Preliminary crystallographic study of CD38 in complex with isatuximab, an FDA-approved antibody drug for treating multiple myeloma

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CD38 is a type II transmembrane glycoprotein highly expressed in diverse hematologic malignancies including B-cell non-Hodgkin lymphoma and multiple myeloma cells. CD38 overexpressed on the surface of multiple myeloma cells has been highlighted as a good target for therapeutic agents. In 2020, the US FDA approved isatuximab, an anti-CD38 monoclonal antibody, for the treatment of multiple myeloma. Here, the recombinant proteins of the ectodomain of CD38 and the Fab fragment of isatuximab were expressed, and their binding complex was purified and crystallized. The crystal diffracted to 1.90 Å resolution and belonged to the space group C2, with unit cell parameters $a = 217.75$, $b = 43.17$, $c = 81.73$ Å, and $\beta = 103.75^\circ$. An asymmetric unit of the crystal contains one molecule of the complex with a VM of $2.33 \text{ Å}^3 \text{ Da}^{-1}$ and a solvent content of 47.31%.

INTRODUCTION

CD38 is an extracellular enzyme expressing both ADP-ribosyl cyclase and cADPR hydrolase activities that generate cyclic adenosine diphosphate (ADP)-ribose ADP ribose (ADPR) and nicotinic acid adenine dinucleotide phosphate from extracellular nicotinamide adenine dinucleotide (NAD⁺) and cytoplasmic nicotinamide adenine dinucleotide phosphate (NADP), thereby producing metabolite that induces intracellular calcium mobilization. This enzyme is expressed on most thymocytes, some activated peripheral blood T and B lymphocytes, plasma cells, NK cells, and DCs. CD38 also functions in cell adhesion, signal transduction, and calcium signaling (Bonello et al., 2018).

Multiple myeloma is hematological cancer characterized by the proliferation of malignant plasma cells in the bone marrow, resulting in the destruction of bone tissue. Immunomodulatory drugs and proteasome inhibitors, such as pomalidomide and carfilzomib, have been used to prolong the survival rates of this disease. Despite the effectiveness of chemotherapy along with autologous stem cell transplantation, the prognosis for patients with multiple myeloma remains poor (Palumbo et al., 2016).

As the expression of CD38 is very high and uniform on myeloma cells, CD38 is a good target for novel therapeutic strategies. Immunotherapy with monoclonal antibodies targeting CD38 has shown to be promising (van de Donk et al., 2018). The anti-tumor effect of anti-CD38 antibodies is related to their ability to induce Antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), and antibody-dependent cell-mediated cytotoxicity (ADCP) of opsonized CD38⁺ cells, which are mediated by Fc-γ receptors (Moreno et al., 2019).

The first anti-CD38 antibody daratumumab (brand name

Darzalex by Janssen Pharmaceuticals) was approved by the U.S. Food and Drug Administration (FDA) in 2015 and by the European Medicines Agency (EMA) in 2016, respectively, for treatment of multiple myeloma (McKeage, 2016). In March 2020, the FDA approved another anti-CD38 antibody Isatuximab (brand name Sarclisa by Sanofi) in combination with pomalidomide and dexamethasone for adult patients with multiple myeloma who have received at least two prior therapies including lenalidomide and a proteasome inhibitor (Dhillon, 2020). Isatuximab can induce direct apoptosis, whereas daratumumab induces apoptosis upon secondary cross-linking (Martin et al., 2019). The epitope mapping of daratumumab using a constrained peptide approach and site-directed mutagenesis showed this antibody binds a unique fine epitope on CD38 (de Weers et al., 2011). The crystal structure of CD38 in complex with SAR650984 revealed its distinct binding site form that of daratumumab (Deckert et al., 2014). Probably, due to the differences in epitopes of these two antibodies, their effectiveness to induce CDC, ADCC, ADCP, or apoptosis varies. Here, we obtained the crystal of the complex of CD38 and the isatuximab Fab, which belongs to the space group C2, and carried out a preliminary crystallographic study to elucidate the exact binding mode and mechanism of action (MOA) of the antibody drug. The exquisite understanding of the MOA of novel antibody drugs can facilitate the design of improved biologics and effective combination therapy in the future.

RESULTS AND DISCUSSION

The ectodomain of the CD38 protein was expressed using Expi293F mammalian cell expression system and secreted into the culture medium. The Fab fragment of isatuximab was overexpressed in the periplasmic region of *Escherichia coli*. The

binding complex of CD38 and the isatuximab Fab was purified to homogeneity by affinity and gel-filtration chromatography (Figure 1A). The crystals of the complex were obtained by a hanging-drop method with a well solution containing 0.2 M Ammonium phosphate monobasic, 0.1 M Tris, 50% (v/v) 2-Methyl-2,4-pentanediol (MPD), pH 8.5 at 20°C and their approximate dimensions were 500 × 70 × 70 μm (Figure 1B). X-ray diffraction data were collected to 1.90 Å resolution and the crystals belonged to space group C2, with unit cell parameters $a = 217.75$, $b = 43.17$, $c = 81.73$ Å, and $\beta = 103.75^\circ$. Data collection statistics are provided in Table 1. The Matthews coefficient was calculated to be $2.33 \text{ \AA}^3 \text{ Da}^{-1}$ with a solvent content of 47.31% (Matthews, 1968), assuming one complex molecule in an asymmetric unit. After phasing by molecular replacement (MR), we could find that there is one molecule of the binding complex in an asymmetric unit with clear electron density (Figure 2). Further studies to refine the structure of isatuximab in complex with CD38 would elucidate the precise epitope and the molecular mechanism of this antibody drug for the treatment of multiple myeloma.

METHODS

Expression and purification of the proteins

The DNA sequence for the ectodomain of CD38 (aa 55-300) was synthesized after codon-optimization for human cell expression (Bioneer, Inc). To maximize protein expression, a Kozak sequence was added as well as sequences encoding an exogenous N-terminal signal peptide derived from human IgG1 heavy chain. For easy purification, C-terminal 6xHis-tag was added. The synthesized gene was cloned into a modified pcDNA3.4 vector using *Bam*I and *Kpn*I restriction sites s (Table 2). CD38 protein was expressed in Expi293F cells grown in suspension using ExpiFectamine™ 293 Transfection Kit (Thermo Fisher Scientific) at 37°C in a humidified 8% CO2 incubator rotating at 130 rpm. Cell culture supernatant was collected five days after transfection and applied to HisTrap HP column (Cytiva)

and washed with five column volumes of wash buffer (20 mM Tris, pH 8.0, 300 mM NaCl, 50 mM imidazole). The protein was then eluted with elution buffer (20 mM Tris, pH 8.0, 300 mM NaCl, 400 mM imidazole). The eluted protein was concentrated for gel filtration chromatography using a HiLoad 16/60 Superdex 200 pg column (Cytiva). The column had previously been equilibrated with gel filtration buffer (20 mM Tris, pH 8.0, 300 mM NaCl). The elution profile of the protein showed a single major peak and the protein quality was evaluated by SDS-PAGE.

The DNA sequence for the Fab fragment of isatuximab was synthesized after codon-optimization for expression in *E. coli* (Bioneer, Inc). The sequence contains the heavy and light chain with the STII signal sequence in each chain for periplasmic secretion and a C-terminal 6xHis-tag in the heavy chain (Lee et al., 2016). The synthesized gene was cloned into a modified pBAD vector using *Nhe*I and *Ap*aI restriction sites (Table 2).

TABLE 1 | Data collection statistics

X-ray source	PLS 11C
Wavelength (Å)	1.0000
Temperature (K)	100
Rotation range per image (°)	1
Total rotation range (°)	180
Exposure time per image (s)	0.5
Space group	C2
a, b, c (Å)	217.75, 43.17, 81.73
α , β , γ (°)	90, 103.75, 90
Resolution range (Å)	50.00-1.90 (1.93-1.90)*
Total No. of reflections	57741
R_{sym} (%)	7.3 (42.5)*
$I/\sigma I$	7.2 (2.4)*
$CC_{1/2}$	0.953 (0.794)*
Completeness (%)	98.1 (97.4)*
Redundancy	3.4 (3.2)*

*Values in parentheses are for the outer resolution shell.

FIGURE 1 | Purification and crystallization of the complex of CD38 and isatuximab Fab. (A) Reducing SDS-PAGE analysis of the purified complex of CD38 and isatuximab Fab fragment containing the heavy and light chain. The lane M contains molecular-weight marker. (B) Crystals of the binding complex of CD38 and isatuximab Fab grown in 0.1 M Tris, 50% (v/v) 2-Methyl-2,4-pentanediol (MPD), pH 8.5 at 20°C.

FIGURE 2 | Molecular replacement solution structure of the complex of CD38 and isatuximab. (A) Phasing by molecular replacement revealed that one complex molecule of CD38 and isatuximab Fab exists in an asymmetric unit of the crystals. (B) The $2fofc$ map (1.6σ contour level) is shown on the complex during refinement, yielding $R/R_{\text{free}} = 0.24/0.30$ at 1.90 Å resolution. In A and B, the heavy and light chain of isatuximab Fab and CD38 are colored purple, cyan, and green, respectively.

TABLE 2 | Macromolecule production

Protein	Isatuximab Fab
Source organism	Isatuximab
DNA source	Gene synthesis (Bioneer, Inc)
Restriction enzyme	<i>NheI</i> and <i>Apal</i>
Cloning vector	pBAD
Expression vector	pBAD
Expression host	<i>E. coli</i> Top10F
Complete amino- acid sequence of the construct produced	<p><Heavy chain> QVQLVQSGAEVAKPGTSVKLSCKASGYTFTDYW MQVWVKQRPQGQGLEWIGTIYPGDGDTGYAQKFQ GKATLTADKSSKTVYMHLSLASEDSAVVYCARG DYYGNSLDYWGQGTSTVTVSSASTKGPSVFPLA PSSKSTSGGTAALGCLVKDYFPEPTVSWNSGAL TSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTY ICNVNHKPSNTKVDKKVEPKSCDKTHHHHHH</p> <p><Light chain> DIVMTQSHLSMSTSLGDPVSIICKASQDVSTVA WYQQKPGQSPRRLIYSASYRIGVDPDRFTGSGA GTDFTFTISSVQAEDLAVYYCQHHYSPPTFGGG TKLEIKRTVAAPSVFIFPPSDEQLKSGTASVWCLLN NFYPREAKVQWKVDNALQSGNSQESVTEQDSK DSTYLSSTLTLSKADYEKHKVYACEVTHQGLSS PVTKSFNRGEC</p>
Protein	CD38
Source organism	Human
DNA source	Gene synthesis (Bioneer, Inc)
Restriction enzyme	<i>BamI</i> and <i>KpnI</i>
Cloning vector	pcDNA3.4
Expression vector	pcDNA3.4
Expression host	Expi293F
Complete amino- acid sequence of the construct produced	RWRQQWWSGPGTTKRFPETVLARCVKYIEHP RHVDCQSVWDAFKGAFISKHPCDITEEDYQPLM KLGQTQVPCNKILLWSRIKDLAQFTQVQRDMFT LEDTLGLYADDLTWCGEFATSKINYQSCPDWRK DCSNNPVSVFWKTVSRRAEAAACDVHVMLDGS RSKIFDKDSTFGSVVEVHNLQPEKVQTLAEAWIHG GREDSRDLCQDPTIKELESISKRNQFSCKNYRP DKFLQCVKNPEDSSCTSEIGTKHHHHHH

The plasmid pBAD-isatuximab Fab was transformed into *E. coli* Top10F (Invitrogen). The cells were grown at 37°C in LB medium supplemented with 50 µg ml⁻¹ ampicillin. When the OD600 of the culture reached 1.0, the protein expression was induced with 0.2% arabinose, and cells were grown at 30°C for 15 h. The cells were harvested by centrifugation, re-suspended in lysis buffer (20 mM Tris, pH 8.0, 200 mM NaCl), and lysed by sonication on ice. After removing cell debris by centrifugation (25,000 × g for 0.5 h at 4°C), the supernatant containing soluble protein was purified using HisTrap HP column (Cytiva) and HiLoad 16/60 Superdex 200 pg column (Cytiva). The elution profile of the protein showed a single major peak and the protein quality was evaluated by SDS-PAGE.

Crystallization, data collection, and structure determination

Gel-filtration fractions containing the complex of CD38 and isatuximab Fab were concentrated to 15 mg ml⁻¹ in 20 mM

Tris, pH 8.0, and 300 mM NaCl. Initial crystallization of the protein was performed with commercially available screening solutions (Hampton Research) by the hanging-drop vapor-diffusion method at 20°C. In the initial crystallization screening experiments, 0.8 µl of protein solution was mixed with 0.8 µl of reservoir solution and equilibrated against 300 µl of the reservoir solution. Crystals were grown with a reservoir solution containing 0.2 M Ammonium phosphate monobasic, 0.1 M Tris, 50% (v/v) 2-Methyl-2,4-pentanediol (MPD), pH 8.5 at 20°C within a week. Crystals were cryoprotected by brief immersion in well solution supplemented with 25% ethylene glycol, and flash frozen in liquid nitrogen. X-ray diffraction data were collected at 100 K on beamline 11C of the Pohang Light Source (PLS) to a resolution of 1.90 Å, integrated and scaled using HKL2000 (HKL Research). The structure was solved by molecular replacement using Phaser with the structures of an antibody Fab (PDB code 5veb, chains A and B) and CD38 (PDB code 5bni) as search models (McCoy et al., 2007). Because of the intrinsic flexibility of the Fab elbow within an antibody, the variable and constant regions of the Fab were separated during MR search. After MR phasing, the electron density corresponding to the complex was prominent in the asymmetric unit. Iterative model building and structure refinement are currently in progress using PHENIX with manual inspection using COOT (Adams et al., 2010; Emsley and Cowtan, 2004).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR INFORMATION

H.T.L., Y.K., U.B.P., and T.J.J. performed the experiments. Y.S.H. wrote the paper. All authors discussed the results and commented on the manuscript.

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