimer and PARP inhibitor

Experimental Example 29. Identification of anticancer effect by administration of combination of mGI-101 and PARP inhibitor (Olaparib)

This experiment was to evaluate the tumor growth inhibitory effect after administration of mGI-101 as a test substance alone or in combination with Olaparib, a PARP inhibitor, in a tumor

model allotransplanted with 4T1 cells (mouse breast cancer cells) into BALB/c mice.

4T1 cells were purchased from ATCC (USA) and cultured in RPMI1640 medium (Gibco) containing 10% FBS (Gibco) and 1% antibiotic/antifungal agent (Gibco). The cultured cells were harvested using trypsin (Gibco) and then suspended in PBS. In order to establish an allotransplanted tumor model, 1×10^5 of 4T1 cells were subcutaneously injected into the back of BALB/c female mice (8-week-old). General symptoms were observed once a day during the engraftment and growth period after cell line transplantation.

A certain period of time after cell inoculation of the tumor grafts of the mice, the tumor volume was measured for animals with no abnormalities in the health condition of the animals, and the mice were randomly selected and assigned, each group including 11 mice. As shown in Table 21, the test groups were configured and the test substances were administered.

[Table 21]

Experimental group		Route of administration	Dosing cycle	Dosage amount	Number of animals
G1	Vehicle control (PBS)	i.p.	QW (once/week)	- mg/kg	11
G2	mGI-101	i.p.	BIW (2 times/week)	3 mg/kg	11
G3	Olaparib	P.O.	5 times/week	30 mg/kg	11
G4	mGI-101 + Olaparib	i.p. + P.O.	mGI-101: BIW (2 times/week), Olaparib: 5 times/week	3 mg/kg + 30 mg/kg	11

Clinical symptoms such as a disease and a behavioral change were observed once a day during the test period, and deceased animals were identified, and the mice were sacrificed when the tumor size reached a size of 4,000 mm³. The size of the 4T1 solid cancer was measured twice a week during the observation period, and the major axis (maximum length, L) and minor axis (perpendicular width, W) of the tumor were measured using a caliper (Digital caliper, Mitutoyo, Japan), and the tumor volume (TV) and the tumor growth inhibition rate (TGI) were calculated by substituting them into the following equations.

[Equation 1]

$$TV (mm^3) = (W^2 \times L)/2$$

[Equation 2]

$$\%TGI (\text{Tumor Growth Inhibition}) = (1 - (T_i - T_0) / (V_i - V_0)) \times 100$$

The tumor volume before administration of each subject was set as the value measured at the time of grouping, and the anti-tumor efficacy was evaluated as compared with the vehicle control.

All statistical calculations were performed using Prism 8.0 (Graph Pad Software Inc,

USA). The comparison of tumor volume measurements was made through two-way analysis of variance followed by Tukey's multiple comparison test. A p value of less than 0.05 was considered significant.

The results of tumor size upon administration of mGI-101 alone or in combination with Olaparib substance against the 4T1 tumor are shown in Fig. 94. As a result of measuring the tumor size, the anticancer effect was observed in the group having received the drug as compared with the control, and according to the results of tumor size levels, the group having received mGI-101 alone and in combination with Olaparib substance exhibited an excellent tumor growth inhibitory effect as compared with the group having received mGI-101 alone.

Experimental Example 30. Identification of anticancer effect by administration of combination of mGI-101 and PARP inhibitor (Talazoparib)

This experiment was to evaluate the effect of killing cancer cells by treating MDA-MB-231 cells (human breast cancer cells) with the test substance GI-101 alone or in combination with the PARP inhibitor Talazoparib substance in an *in vitro* environment.

MDA-MB-231 cells were purchased from the Korea cell line bank and cultured in RPMI1640 medium (Gibco) containing 10% FBS (Gibco) and 1% antibiotic/antifungal agent (Gibco). For use in cancer cell killing test, the cells were harvested using trypsin (Gibco), and then suspended in RPMI1640 medium, and then dead cells and debris were removed using Ficoll (GE Healthcare Life Sciences) solution. The cells suspended in RPMI1640 medium were carefully layered on ficoll solution. The cell layer with a low specific gravity formed by centrifuging at room temperature at 350×g for 20 minutes was collected with a pipette, washed with PBS (Gibco), and then centrifuged at room temperature at 350×g for 5 minutes. The separated cell layer was made into a suspension of 2×10^5 cells/mL with FBS-free RPMI1640 medium. The cancer cell suspension was stained at 37°C for 1 hour using CELLTRACKER™ Deep Red Dye (Thermo) in order to track proliferation of cancer cells or inhibition of the proliferation. After staining, it was centrifuged at 1300 rpm for 5 minutes, and then it was washed with FBS-free RPMI1640 medium, and then suspended in RPMI1640 medium containing 5% human AB serum (Sigma) to a concentration of 2×10^5 cells/mL. The cancer cell suspension was added to each well of a 96-well microplate (Corning) by 50 μ l (1×10^4 cells), and then stabilized in an incubator (37°C, 5% CO₂) for 1 hour.

Human peripheral blood mononuclear cells (PBMCs) were used in order to identify the effect of killing cancer cells by GI-101. The human PBMCs were purchased from Zen-Bio, and the PBMCs stored frozen were placed in a 37°C water bath, and thawed as quickly as possible, and then transferred to RPMI1640 medium (Gibco) containing 10% FBS (Gibco) and 1% antibiotic/antifungal agent (Gibco), and centrifuged at 1300 rpm for 5 minutes. The separated cell layer was suspended in RPMI1640 medium, and then dead cells and debris were removed

using Ficoll (GE Healthcare Life Sciences) solution in the same manner as the cancer cell line. The cells suspended in RPMI1640 medium were carefully layered on ficoll solution. The cell layer with a low specific gravity formed by centrifuging at room temperature at 350×g for 20 minutes was collected with a pipette, washed with PBS (Gibco), and then centrifuged at room temperature at 350×g for 5 minutes. The separated cell layer was suspended in RPMI1640 medium containing 5% human AB serum (Sigma) to a concentration of 5×10^5 cells/mL. The PBMC suspension was dispensed 50 µl into each well of a 96-well microplate (Corning) in which cancer cell line has been dispensed, depending on the conditions.

In order to identify the effect of killing the cells, a CytoTox Green reagent (INCUCYTE™ CytoTox Green, Satorius) that binds to the DNA of cells to be killed was prepared in 1 µl per 1 mL of RPMI1640 medium containing 5% human AB serum (Sigma). The prepared medium was used for dilution of the test substance, and the effect of killing the cells could be quantitatively identified by staining the cells to be killed when the test substance was co-cultured with cancer cell lines and PBMCs.

Talazoparib test substance was diluted using RPMI1640 medium containing a CytoTox Green reagent, and then used in the experiment at a final concentration of 0.57 nM (50 µl) per well of a 96-well microplate. GI-101 was diluted by 1/3 using RPMI1640 medium containing a CytoTox Green reagent, and then used in the experiment at final concentrations of 0.4 nM, 1.2 nM, 3.7 nM, 11.1 nM, 33.3 nM, and 100 nM by 50 µl per well of a 96-well microplate.

The prepared test substance was placed in each well of a 96-well microplate in which cancer cell lines and PBMCs were dispensed depending on the conditions, and cultured in an incubator (37°C, 5% CO₂) for 24 hours, and the proliferation or death of cancer cells was observed through the real-time cell imaging analysis equipment IncuCyte S3 (Satorius). The death of cancer cells was quantified by the integrated intensity of the cells stained in green with a CytoTox Green reagent.

As a result, it was identified that the group having received a combination of GO-101 and Talazoparib exhibited the excellent effect of killing cancer cells as compared with the group having received each drug alone.

X. Identification of anticancer effect according to administration of combination of fusion protein dimer and DNA methyltransferase inhibitor

Experimental Example 31. Identification of anticancer effect by administration of combination of mGI-101 and Guadecitabine in mice transplanted with mouse-derived colorectal cancer cells

This experiment was to evaluate the tumor growth inhibitory effect after administration of mGI-101 as a test substance alone or in combination with Guadecitabine substance that inhibits

DNA methylation in a tumor model allotransplanted with CT26 cells (murine colon carcinoma cells) into BALB/c mice.

CT26 cells were purchased from ATCC (USA) and cultured in RPMI1640 medium (Gibco) containing 10% FBS (Gibco) and 1% antibiotic/antifungal agent (Gibco). The cultured cells were harvested using trypsin (Gibco) and then suspended in PBS. In order to establish an allotransplanted tumor model, 5×10^5 of CT26 cells were subcutaneously injected into the right flank of BALB/c female mice (8-week-old) (Fig. 97).

The tumor grafts of the mice were identified about day 7 after cell inoculation, and the mice were randomly assigned based on tumor volume ($50\text{-}120 \text{ mm}^3$), each group including 13 mice. As shown in Table 22, the test groups were configured and the test substances were administered.

[Table 22]

Experimental group		Route of administration	Dosing cycle	Dosage amount	Number of animals
G1	Vehicle control (PBS)	i.p.	QW (once/week)	- mg/kg	13
G2	mGI-101	i.v.	QW (once/week)	0.6 mg/kg	13
G3	mGI-101	i.v.	QW (once/week)	3 mg/kg	13
G4	Guadecitabine	i.p.	total 4 times, 4 consecutive days	50 μg	13
G5	mGI-101 + Guadecitabine	i.v. + i.p.	mGI-101: QW (once/week),	0.6 mg/kg + 50 μg	13
G6	mGI-101 + Guadecitabine	i.v. + i.p.	Guadecitabine: total 4 times, 4 consecutive days	3 mg/kg + 50 μg	13

Clinical symptoms such as a disease and a behavioral change were observed once a day during the test period, and deceased animals were identified, and the mice were sacrificed when the tumor size reached a size of $4,000 \text{ mm}^3$. The size of the CT26 solid cancer was measured using a tumor 3D scanner (TM900, Peria, Belgium). For each experimental group, the average loss and percentage change of body weight and the average tumor growth inhibition were calculated. The anti-tumor efficacy was evaluated as compared with the vehicle control. All statistical calculations were performed using Prism 8.0 (Graph Pad Software Inc, USA). The

comparison of tumor volume measurements was made through two-way analysis of variance followed by Tukey's multiple comparison test. A *p* value of less than 0.05 was considered significant.

The results of tumor size upon administration of mGI-101 alone or in combination with Guadecitabine substance against the CT26 tumor are shown in Figs. 97 and 100. As a result of measuring the tumor size, the anticancer effect was observed in the group having received the drug as compared with the control, and the group having received a combination of mGI-101 and Guadecitabine exhibited a more excellent tumor growth inhibitory effect as compared with the group having received mGI-101 alone. The tumor size of the group having received a combination of mGI-101 (3 mg/kg) + Guadecitabine on day 7 after the start of administration of the test substance was statistically significantly lower than that of the control ($p < 0.05$). On day 10 after the start of administration of the test substance, the tumor size level of the group having received mGI-101 (0.6 mg/kg) alone tended to be lower than that of the control, and the tumor size level of the group having received mGI-101 (3 mg/kg) alone was statistically significantly lower than that of the control ($p < 0.05$). The tumor size levels of the group having received a combination of mGI-101 (0.6 mg/kg) + Guadecitabine and the group having received a combination of mGI-101 (3 mg/kg) + Guadecitabine were statistically significantly lower than that of the control ($p < 0.01$, $p < 0.001$).

Individual tumor sizes for each test group are shown in Figs. 99 and 102. According to the results of individual tumor sizes, the group having received mGI-101 alone also exhibited the tumor growth inhibitory effect, and the group having received a combination mGI-101 and Guadecitabine substance exhibited the most excellent tumor growth inhibitory effect.

At the end of the experiment, mice with the tumor growth inhibition rate of 30%, 50%, or 80% or more are as shown in Figs. 99 and 101. It was identified that subjects with the tumor growth inhibition rate of 30%, 50%, 80% or more were the most in the group having received a combination of mGI-101 (3 mg/kg) + Guadecitabine.

XI. Identification of anticancer effect according to administration of combination of fusion protein dimer and anticancer chemotherapeutic agent (antineoplastic or cytotoxic agent)

Experimental Example 32. Identification of anticancer effect by administration of combination of mGI-101, anti-PD-L1 antibody, and Docetaxel in mice transplanted with mouse-derived breast cancer cells

This experiment was to evaluate the anticancer efficacy according to administration of the test substance mGI-101 alone or in combination with Docetaxel (Selleck Chemicals, cat.no. S1148) and the anti-PD-L1 antibody (BioXcell, cat.no. BE0101) in a tumor model allotransplanted with 4T1 cells (mouse breast cancer cells) into BALB/c mice.

4T1 cells were purchased from ATCC (USA) and cultured in RPMI1640 medium (Gibco) containing 10% FBS (Gibco) and 1% antibiotic/antifungal agent (Gibco). The cultured cells were harvested using trypsin (Gibco), and then a cell suspension was prepared with PBS and stored on ice until injected into mice. In order to establish an allotransplanted tumor model, after identifying the location of the second mammary fat pad from the upper right of the ventral region of BALB/c female mice (8-week-old), 4T1 cell line prepared inside the mammary fat pad was injected at 4×10^4 cells/40 μ l/head.

A certain period of time after cell inoculation of the tumor grafts of the mice, the tumor volume was measured for animals with no abnormalities in the health condition of the animals, and the subjects were selected so that the average of each group reached less than 70-100 mm³, and the selected animals were assigned as evenly as possible based on tumor volume and body weight, each group including 10 animals. As shown in Table 23, the test groups were configured and the test substances were administered.

Docetaxel was dissolved in 100% DMSO, and then the volume was adjusted with 5% of DMSO, 30% of PEG300, 5% of Tween 80, and 60% of distilled water for injection (DMSO : PEG300 : Tween 80 : distilled water for injection = 5% : 30% : 5% : 60% (v:v:v:v)) and prepared.

The Anti-PD-L1 antibody was prepared and administered using 1X PBS in consideration of dosage amount and volume.

[Table 23]

Experimental group		Route of administration	Dosing cycle	Dosage amount	Number of animals
G1	Vehicle control (PBS)	i.p.	QW (once/week)	- mg/kg	10
G2	mGI-101 + aPD-L1	i.p. + i.p.	mGI-101: QW (once/week),	3 mg/kg + 10 mg/kg	10
G3	Docetaxel	i.p.	aPD-L1: QW (once/week),	15 mg/kg	10
G4	Docetaxel + mGI-101 + aPD-L1	i.p. + i.p. + i.p.	Docetaxel: QW (once/week)	15 mg/kg + 3 mg/kg + 10 mg/kg	10

Clinical symptoms such as a disease and a behavioral change were observed once a day during the test period, and deceased animals were identified, and the mice were sacrificed when the tumor size reached a size of 4,000 mm³. The size of the 4T1 solid cancer was measured twice a week during the observation period, and the major axis (maximum length, L) and minor axis (perpendicular width, W) of the tumor were measured using a caliper (Digital caliper, Mitutoyo, Japan), and the tumor volume (TV) and the tumor growth inhibition rate (TGI) were calculated by

substituting them into the following equations.

[Equation 1]

$$TV (mm^3) = (W^2 \times L)/2$$

[Equation 2]

$$\%TGI (\text{Tumor Growth Inhibition}) = (1 - (T_i - T_0) / (V_i - V_0)) \times 100$$

The tumor volume before administration of each subject was set as the value measured at the time of grouping, and the anti-tumor efficacy was evaluated as compared with the vehicle control.

All statistical calculations were performed using Prism 8.0 (Graph Pad Software Inc, USA). The comparison of tumor volume measurements was made through two-way analysis of variance followed by Tukey's multiple comparison test. A p value of less than 0.05 was considered significant.

After administration of mGI-101 alone or in combination with Docetaxel and anti-PD-L1 antibody to mice transplanted with mouse-derived breast cancer cells, the results of measuring the tumor size are shown in Fig. 103. As a result of measuring the tumor size, on day 14 after the start of administration of the test substance, as compared with the control, the statistically significant anticancer effect was observed in the group having received Docetaxel and the group having received a combination of Docetaxel + mGI-101 + aPD-L1 ($p < 0.05$, $p < 0.01$).

Individual tumor sizes for each test group are shown in Fig. 105.

Fig. 104 illustrates a tumor growth inhibition rate when mGI-101 is administered alone or in combination with Docetaxel and an anti-PD-L1 antibody in mice transplanted with 4T1 at the end of the test. The vehicle control exhibited a tumor growth inhibition rate of 30% or more, 50% or more in no mouse. The group having received a combination of mGI-101 + aPD-L1 exhibited a tumor growth inhibition rate of 30% or more in 1 mouse, and 50% or more in no mouse. The group having received Docetaxel exhibited a tumor growth inhibition rate of 30% or more in 5 mice, and 50% or more in 1 mouse. The group having received a combination of Docetaxel + mGI-101 + aPD-L1 exhibited a tumor growth inhibition rate of 30% or more in 6 mice, and 50% or more in 2 mice.

Experimental Example 33. Identification of anticancer effect by administration of combination of mGI-101 and Paclitaxel in mice transplanted with mouse-derived breast cancer cells

This experiment was to evaluate the tumor growth inhibitory effect after administration of mGI-101 as a test substance alone or in combination with Paclitaxel substance in a tumor model allotransplanted with EMT6 cells (mouse breast cancer cells) into BALB/c mice.

EMT6 cells were purchased from ATCC (USA) and cultured in Waymouth MB 751/1 medium (WELGENE) containing 10% FBS (Gibco) and 1% antibiotic/antifungal agent (Gibco).

The cultured cells were harvested using trypsin (Gibco), and then a high-concentration cell suspension (2×10^6 cells/0.4 mL) for 10 mice was prepared with PBS and stored on ice until injected into mice. In order to establish an allotransplanted tumor model, the skin was incised slightly away from the 4th nipple position from the upper right of the ventral region of BALB/c female mice (7-week-old), and the location of mammary fat pad at the incised site was identified, and then EMT6 cell line prepared inside the mammary fat pad was injected at 2×10^5 cells/40 μ L/head.

A certain period of time after cell inoculation of the tumor grafts of the mice, the tumor volume was measured for animals with no abnormalities in the health condition of the animals, and the subjects were selected so that the average of each group reached less than 70-100 mm³, and the selected animals were assigned as evenly as possible based on tumor volume and body weight, each group including 10 animals. As shown in Table 24 and Fig. 106, the test groups were configured and the test substances were administered.

[Table 24]

Experimental group		Route of administration	Dosing cycle	Dosage amount	Number of animals
G1	Vehicle control (PBS)	i.v.	QW (once/week)	- mg/kg	10
G2	mGI-101	i.v.	QW (once/week)	3 mg/kg	10
G3	Paclitaxel (PTX)	i.p.	total 7 times, 7 consecutive days	10 mg/kg	10
G4	mGI-101 + Paclitaxel	i.v. + i.p.	QW (once/week) + total 7 times, 7 consecutive days	3 mg/kg + 10 mg/kg	10

Clinical symptoms such as a disease and a behavioral change were observed once a day during the test period, and deceased animals were identified, and the mice were sacrificed when the tumor size reached a size of 4,000 mm³. The size of the EMT6 solid cancer was measured twice a week during the observation period, and the major axis (maximum length, L) and minor axis (perpendicular width, W) of the tumor were measured using a caliper (Digital caliper, Mitutoyo, Japan), and the tumor volume (TV) and the tumor growth inhibition rate (TGI) were calculated by substituting them into the following equations.

[Equation 1]

$$TV \text{ (mm}^3\text{)} = (W^2 \times L)/2$$

[Equation 2]

$$\%TGI \text{ (Tumor Growth Inhibition)} = (1 - (T_i - T_0) / (V_i - V_0)) \times 100$$

The tumor volume before administration of each subject was set as the value measured at the time of grouping, and the anti-tumor efficacy was evaluated as compared with the vehicle control.

All statistical calculations were performed using Prism 8.0 (Graph Pad Software Inc, USA). The comparison of tumor volume measurements was made through two-way analysis of variance followed by Tukey's multiple comparison test. A *p* value of less than 0.05 was considered significant.

The results of tumor size upon administration of mGI-101 alone or in combination with Paclitaxel substance against the EMT6 tumor are shown in Fig. 107. As a result of measuring the tumor size, the anticancer effect was observed in the group having received the drug as compared with the control, and the tumor size level of the group having received mGI-101 alone and in combination with Paclitaxel substance on day 11 after the start of administration of the substance was statistically significantly lower than that of the control ($p < 0.01$, $p < 0.001$). The tumor size of all the group having received Paclitaxel, the group having received mGI-101 alone, and the group having received a combination of mGI-101 and Paclitaxel substance on day 13 after the start of administration of the substance was statistically significantly lower than that of the control ($p < 0.05$, $p < 0.01$, $p < 0.0001$).

Individual tumor sizes for each test group are shown in Fig. 109. According to the results of individual tumor sizes, on day 13 after the start of administration of the test substance, the group having received mGI-101 alone exhibited complete remission in 2 mice, and the group having received a combination of mGI-101 and Paclitaxel exhibited complete remission in 1 mouse.

Fig. 108 illustrates a tumor growth inhibition rate when mGI-101 and Paclitaxel are administered in combination in mice transplanted with EMT6. The vehicle control exhibited a tumor growth inhibition rate of 30% or more in 2 mice, 50% or more in 1 mouse, and 80% or more in no mouse. The group having received mGI-101 exhibited a tumor growth inhibition rate of 30% or more in 6 mice, 50% or more in 5 mice, and 80% or more in 5 mice. The group having received Paclitaxel exhibited a tumor growth inhibition rate of 30% or more in 7 mice, 50% or more in 4 mice, and 80% or more in 2 mice. The group having received a combination of mGI-101 + Paclitaxel exhibited a tumor growth inhibition rate of 30% or more in 7 mice, 50% or more in 5 mice, and 80% or more in 4 mice.

XII. Identification of anticancer effect according to administration of combination: mGI-101 + anti-PD-1 + Pemetrexed + Cisplatin (Chemotherapy, Maintaining therapy)

Experimental Example 34. Identification of anticancer effect by administration of

combination of mGI-101 and anticancer chemotherapeutic agent and anti-PD-1 antibody in mice transplanted with mouse-derived lung cancer cells

This experiment was to evaluate the tumor growth inhibitory effect after intraperitoneal administration of mGI-101 as a test substance alone or in combination with an anticancer chemotherapeutic agent, Cisplatin (Selleck Chemicals, cat.no. S1166), Pemetrexed (Selleck Chemicals, cat.no. S1135), and an anti-PD-1 antibody (BioXcell, cat.no. BE0146) as a standard therapeutic agent in a tumor model allotransplanted with TC1 cells, lung cancer cell line, into C57BL/6 mice. Mouse-derived lung cancer cell line, TC1, was purchased from ATCC (USA) and used for the test.

TC1 cells were cultured in RPMI1640 medium (Gibco) containing 10% FBS (Gibco) and 1% antibiotic/antifungal agent (Gibco). The cultured cells were harvested using trypsin (Gibco), and then 1×10^6 of TC1 cells were subcutaneously injected into the right flank of C57BL/6 female mice (7-week-old) in order to establish an allotransplanted tumor model.

The mice were randomly assigned based on tumor volume ($\sim 100 \text{ mm}^3$), each group including 13 to 19 mice. The tumor grafts were identified about day 2 after cell inoculation. The test groups were configured as shown in Table 25, and the test substances were administered according to the schedule shown in Fig. 110.

The test was divided into the 1st line treatment and the anticancer maintenance therapy. For the 1st line treatment, cisplatin (CDDP) at 5 mg/kg, pemetrexed at 100 mg/kg, and an anti-PD-1 antibody at 10 mg/kg were intraperitoneally administered twice a week, respectively, and in the case of the group having received a combination with mGI-101, it was intraperitoneally administered once a week, i.e., a total of 3 times, at 3 mg/kg.

In the anticancer maintenance therapy, mGI-101 was administered alone or mGI-101 was administered in combination with an anticancer chemotherapeutic agent and an anti-PD-1 antibody. [Table 25]

Experimental group		Route of administration, dosing cycle	Dosage amount	Number of animals
G1	mouse IgG4	i.p. BIW	3 mg/kg	13
G2	1) 1 st line: Cisplatin + Pemetrexed + anti-PD-1 antibody 2) Maintenance therapy: Pemetrexed + anti-PD-1 antibody	mGI-101: i.p. QW Cisplatin: i.p. BIW Pemetrexed: i.p. BIW anti-PD-1 antibody: i.p. BI	mGI-101: 3 mg/kg Cisplatin: 5 mg/kg Pemetrexed: 100 mg/kg anti-PD-1 antibody: 10 mg/k	19

G3	1) 1 st line: Cisplatin + Pemetrexed + anti-PD-1 antibody 2) Maintenance therapy: Pemetrexed + anti-PD-1 antibody + mGI-101			13
G4	1) 1 st line: Cisplatin + Pemetrexed + anti-PD-1 antibody 2) Maintenance therapy: mGI-101			13
G5	1) 1 st line: Cisplatin + Pemetrexed + anti-PD-1 antibody + mGI-101 2) Maintenance therapy: Pemetrexed + anti-PD-1 antibody + mGI-101			15

Clinical symptoms such as a disease and a behavioral change were observed once a day during the test period, and deceased animals were identified, and the mice were sacrificed when the tumor size reached a size of 4,000 mm³. The size of the TC1 solid cancer was measured using a tumor 3D scanner (TM900, Peria, Belgium). For each experimental group, the average loss and percentage change of body weight and the average tumor growth inhibition were calculated. The anti-tumor efficacy was evaluated as compared with the mouse IgG4 control. All statistical calculations were performed using Prism 8.0 (Graph Pad Software Inc, USA). The comparison of tumor volume measurements was made through two-way analysis of variance followed by Bonferroni's multiple comparison test. A p value of less than 0.05 was considered significant.

The results of administration of mGI-101 alone or in combination with an anticancer chemotherapeutic agent and an anti-PD-1 antibody against the TC1 tumor are shown in Fig. 111. The anticancer effect was observed in the group having received the drug as compared with the control, and the difference in tumor size was noticeable during the test period of 24 days. In the case of the 1st line treatment, the group having received mGI-101 in combination with an anticancer chemotherapeutic agent and an anti-PD-1 antibody exhibited a more excellent tumor growth inhibitory effect than that of the group having received only an anticancer chemotherapeutic agent and an anti-PD-1 antibody. In the case of the anticancer maintenance therapy, the group having received mGI-101 alone exhibited as much anticancer effect as the group having received an anticancer chemotherapeutic agent and an anti-PD-1 antibody, and the group having received mGI-101 in combination with an anticancer chemotherapeutic agent and an anti-

PD-1 antibody exhibited a more excellent tumor growth inhibitory effect.

Individual tumor sizes for each test group are shown in Figs. 112 to 117. According to the results of individual tumor sizes, in the 1st line treatment and the anticancer maintenance therapy, the group having received a combination with mGI-101 exhibited the most excellent tumor growth inhibitory effect.

Fig. 118 illustrates a result obtained by analyzing the survival rate of the mice according to the administration of a combination with mGI-101 during the 1st line treatment and the anticancer maintenance therapy in mice transplanted with TC1 cells. During the 1st line treatment and the anticancer maintenance therapy, a survival rate of 100% was identified in the group having received a combination with mGI-101. The average body weight of each test group is shown in Table 26.

[Table 26]

	Treatment		Body weight (g) on days											
	Treatment		-2	1	3	5	7	10	12	13	15	20	23	26
1	mouse IgG4	Mean	17.5 8	17.7 4	18.2 4	17.5 8	17.8 0	18.2 3	18.0 2	18.9 4	19.6 2	20.3 0	21.2 6	20.4 8
1	mouse IgG4	SEM	1.00	1.07	0.95	1.14	1.08	1.09	1.01	1.08	1.32	1.35	1.61	1.71
2	1 st line: Cisplatin + Pemetrexed + anti-PD-1 Maintenance: Pemetrexed + anti- PD-1	Mean	17.5 8	17.8 1	18.1 3	17.2 4	17.4 1	18.1 0	17.7 7	18.5 7	19.2 9	20.4 2	20.6 9	20.6 1
2	1 st line: Cisplatin + Pemetrexed +anti-PD-1 Maintenance: Pemetrexed + anti- PD-1	SEM	0.82	0.77	0.98	0.94	1.15	1.56	1.47	1.56	1.71	1.91	2.01	1.61
3	1 st line: Cisplatin + Pemetrexed + anti-PD-1 Maintenance: Pemetrexed + anti- PD-1 + mGI-101	Mean	17.4 2	17.7 3	18.0 6	17.4 2	17.7 8	18.5 4	18.1 1	19.0 1	19.2 3	19.7 2	20.2 8	20.1 8

3	1 st line: Cisplatin + Pemetrexed + anti-PD-1 Maintenance: Pemetrexed + anti-PD-1 + mGI-101	SEM	1.25	1.08	1.39	1.66	1.70	1.57	1.42	1.70	1.44	1.88	1.88	1.90
4	1 st line: Cisplatin + Pemetrexed + anti-PD-1 Maintenance: mGI-101	Mean	17.10	17.70	17.58	16.58	16.83	17.41	17.49	18.06	18.67	19.29	19.18	19.15
4	1 st line: Cisplatin + Pemetrexed + anti-PD-1 Maintenance: mGI-101	SEM	1.05	1.04	1.04	1.18	1.19	1.02	1.02	1.22	1.39	1.23	1.63	1.59
5	1 st line: Cisplatin + Pemetrexed + anti-PD-1 + mGI-101 Maintenance: Pemetrexed + anti-PD-1 + mGI-101	Mean	17.53	18.64	18.92	18.72	18.71	19.82	19.67	20.59	20.76	21.56	21.85	20.50
5	1 st line: Cisplatin + Pemetrexed + anti-PD-1 Maintenance: Pemetrexed + anti-PD-1 + mGI-101	SEM	0.99	0.85	1.01	0.96	0.89	1.18	1.15	0.94	1.34	1.67	1.69	0.71

XIII. Identification of anticancer effect according to administration of combination of fusion protein dimer and anti-HER antibody

Experimental Example 35. Identification of anticancer effect by administration of combination of GI-101 and Trastuzumab

This experiment was to evaluate the tumor growth inhibitory effect after administration of mGI-101 as a test substance alone or in combination with Herceptin (Trastuzumab) substance

in a tumor model xenotransplanted with BT-474 cells (human breast cancer cells) into BALB/c nu/nu mice.

BT-474 cells were purchased from ATCC (USA) and cultured in RPMI1640 medium (Welgene) containing 10% FBS (Gibco) and 1% antibiotic/antifungal agent (Gibco). The cultured cells were harvested using trypsin (Gibco), and then a cell line was prepared by diluting in a medium to a concentration of 5.0×10^6 cells/0.05 mL.

In order to establish a xenotransplanted tumor model for BT-474 cells, it was subcutaneously injected into the left flank of BALB/c nu/nu female mice (7-week-old) by pulling the leather skin of the mice to make a space using a pellet transplant trochar (MP-182, Innovative Research of America, USA) so that it was located under the skin of the left flank. 7 days after the injection of the estrogen pellet, the prepared BT-474 cell suspension (5×10^6 cells/0.05 mL) was dispensed, and 0.05 mL MATRIGEL matrix phenol red-free (356237, BD) was added, and the prepared solution was filled into a disposable syringe, and transplantation of the solution was performed by subcutaneous administration at 0.1 mL/head in the right dorsal region of the animals.

A certain period of time after cell inoculation of the tumor grafts of the mice, the tumor volume was measured for animals with no abnormalities in the health condition of the animals, and the subjects were selected so that the average of each group reached less than 60-120 mm³, and the selected animals were assigned as evenly as possible based on tumor volume and body weight, each group including 10 animals. As shown in Table 27, the test groups were configured and the test substances were administered.

[Table 27]

Experimental group		Route of administration	Dosing cycle	Dosage amount	Number of animals
G1	Vehicle control (PBS)	i.v.	QW (once/week)	- mg/kg	10
G2	mGI-101	i.v.	QW (once/week)	3 mg/kg	10
G3	Herceptin	i.p.	QW (once/week)	1 mg/kg	10
G4	mGI-101 + Herceptin	i.v. + i.p.	mGI-101: QW (once/week), Herceptin: QW (once/week)	3 mg/kg + 1 mg/kg	10

Clinical symptoms such as a disease and a behavioral change were observed once a day during the test period, and deceased animals were identified, and the mice were sacrificed when the tumor size reached a size of 4,000 mm³. The size of the solid cancer was measured twice a

week during the observation period, and the major axis (maximum length, L) and minor axis (perpendicular width, W) of the tumor were measured using a caliper (Digital caliper, Mitutoyo, Japan), and the tumor volume (TV) and the tumor growth inhibition rate (TGI) were calculated by substituting them into the following equations.

[Equation 1]

$$TV (mm^3) = (W^2 \times L)/2$$

[Equation 2]

$$\%TGI (\text{Tumor Growth Inhibition}) = (1 - (T_i - T_0) / (V_i - V_0)) \times 100$$

The tumor volume before administration of each subject was set as the value measured at the time of grouping.

All statistical calculations were performed using Prism 8.0 (Graph Pad Software Inc, USA). The comparison of tumor volume measurements was made through two-way analysis of variance followed by Tukey's multiple comparison test. A p value of less than 0.05 was considered significant.

The results of measuring the tumor size after administration of mGI-101 alone or in combination with Herceptin to mice transplanted with human-derived breast cancer cells are shown in Fig. 119. In the case of the group having received mGI-101 alone, a statistically significant reduction in tumor size was shown on day 28 after the start of administration of the test substance as compared with the vehicle control ($p < 0.05$). In the case of the group having received Herceptin alone, a statistically significant reduction in tumor size was shown on day 14, day 18, and day 21 after the start of administration of the test substance as compared with the vehicle control (in the case of day 14 and day 18, $p < 0.05$; in the case of day 21, $p < 0.01$), but the tumor size tended to be increased rapidly on day 25 and day 28. In the case of the group having received a combination of mGI-101 + Herceptin, a statistically significant reduction in tumor size was shown from day 14 to day 28 after the start of administration of the test substance as compared with the vehicle control, and a statistically significant reduction in tumor size was shown on day 28 after the start of administration of the test substance as compared with the group having received Herceptin alone ($p < 0.01$).

Individual tumor sizes for each test group are shown in Fig. 121. According to the results of individual tumor sizes, the group having received the test substance exhibited a tumor growth inhibitory effect as compared with the vehicle control, and in particular, the group having received a combination of mGI-101 + Herceptin exhibited an excellent tumor growth inhibitory effect as compared with the group having received mGI-101 alone.

Fig. 120 illustrates a tumor growth inhibition rate when mGI-101 is administered alone or in combination with Herceptin in tumor mice xenotransplanted with BT-474 at the end of the test. The vehicle control exhibited a tumor growth inhibition rate of 30% or more in 3 mice, 50% or

more in 3 mice, and 80% or more in no mouse. The group having received mGI-101 alone exhibited a tumor growth inhibition rate of 30% or more in 5 mice, 50% or more in 1 mouse, and 80% or more in no mouse. The group having received Herceptin alone exhibited a tumor growth inhibition rate of 30% or more in 3 mice, 50% or more in 2 mice, and 80% or more in 1 mouse. The group having received a combination of mGI-101 + Herceptin exhibited a tumor growth inhibition rate of 30% or more in 5 mice, 50% or more in 4 mice, and 80% or more in no mouse.

Experimental Example 36. Identification of anticancer effect by administration of combination of GI-101 and Pertuzumab

This experiment was to evaluate the effect of killing cancer cells by treating HCT116 cells (human colon cancer cells) with the test substance GI-101 alone or in combination with Pertuzumab substance in an *in vitro* environment.

HCT116 cells were purchased from the Korea cell line bank and cultured in McCoy's 5A medium (ATCC) containing 10% FBS (Gibco) and 1% antibiotic/antifungal agent (Gibco). For use in cancer cell killing test, the cells were harvested using trypsin (Gibco), and then suspended in McCoy's 5A medium, and then dead cells and debris were removed using Ficoll (GE Healthcare Life Sciences) solution. The cells suspended in McCoy's 5A medium were carefully layered on ficoll solution. The cell layer with a low specific gravity formed by centrifuging at room temperature at 350×g for 20 minutes was collected with a pipette, washed with PBS (Gibco), and then centrifuged at room temperature at 350×g for 5 minutes. The separated cell layer was made into a suspension of 2×10^5 cells/mL with FBS-free RPMI1640 medium. The cancer cell suspension was stained at 37°C for 1 hour using CELLTRACKER™ Deep Red Dye (Thermo) in order to track proliferation of cancer cells or inhibition of the proliferation. After staining, it was centrifuged at 1300 rpm for 5 minutes, and then it was washed with FBS-free RPMI1640 medium, and then suspended in RPMI1640 medium containing 5% human AB serum (Sigma) to a concentration of 2×10^5 cells/mL. The cancer cell suspension was added to each well of a 96-well microplate (Corning) by 50 μ L (1×10^4 cells), and then stabilized in an incubator (37°C, 5% CO₂) for 1 hour.

In order to identify the effect of killing cancer cells through antibody-dependent cellular cytotoxicity (ADCC) by the test substance, natural killer cells (NK cells) were isolated from human peripheral blood mononuclear cells (PBMCs) using a CD56+CD16+ NK cell isolation kit (Miltenyi Biotec) and used. In isolated NK cells, dead cells and debris were removed using Ficoll (GE Healthcare Life Sciences) solution in the same manner as the cancer cell line. The cells suspended in RPMI1640 medium were carefully layered on ficoll solution. The cell layer with a low specific gravity formed by centrifuging at room temperature at 350×g for 20 minutes was collected with a pipette, washed with PBS (Gibco), and then centrifuged at room temperature at 350×g for 5 minutes. The separated cell layer was suspended in RPMI1640 medium containing

5% human AB serum (Sigma) to a concentration of 2×10^5 cells/mL. The PBMC suspension was dispensed 50 μ l into each well of a 96-well microplate (Corning) in which cancer cell line has been dispensed, depending on the conditions.

In order to identify the effect of killing the cells, a CytoTox Green reagent (INCUCYTE™ CytoTox Green, Satorius) that binds to the DNA of cells to be killed was prepared in 1 μ l per 1 mL of RPMI1640 medium containing 5% human AB serum (Sigma). The prepared medium was used for dilution of the test substance, and the effect of killing the cells could be quantitatively identified by staining the cells to be killed when the test substance was co-cultured with cancer cell lines and PBMCs.

Pertuzumab was diluted using RPMI1640 medium containing a CytoTox Green reagent, and then used in the experiment at a final concentration of 16.9 nM (50 μ l) per well of a 96-well microplate.

GI-101 was diluted by 1/3 using RPMI1640 medium containing a CytoTox Green reagent, and then used in the experiment at final concentrations of 0.4 nM, 1.2 nM, 3.7 nM, 11.1 nM, 33.3 nM, and 100 nM by 50 μ l per well of a 96-well microplate.

The prepared test substance was placed in each well of a 96-well microplate in which cancer cell lines and PBMCs were dispensed depending on the conditions, and cultured in an incubator (37°C, 5% CO₂) for 24 hours, and the proliferation or death of cancer cells was observed through the real-time cell imaging analysis equipment IncuCyte S3 (Satorius). The death of cancer cells was quantified by the integrated intensity of the cells stained in green with a CytoTox Green reagent.

Fig. 122 illustrates results obtained by measuring the degree of killing cancer cells after the HCT116 cells were treated with GI-101 at a concentration of 0.4 nM, 1.2 nM, 3.7 nM, 11.1 nM, 33.3 nM and 100 nM, respectively. In the case of co-culturing only cancer cells and NK cells and in the case of treatment of Pertuzumab alone, the effect of killing cancer cells was not shown as in the case of culturing only cancer cells. In the case of treatment with GI-101 alone and with a combination of GI-101 + Pertuzumab, an excellent effect of killing cancer cells was shown, and in the case of treatment with a combination of GI-101 + Pertuzumab, the most excellent effect of killing cancer cells was shown.

XIV. Identification of anticancer effect according to administration of combination of fusion protein dimer and CDK4/6 inhibitor

Experimental Example 37. Identification of anticancer effect by administration of combination of GI-101 and Abemaciclib

This experiment was to evaluate the tumor growth inhibitory effect after administration of mGI-101 as a test substance alone or in combination with Abemaciclib substance in a tumor

model allotransplanted with 4T1 cells (mouse breast cancer cells) into BALB/c mice.

4T1 cells were purchased from ATCC (USA) and cultured in RPMI1640 medium (Gibco) containing 10% FBS (Gibco) and 1% antibiotic/antifungal agent (Gibco). The cultured cells were harvested using trypsin (Gibco) and then suspended in PBS. In order to establish an allotransplanted tumor model, 1×10^5 of 4T1 cells were subcutaneously injected into the back of BALB/c female mice (8-week-old). General symptoms were observed once a day during the engraftment and growth period after cell line transplantation.

A certain period of time after cell inoculation of the tumor grafts of the mice, the tumor volume was measured for animals with no abnormalities in the health condition of the animals, and the mice were randomly selected and assigned, each group including 11 mice. As shown in Table 28, the test groups were configured and the test substances were administered.

[Table 28]

Experimental group		Route of administration	Dosing cycle	Dosage amount	Number of animals
G1	Vehicle control (PBS)	i.p.	QW (once/week)	- mg/kg	11
G2	mGI-101	i.p.	BIW (2 times/week)	3 mg/kg	11
G3	Abemaciclib	p.o.	6 times/week	50 mg/kg	11
G4	mGI-101 + Abemaciclib	i.p. + p.o.	mGI-101: BIW (2 times/week), Abemaciclib: 6 times/week	3 mg/kg + 50 mg/kg	11

The death of the mouse, the type of general symptoms, the date of onset, and the severity of symptoms were observed once a day during the test period, and recorded for each subject. The size of the Renca solid cancer was measured twice a week during the observation period, and the major axis (maximum length, L) and minor axis (perpendicular width, W) of the tumor were measured using a vernier caliper, and the tumor volume (TV) and the tumor growth inhibition rate (TGI) were calculated by substituting them into the following equations.

[Equation 1]

$$TV (mm^3) = (W^2 \times L)/2$$

[Equation 2]

$$\%TGI (\text{Tumor Growth Inhibition}) = (1 - (T_i - T_0) / (V_i - V_0)) \times 100$$

The tumor volume before administration of each subject was set as the value measured at the time of grouping, and the anti-tumor efficacy was evaluated as compared with the vehicle

control.

All statistical calculations were performed using Prism 8.0 (Graph Pad Software Inc, USA). The comparison of tumor volume measurements was made through two-way analysis of variance followed by Tukey's multiple comparison test. A p value of less than 0.05 was considered significant.

The results of measuring the tumor size after administration of mGI-101 alone or in combination with Abemaciclib substance in mice transplanted with mouse-derived breast cancer cells are shown in Fig. 123. In the case of the group having received a combination of mGI-101 (BIW) + Abemaciclib, the tumor growth inhibition tended to be highest.

Individual tumor sizes for each test group are shown in Fig. 125. According to the results of individual tumor sizes, the group having received a combination of mGI-101 (BIW) + Abemaciclib exhibited the greatest tumor growth inhibitory effect.

Fig. 124 illustrates a tumor growth inhibition rate when mGI-101 and Abemaciclib are administered in combination in mice transplanted with 4T1. The vehicle control exhibited a tumor growth inhibition rate of 30% or more in 3 mice, and 50% or more and 80% or more in no mouse. The group having received mGI-101 (BIW) exhibited a tumor growth inhibition rate of 30% or more in 3 mice, 50% or more in 1 mouse, and 80% or more in no mouse. The group having received Abemaciclib exhibited a tumor growth inhibition rate of 30% or more in 4 mice, 50% or more in 1 mouse, and 80% or more in 1 mouse. The group having received a combination of mGI-101 (BIW) + Abemaciclib exhibited a tumor growth inhibition rate of 30% or more in 7 mice, 50% or more in 5 mice, and 80% or more in 1 mouse.

Experimental Example 38. Identification of anticancer effect by administration of combination of GI-101 and Ribociclib

This experiment was to evaluate the effect of killing cancer cells by treating MDA-MB-231 cells (human breast cancer cells) with the test substance GI-101 alone or in combination with Ribociclib substance in an *in vitro* environment.

MDA-MB-231 cells were purchased from the Korea cell line bank and cultured in RPMI1640 medium (Gibco) containing 10% FBS (Gibco) and 1% antibiotic/antifungal agent (Gibco). For use in cancer cell killing test, the cells were harvested using trypsin (Gibco), and then suspended in RPMI1640 medium, and then dead cells and debris were removed using Ficoll (GE Healthcare Life Sciences) solution. The cells suspended in RPMI1640 medium were carefully layered on ficoll solution. The cell layer with a low specific gravity formed by centrifuging at room temperature at 350×g for 20 minutes was collected with a pipette, washed with PBS (Gibco), and then centrifuged at room temperature at 350×g for 5 minutes. The separated cell layer was made into a suspension of 2×10^5 cells/mL with FBS-free RPMI1640 medium. The cancer cell suspension was stained at 37°C for 1 hour using CELLTRACKER™

Deep Red Dye (Thermo) in order to track proliferation of cancer cells or inhibition of the proliferation. After staining, it was centrifuged at 1300 rpm for 5 minutes, and then it was washed with FBS-free RPMI1640 medium, and then suspended in RPMI1640 medium containing 5% human AB serum (Sigma) to a concentration of 2×10^5 cells/mL. The cancer cell suspension was added to each well of a 96-well microplate (Corning) by 50 μ l (1×10^4 cells), and then stabilized in an incubator (37°C, 5% CO₂) for 1 hour.

Human peripheral blood mononuclear cells (PBMCs) were used in order to identify the effect of killing cancer cells by GI-101. The human PBMCs were purchased from Zen-Bio, and the PBMCs stored frozen were placed in a 37°C water bath, and thawed as quickly as possible, and then transferred to RPMI1640 medium (Gibco) containing 10% FBS (Gibco) and 1% antibiotic/antifungal agent (Gibco), and centrifuged at 1300 rpm for 5 minutes. The separated cell layer was suspended in RPMI1640 medium, and then dead cells and debris were removed using Ficoll (GE Healthcare Life Sciences) solution in the same manner as the cancer cell line. The cells suspended in RPMI1640 medium were carefully layered on ficoll solution. The cell layer with a low specific gravity formed by centrifuging at room temperature at 350×g for 20 minutes was collected with a pipette, washed with PBS (Gibco), and then centrifuged at room temperature at 350×g for 5 minutes. The separated cell layer was suspended in RPMI1640 medium containing 5% human AB serum (Sigma) to a concentration of 5×10^5 cells/mL. The PBMC suspension was dispensed 50 μ l into each well of a 96-well microplate (Corning) in which cancer cell line has been dispensed, depending on the conditions.

In order to identify the effect of killing the cells, a CytoTox Green reagent (INCUCYTE™ CytoTox Green, Satorius) that binds to the DNA of cells to be killed was prepared in 1 μ l per 1 mL of RPMI1640 medium containing 5% human AB serum (Sigma). The prepared medium was used for dilution of the test substance, and the effect of killing the cells could be quantitatively identified by staining the cells to be killed when the test substance was co-cultured with cancer cell lines and PBMCs.

Ribociclib test substance was diluted using RPMI1640 medium containing a CytoTox Green reagent, and then used in the experiment at a final concentration of 913 nM (50 μ l) per well of a 96-well microplate. GI-101 was diluted by 1/3 using RPMI1640 medium containing a CytoTox Green reagent, and then used in the experiment at a final concentration 100 nM by 50 μ l per well of a 96-well microplate.

The prepared test substance was placed in each well of a 96-well microplate in which cancer cell lines and PBMCs were dispensed depending on the conditions, and cultured in an incubator (37°C, 5% CO₂) for 24 hours, and the proliferation or death of cancer cells was observed through the real-time cell imaging analysis equipment IncuCyte S3 (Satorius). The death of cancer cells was quantified by the integrated intensity of the cells stained in green with a CytoTox

Green reagent.

Fig. 126 illustrates the effect of killing cancer cells in a condition of GI-101 at 100 nM. In the case of co-culturing only cancer cells and PBMCs, the effect of killing cancer cells was identified, and tended to be higher than that of treatment with GI-101 alone. In the case of treatment with Ribociclib alone and treatment with a combination of GI-101 + Ribociclib, an excellent effect of killing cancer cells was shown as compared with the case of co-culturing only cancer cells and PBMCs, and in the case of treatment with a combination of GI-101 + Ribociclib, the most excellent effect of killing cancer cells was shown.

XV. Identification of anticancer effect according to administration of combination of fusion protein dimer and STING agonist

Experimental Example 39. Identification of anticancer effect by administration of combination of mGI-101 and DMXAA

This experiment was to evaluate the anticancer efficacy according to administration of mGI-101 as a test substance alone or in combination with DMXAA substance, a STING agonist, in a tumor model allotransplanted with MC38 cells (mouse colon cancer cells) into C57BL/6 mice.

In order to establish an allotransplanted tumor model, 5×10^5 of MC38 cells were subcutaneously injected into C57BL/6 mice. The tumor grafts of the mice were identified to be a size of 100-200 mm³ about day 10 after cell inoculation, and the mice were assigned, each group including 5 mice. The test groups were configured as shown in Table 29, and the test substances were administered according to the schedule shown in Fig. 127.

[Table 29]

Experimental group		Route of administration	Dosing cycle	Dosage amount	Number of animals
G1	Vehicle control (PBS)		mGI-101: BIW	- mg/kg	5
G2	mGI-101	i.p.	(2 times/week),	6 mg/kg	5
G3	DMXAA	I.T.	DMXAA: Day	450 µg	5
G4	mGI-101 + DMXAA	i.p. + I.T.	10 and 13	6 mg/kg + 450 µg	5

The size of the MC38 solid cancer was measured three times a week during the observation period, and the major axis (maximum length, L) and minor axis (perpendicular width, W) of the tumor were measured using a caliper, and the tumor volume (TV) was calculated by substituting them into the following equation, and once the tumor size was no less than 2 cm, the mice were sacrificed.

[Equation 1]

$$TV \text{ (mm}^3\text{)} = (W^2 \times L)/2$$

The survival rate upon administration of mGI-101 alone or in combination with DMXAA substance against the MC38 tumor is shown in Fig. 128. In the case of the vehicle control, all mice died before 40 days after tumor injection. In the case of the group having received mGI-101 alone, the survival rate on day 60 after tumor injection was 20%; and in the case of the group having received DMXAA, a STING agonist, alone, the survival rate on day 60 after tumor injection was 60%; and in the case of the group having received a combination of mGI-101 + DMXAA, the survival rate on day 60 after tumor injection was 80%, indicating that the survival rate was higher than that of the other groups.

Individual tumor sizes for each test group are shown in Figs. 129 to 133. The group having received a combination of mGI-101 + DMXAA exhibited the greatest tumor growth inhibitory effect.

Claims

[Claim 1]

A pharmaceutical composition for preventing or treating cancer, comprising, as active ingredients:

a fusion protein dimer comprising a CD80 protein or a fragment thereof, and an IL-2 protein or a variant thereof; and
an anticancer agent.

[Claim 2]

The pharmaceutical composition of claim 1, wherein the CD80 protein or fragment thereof and the IL-2 protein or variant thereof are bound to each other via a linker.

[Claim 3]

The pharmaceutical composition of claim 1, wherein the IL-2 protein has the amino acid sequence of SEQ ID NO: 10.

[Claim 4]

The pharmaceutical composition of claim 1, wherein the CD80 has the amino acid sequence of SEQ ID NO: 11.

[Claim 5]

The pharmaceutical composition of claim 1, wherein the anticancer agent is any one selected from the group consisting of an anticancer chemotherapeutic agent, a target anticancer agent, an anticancer virus, an antibody therapeutic agent, a cell therapeutic agent, and an immune checkpoint inhibitor.

[Claim 6]

The pharmaceutical composition of claim 5, wherein the anticancer chemotherapeutic agent is any one selected from the group consisting of an alkylating agent, a microtubule inhibitor, an anti-metabolite, and a topoisomerase inhibitor.

[Claim 7]

The pharmaceutical composition of claim 6, wherein the anticancer chemotherapeutic agent is any one selected from the group consisting of Mechlorethamine, Cyclophosphamide, Ifosfamide, Melphalan, Chlorambucil, Thiotepa, Altretamine, Procarbazine, Busulfan,

Streptozocin, Carmustine, Lomustine, Dacarbazine, Cisplatin, Carboplatin, Oxaliplatin, Docetaxel, Velban, Oncovin, Navelbine, Fluorouracil, Capecitabine, Cytarabine, Gemcitabine, Fludarabine, Methotrexate, Pemetrexed, Hycamtin, Camptosar, Vepesid, Paclitaxel, Blenoxane, Adriamycin, and Cerubidine.

[Claim 8]

The pharmaceutical composition of claim 5, wherein the target anticancer agent targets any one protein selected from the group consisting of EGFR, VEGFR, CD20, CD38, RNAK-L, BTK, Bcr-abl, PDGFR/FGFR family, MEK/RAF, HER2/Neu, Ubiquitin, JAK, ALK, PARP, TGF β R, Proteasome, Bcl-2, C-Met, VR1, VR2, VR3, c-kit, AXL, RET, Braf, DNMT, CDK4/6, and STING.

[Claim 9]

The pharmaceutical composition of claim 8, wherein the target anticancer agent is any one selected from the group consisting of Cetuximab, Trastuzumab, Pertuzumab, Axitinib, Lenvatinib, Bevacizumab, Ramucirumab, Aflibercept, Rituximab, Obinutuzumab, Daratumumab, Denosumab, Ibrutinib, Dasatinib, Nilotinib, Imatinib, Bosutinib, Galunisertib, Vactosertib, Nintedanib, Sunitinib, Sorafenib, Cabozantinib, Regorafenib, Masitinib, Semaxanib, Tivozanib, Vandetanib, Pazopanib, Trametinib, Dabrafenib, Afatinib, Lapatinib, Neratinib, Lenalidomide, Ixazomib, Ruxolitinib, Lestaurtinib, Pacritinib, Cobimethinib, Selumetinib, Trametinib, Binimetinib, Alectinib, Crizotinib, Venetoclax, Crizotinib, Cabozantinib, Bemcentinib, Gilteritinib, Selpercatinib, Pralsetinib, Vemurafenib, Olaparib, Talazoparib, Niraparib, Rucaparib, Azacitidine, Decitabine, Guadecitabine, Abemaciclib, Ribociclib, Palbociclib, CDNs, SB11285, and DMXAA.

[Claim 10]

The pharmaceutical composition of claim 5, wherein the anticancer virus is any one selected from the group consisting of Talimogenem and Laherparepvec.

[Claim 11]

The pharmaceutical composition of claim 5, wherein the antibody therapeutic agent is any one selected from the group consisting of Cetuximab, Trastuzumab, Pertuzumab, Panitumumab, Emtansine, Rituximab, Daratumumab, Denosumab, Ibritumomab, Tositumomab, Brentuximab, Ofatumumab, Obinutuzumab, Necitumumab, Bevacizumab, Ramucirumab, Nivolumab, Pembrolizumab, Atezolizumab, Durvalumab, and Ipilimumab.

[Claim 12]

The pharmaceutical composition of claim 5, wherein the cell therapeutic agent is any one selected from the group consisting of Tisagenlecleucel and Axicabtagene Ciloleucel.

[Claim 13]

The pharmaceutical composition of claim 5, wherein the immune checkpoint inhibitor is any one selected from the group consisting of an anti-CTLA-4 antibody, an anti-PD-1 antibody, an anti-PD-L1 antibody, an anti-PD-L2 antibody, an anti-B7-H4 antibody, an anti-HVEM antibody, an anti-TIM3 antibody, an anti-GAL9 antibody, an anti-LAG3 antibody, an anti-VISTA antibody, an anti-KIR antibody, an anti-BTLA antibody, and an anti-TIGIT antibody.

[Claim 14]

The pharmaceutical composition of claim 13, wherein the immune checkpoint inhibitor is any one selected from the group consisting of Ipilimumab, Pembrolizumab, Nivolumab, Cemiplimab, Atezolizumab, Avelumab, and Durvalumab.

[Claim 15]

The pharmaceutical composition of claim 1, wherein the cancer is any one selected from the group consisting of gastric cancer, liver cancer, lung cancer, colorectal cancer, breast cancer, prostate cancer, ovarian cancer, pancreatic cancer, cervical cancer, thyroid cancer, laryngeal cancer, acute myeloid leukemia, brain tumor, neuroblastoma, retinoblastoma, head and neck cancer, salivary gland cancer, and lymphoma.

[Claim 16]

The pharmaceutical composition of claim 1, wherein the anticancer agent is an anticancer chemotherapeutic agent and a target anticancer agent; an anticancer chemotherapeutic agent and an immune checkpoint inhibitor; or an anticancer chemotherapeutic agent, a target anticancer agent, and an immune checkpoint inhibitor.

[Claim 17]

A composition for anticancer maintenance therapy, comprising, as an active ingredient:
a fusion protein dimer comprising a CD80 protein or a fragment thereof and an IL-2 protein or a variant thereof.

[Claim 18]

The composition of claim 17, wherein the composition further comprises an anticancer agent.

[Claim 19]

A kit for preventing or treating cancer, comprising, as active ingredients:
a fusion protein dimer comprising a CD80 protein or a fragment thereof and an IL-2 protein or a variant thereof; and
an anticancer agent.

[Claim 20]

A kit for anticancer maintenance therapy, comprising, as active ingredients:
a fusion protein dimer comprising a CD80 protein or a fragment thereof and an IL-2 protein or a variant thereof; and
an anticancer agent.

[Claim 21]

A method for preventing or treating cancer, comprising:
administering, to a subject, a fusion protein dimer comprising a CD80 protein or a fragment thereof and an IL-2 protein or a variant thereof, and an anticancer agent.

[Claim 22]

The method of claim 21, wherein the fusion protein dimer and the anticancer agent are administered simultaneously and/or sequentially.

[Claim 23]

Use of a composition comprising a fusion protein dimer comprising a CD80 protein or a fragment thereof and an IL-2 protein or a variant thereof, and an anticancer agent for the prevention or treatment of cancer.