Supplementary Information for

Synthesis and biological evaluation of a monocyclic Fc-binding antibody-recruiting molecule for cancer immunotherapy

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Table of Contents

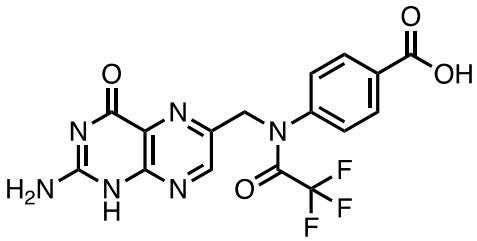
Materials and Methods

Chemicals

Reagents and solvents were purchased from Wako Pure Chemical Industries (Osaka, Japan), Sigma-Aldrich (MO, USA), Watanabe Chemical Industries (Hiroshima, Japan), Nacalai tesque (Kyoto, Japan), Kanto Chemical Tokyo (Tokyo, Japan), and Tokyo Chemical Industries (Tokyo, Japan). Mass spectra were obtained on a Waters MICRO MASS LCT-premier mass spectrometer. ^{1}H NMR spectra were measured in DMSO-d_6 solution and referenced to TMS (0.00 ppm) using Bruker DPX-400 (400 MHz). When peak multiplicities are reported, the following abbreviations are used: bs, broad singlet; s, singlet; d, doublet.

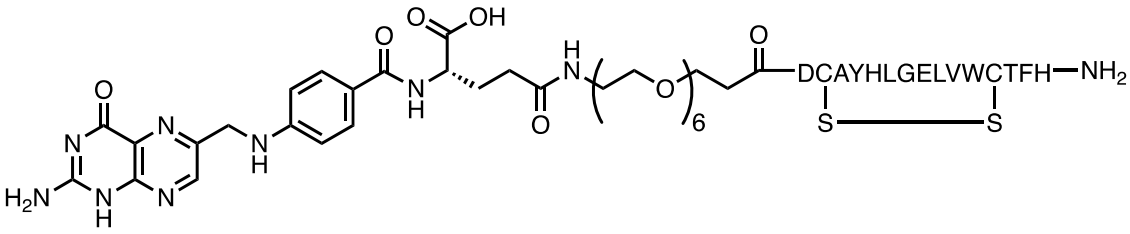
Synthesis

***N*^{10}-(trifluoroacetyl)pteroic acid**



N^{10}-(Trifluoroacetyl)pteroic acid was synthesized according to a previous protocol with minor modifications.[1] Pteroic acid (100 mg) and trifluoroacetic anhydride (2 mL) were added to a shield tube, and the mixture was stirred for 21 h at 60 °C under an argon atmosphere. After cooling to room temperature, the solvent was removed under vacuo. Then, Et_2O was added to the residual oil at 0 °C, and the precipitate was collected by centrifugation and decantation and washed with 1% TFA (trifluoroacetic acid) in H_2O. The collected precipitate was dissolved in DMF:MeOH:EtOH (6:10:3) at 50 °C and recrystallized by adding water as a poor solvent to obtain the title compound (yellow solid, yield 16%). Characterization was consistent with a previous report of this compound:[2] ^{1}H NMR (400 MHz, DMSO-d_6): 11.59 (bs, 1H), 8.62 (s, 1H), 7.96 (d, J = 8.16, 2H), 7.62 (d, J = 7.78, 2H), 7.00 (bs, 2H), 5.12 (s, 2H); HRMS (ESI) *m/z* calcd for $C_{16}H_{11}N_6O_4F_3$ $[M+Na]^+$ 431.0692, found 431.0689.

Reo-3



The peptide chain was elongated by the general Fmoc-based solid-phase peptide synthesis method according to a previous method with minor modifications[3] using an automated peptide synthesizer (Prelude, Protein Technologies Inc. (Tucson, AZ, USA)). After swelling the rink amide resin (40 μmol) with DMF for 30 min, the Fmoc group was deprotected by 20% piperidine/DMF for 10 min twice, and Fmoc-amino acids were coupled with HATU (*O*-(7-aza-1H-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, 5eq.) and HOAt (1-hydroxy-7-azabenzotriazole, 5eq.) in the presence of *N*,*N*-diisopropylethylamine (10 eq.) in DMF for 30 min. These reactions were repeated to lengthen the peptide chain. Then, the

coupling reaction of (Fmoc-amino)-PEG_6-carboxylic acid, Fmoc-Glu-*O*tBu and N^{10}-(trifluoroacetyl)pteroic acid was manually performed using three equivalents of HATU, HOAt and *N*,*N*-diisopropylethylamine. To deprotect the trifluoroacetyl group, the resin was treated with 2% hydrazine[4] in DMF for 5 min thrice and washed with DMF, MeOH and Et_2O. After drying in vacuo, cleavage from resin and final deprotection were performed by treating with a TFA cocktail (TFA:triisopropylsilane:1,3-dimethoxybenzene = 40:1:2) for 3 h. The crude material was precipitated with Et_2O, washed twice and dried. To form the disulfide bond, the peptide was dissolved in 20% DMSO in 100 mM sodium phosphate buffer (pH = 7.4), and stirred for 18 h at room temperature. The reaction mixture was purified directly by RP-HPLC [SunFire PrepC18 OBD 19 × 150 mm (5 μm)] to obtain the desired compound (white solid, yield 4%). The purity was analyzed by RP-HPLC with the following conditions: gradient: 0.1% TFA aq./CH_3CN = 90:10 to 50:50 over 40 min; flow rate: 0.9 mL/min, λ = 230 nm; column: COSMOSIL 5C18-AR-Ⅱ4.6ID × 150 mm. HRMS (ESI) *m/z* calcd for $C_{116}H_{157}N_{29}O_{33}S_2$ $[M+H]^+$ 2549.1018, found 2549.1008, purity >99%. Fc-ARM2 was synthesized and purified as reported previously.[5]

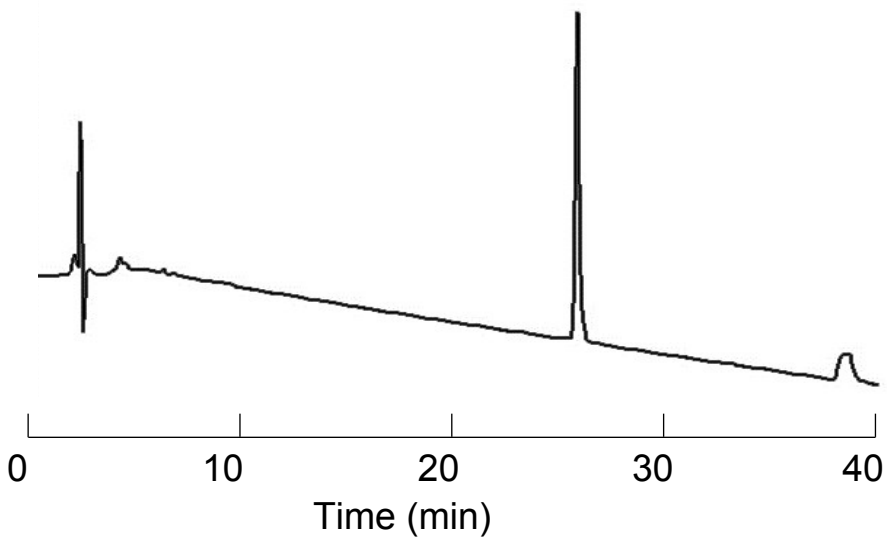


Fig. S1 RP-HPLC chromatogram of Reo-3. For calculating the purity, the peak derived from the column used (retention time = 38 min) and injection noise (retention time = 0–5 min) were excluded.

Surface plasmon resonance (SPR)

SPR measurements were performed as reported previously.[5] A Biacore T-200 (GE Healthcare, IL, USA) was used. Trastuzumab was immobilized on a CM5 chip (GE healthcare) via amine

coupling for 2000 RU according to the manufacturer's instructions. Reo-3 was analyzed at a flow rate of 50 μL/min in running buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.05% surfactant P20, pH 7.4). To solubilize Reo-3 in the running buffer, a maximum of 0.001% of DMSO was added. The chip was exposed to 10 mM Gly-HCl (pH 2.0) for 5 seconds for regeneration between each measurement. Non-linear kinetic analysis was conducted.

Cell culture

Cell lines used in this study were cultured as reported previously.[5] IGROV-1 human ovarian carcinoma cells were kindly provided by Dr. T. Matsuyama (Kagoshima University). KHYG-1/CD16a-158 V cells were kindly provided by Dr. Y. Mishima (Japanese Foundation for Cancer Research, Japan).[6] IGROV-1 cells were cultured in folate-free RPMI-1640 culture medium (Invitrogen, CA, USA). KHYG- 1/CD16a-158 V cells were cultured in RPMI-1640 medium (Nacalai Tesque) containing 10 ng mL^{-1} recombinant human IL-2 (Peprotech, NJ, USA). All of the media were supplemented with 10% heat inactivated FBS (Thermo Fisher Scientific, MA, USA), 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 0.25 μg mL^{-1} amphotericin B (Thermo Fisher Scientific). Cells were cultured in a humidified atmosphere containing 5% CO2 and 95% air at 37 °C. The cell lines were checked negative for mycoplasma contamination using a MycoAlert mycoplasma detection kit (Lonza, Basel, Switzerland).

Fluorescent microscopy

IGROV-1 cells were analyzed by fluorescent microscopy as reported previously.[5] IGROV-1 cells were seeded at 1×10^4 cells/well in a folate-free RPMI-1640 medium onto a 96-well glass bottom microplate (Greiner Bio-One, Kremsmünster, Austria) and incubated for 24 h. The cells were washed twice with 100 μL of PBS (-), and then incubated with Reo-3 (100 nM) and FITC-labeled hIgG (500 nM, Sigma–Aldrich) in folate-free RPMI-1640 medium containing 1% FBS for 30 min at 4 °C. For competition experiments, folic acid (100 μM, Sigma–Aldrich) was added simultaneously with IgG-FITC and Reo-3. After washing, cells were stained with Hoechst 33342 (Life Technologies, CA, USA) and analyzed using a BZ-8000 fluorescent microscope (Keyence, Osaka, Japan).

Flow cytometry

Flow cytometric analyses were performed as reported previously.[5] IGROV-1 cells were harvested with Accutase (PAN-Biotech, Aidenbach, Germany) and washed with PBS (-)

containing 2% FBS. After cell counting, the IGROV-1 cells were re-suspended in 2% FBS/PBS (-) to a density of 5×10^5 cells/mL. Fc-ARMs (final concentration of 10 nM) and IgG-FITC (final concentration ~ 100 nM) were added and the cells were further incubated for 30 min on ice. After two washes with 2% FBS/PBS (-), cells were resuspended in 200 μL of 2% FBS/PBS (-) and analyzed using an EC800 cell analyzer (Sony, Tokyo, Japan).

Antibody-dependent cell-mediated cytotoxicity (ADCC) assay

ADCC assays were performed as reported previously.[5] IGROV-1 cells in tissue culture dishes were washed in 2 mL of PBS (-), detached with Accutase, and washed with folate-free RPMI-1640 medium containing 1% FBS. After cell counting, IGROV-1 cells were re-suspended in the folate-free RPMI-1640 medium containing 1% FBS diluted to 1×10^5 cells/mL. This suspension was seeded into 96-well U-bottom plates (Greiner Bio-One) at 5000 cells/well (50 μL/well). IVIG (Japan Blood Products Organization, Tokyo, Japan) and Fc-ARMs (50 μL, final concentration of each reagent is indicated in the figure legend) were added to the wells. Subsequently, KHYG-1/CD16a-158V was added (100 μL/well at the indicated effector/target ratio) and the plates were centrifuged (200 × g, 5 min). After incubation at 37 °C under 5% CO_2 for 16 h, the plates were centrifuged and 100 μL of the supernatant was transferred to a new 96-well F-bottom plate. ADCC was evaluated using the Cytotoxicity LDH Assay Kit-WST (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. The absorbance at 490 nm was measured using the Wallac 1420 ARVOsx (Perkin Elmer, MA, USA). The percentage of cytotoxicity was calculated using the following formula:

% cytotoxicity = (Abs_{490} sample - Abs_{490} effector spontaneous - Abs_{490} target spontaneous) ×100/(Abs_{490} target max - Abs_{490} target spontaneous)

For competition experiments, folic acid (10 μM) was added simultaneously with IVIG and Fc-ARM.

Statistical Analysis

Statistical analyses were carried out as reported previously.[5] In brief, Prism software (v8, GraphPad, CA, USA) was used for analysis of statistically significant differences among experimental controls. Where one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used, the similarity of data variance between groups was confirmed by a Brown–Forsythe test. Two-tailed Welch's t-test was used for single comparisons. The symbols * and ** indicate P values less than 0.05 and 0.01, respectively; N.S., not significant.

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