RSC Medicinal Chemistry

ROYAL SOCIETY OF CHEMISTRY

RESEARCH ARTICLE

View Article Online
View Journal | View Issue

Cite this: *RSC Med. Chem.*, 2021, **12**, 406

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

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Received 2nd October 2020,
Accepted 10th January 2021

DOI: 10.1039/d0md00337a

rsc.li/medchem

Antibody-recruiting molecules (ARMs) are bispecific molecules composed of an antibody-binding motif and a target-binding motif that redirect endogenous antibodies to target cells to elicit immune responses. To enhance the translational potential of ARMs, it is crucial to design antibody/target-binding motifs that have strong affinity and are easy to synthesize. Here, we synthesized a novel Fc-binding ARM (Fc-ARM) that targets folate receptor (FR)-positive cancer cells, Reo-3, using a recently developed monocyclic peptide 15-Lys8Leu, which binds strongly to the Fc region of an antibody. Reo-3 bound to the Fc region of the antibody with a K_d of 5.8 nM, and recruited a clinically used antibody mixture to attack FR-positive IGROV-1 cells as efficiently as Fc-ARM2, in which a bicyclic Fc-binding peptide was used. These results indicate that 15-Lys8Leu, which can be synthesized readily, is suitable for various applications including the development of Fc-ARMs.

Introduction

Small synthetic alternatives to therapeutic antibodies can potentially solve the intrinsic problems of antibodies such as immunogenicity[1,2] and stability.[3] As one alternative, antibody-recruiting small molecules (ARMs) are capable of bridging an endogenous antibody and a target antigen expressed on the surface of target cells, resulting in immune-mediated clearance of the target cells.[4] In general, ARMs are composed of an antigen recognized by endogenous antibodies and a targeting ligand. A variety of ARMs targeting bacteria,[5,6] virus-infected cells,[7–9] and cancer cells[10–16] have been reported. We have demonstrated previously that Fc-binding ARMs (Fc-ARMs), which are composed of an Fc-binding peptide and a targeting ligand, can eliminate folate receptor (FR)- or prostate-specific membrane antigen-positive cancer cells *via* a mechanism called antibody-dependent cell-mediated cytotoxicity (ADCC) (Fig. 1A).[17,18] Taking advantage of the relatively conserved structure of the Fc region of antibodies, our approach recruits the majority of endogenous antibodies for targeted immune responses without antigen–antibody interactions. We also showed that the Fc affinity of Fc-ARMs positively regulates ADCC efficacy,[17] suggesting that the development of potent Fc-binders is highly beneficial for the advancement of Fc-ARMs as novel immunotherapeutics. Additionally, we have conducted a structure–activity relationship study and discovered a novel monocyclic peptide, 15-Lys8Leu, which has an equilibrium dissociation constant (K_d) of 8.19 nM against the Fc region of an antibody.[19] 15-Lys8Leu has great potential in various applications because of its comparable Fc affinity and ease of synthesis when compared with that of bicyclic Fc-binding peptides.[19]

Here, we used 15-Lys8Leu for the synthesis of a new Fc-ARM that targets the FR, named Reo-3 (Fig. 1B). 15-Lys8Leu and folic acid (FA) as the targeting ligand of the FR were linked with an oligo-ethylene glycol linker. We tested the Fc affinity of this new Fc-ARM, the ability to recruit antibodies to the surface of FR positive cancer cells, and ADCC efficacy in comparison with Fc-ARM2, a previously reported molecule

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/d0md00337a

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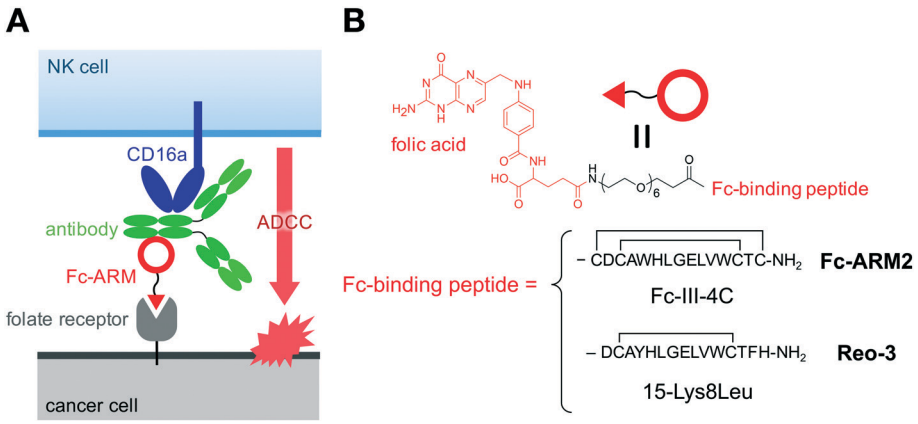

Fig. 1 The Fc-binding antibody-recruiting molecules used in this study. (A) Schematic representation of the mechanism of action of Fc-ARMs that target folate receptor-positive cancer cells. (B) Molecular structures of the Fc-ARMs used.

that possesses a bicyclic Fc-III-4C peptide (K_d value is reportedly 2.45 nM)[20] as an Fc-binder.[17]

Results and discussion

Synthesis and evaluation of Fc-affinity

Reo-3 was synthesized by the general Fmoc-based solid-phase peptide synthesis method,[21] purified by reversed-phase high-performance liquid chromatography (RP-HPLC, Fig. S1†) and identified by electrospray ionization mass spectrometry (ESI-MS). The intramolecular disulfide bond in Reo-3 was simply formed by DMSO oxidation of the crude product, which facilitates a single purification step that differs from the Fc-III-4C-based peptide[19] with two disulfide bonds. We first evaluated the Fc affinity of Reo-3 by surface plasmon resonance (SPR) binding analysis (Fig. 2). The humanized IgG1 monoclonal antibody, trastuzumab, was immobilized onto SPR chips, and Fc-ARMs were flowed over the chip surface. The result showed that Reo-3 has a K_d of 5.8 nM against the Fc region, which is very close to that of Fc-ARM2 (K_d = 6.1 nM).[17] The binding affinity of Reo-3 is 1.4 times higher because of the slower association and dissociation steps (k_{on} = 9.0 × 10^5 M^{-1} s^{-1}, k_{off} = 0.0052 s^{-1}) when compared with the parental 15-Lys8Leu (k_{on} = 1.5 × 10^6 M^{-1} s^{-1}, k_{off} = 0.012 s^{-1}).[19] This may be caused by the bulkiness and flexibility of the additional FA and linker moieties that suppress fast association of the peptide and interact with the antibody surface after the peptide binds to the Fc region.

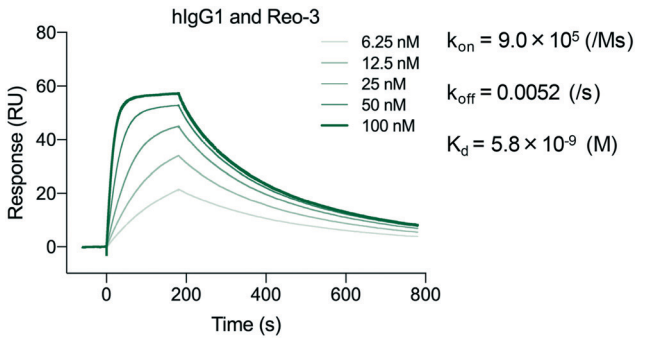

Fig. 2 SPR measurement of the Fc affinity of Reo-3. Equilibrium dissociation constant (K_d) of Reo-3 to trastuzumab was determined by SPR analysis. k_{on} and k_{off} are also shown.

Antibody recruitment

We next tested if Reo-3 recruits antibodies to FR-positive IGROV-1 cancer cells. Fluorescent microscopy revealed that Reo-3 successfully recruited human IgG antibodies labeled with FITC (IgG-FITC) to IGROV-1 cells (Fig. 3A). Excess FA diminished the fluorescence on the cell surface, indicating that antibody recruitment is mediated by specific binding of Reo-3 to the FR. We also quantitatively evaluated the efficiency of antibody recruitment by Reo-3 using flow cytometry. The results showed that Reo-3 recruits antibodies to IGROV-1 cells with similar efficiency when compared with that of Fc-ARM2 (Fig. 3B), in accord with the aforementioned K_d values.

Cell-mediated cytotoxicity

Finally, we evaluated the ADCC efficacy of antibodies recruited by Reo-3. IGROV-1 cells were first treated with 10 nM of an Fc-ARM (Fc-ARM2 or Reo-3) and 100 nM of intravenous immunoglobulin (IVIG, a clinically used mixture of human IgG from donor sera). Then, IGROV-1 cells (target) were co-cultured with natural killer (NK) cells (effector) at indicated effector/target ratios for 16 h. Lactose dehydrogenase (LDH) released from lysed target cells was quantified (Fig. 4). "Reo-3" and "IVIG" did not induce ADCC. In contrast, both "Fc-ARM2 + IVIG" and "Reo-3 + IVIG" showed similar efficacy of target cell lysis in an effector/target ratio-dependent manner. Excess FA reduced cytotoxicity of Reo-3 + IVIG significantly. Taken together, these results demonstrate that Reo-3 can recruit endogenous antibodies to eliminate cancer cells *in vitro*, and the efficacy is comparable with antibodies recruited by bicyclic Fc-ARM2.

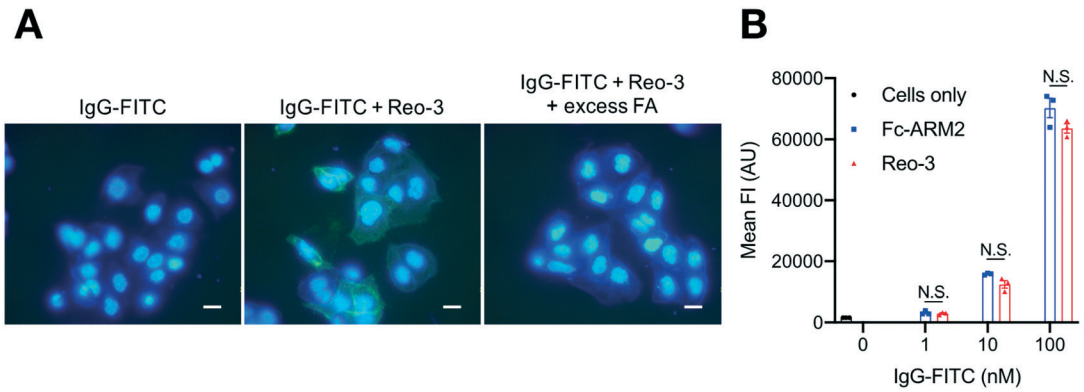

Fig. 3 Antibody recruitment to FR$^+$ IGROV-1 cells by Reo-3 and Fc-ARM2. (A) IgG-FITC (500 nM), Reo-3 (100 nM) and FA (100 μM) were used. IgG-FITC, IgG-FITC + Reo-3, or IgG-FITC + Reo-3 + excess FA were added to the IGROV-1 cells and incubated. After a washing step and staining with Hoechst 33342, the cells were analyzed by fluorescent microscopy. Scale bar = 20 μm. (B) The IGROV-1 cells were treated with 10 nM of Fc-ARM (Fc-ARM2 or Reo-3) and increasing concentrations of IgG-FITC (1 to 100 nM). After washing, the cells were analyzed by flow cytometry (n = 3, mean ± SEM). Statistical analyses were carried out using two-tailed Welch's t-test. N.S. = not significant.

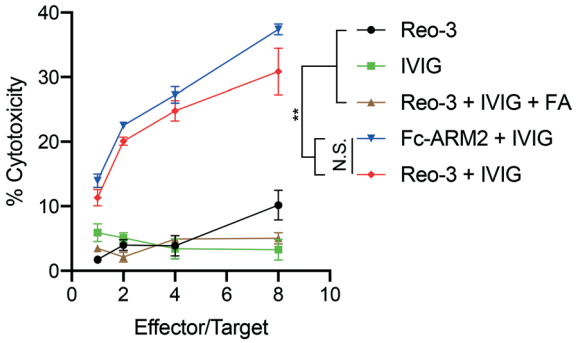

Fig. 4 Antibody-dependent cell-mediated cytotoxicity assay. IGROV-1 cells (5000 cells/well) were treated with 10 nM Fc-ARM (Fc-ARM2 or Reo-3) and 100 nM IVIG. Then, 5000–40 000 cells/well of KHYG-1/CD16a-158 V cells were added and the cells were co-cultured for 16 h. LDH released from lysed cells was quantified (n = 3, mean ± SEM). FA (10 μM) was used to inhibit the binding of Fc-ARMs to the FR. Statistically significant differences between Fc-ARM (Fc-ARM2 or Reo-3) + IVIG and all of the other groups were observed at all effector/target ratios. Statistical analyses were carried out using one-way ANOVA with Tukey's multiple comparison test. $^{**}p < 0.01$; N.S. = not significant.

Conclusions

In summary, we developed a new Fc-ARM named Reo-3, which contains a monocyclic Fc-binding peptide 15-Lys8Leu. Reo-3 showed strong affinity for the Fc region of the human IgG1 antibody (K_d = 5.8 nM). Reo-3 recruited IVIG to induce ADCC against FR-positive cancer cells as effective as Fc-ARM2, which has a bicyclic Fc-binding peptide. 15-Lys8Leu is easily synthesized because of its relatively short amino acid sequence and monocyclic structure, and is one of the strongest binding peptides to the Fc region.[19] Thus, 15-Lys8Leu has significant potential for use in various applications, including the development of Fc-ARMs and non-covalent antibody-drug conjugates (ADCs),[22] purification of antibodies and preparations of homogenous ADCs.[23] The straightforward synthesis of the pivotal unit of the Fc-ARM should accelerate application studies targeting other molecules and diverse derivatization for discovery of more potent Fc-ARMs.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgements

This work was in part supported by the Japanese Society for the Promotion of Sciences (JSPS) KAKENHI Grant-in-Aid for Challenging Research (Exploratory) (Grant number: 18K19148 to Y. K.), and Grant-in-Aid for Scientific Research (B) (Grant number: 19H03356 to Y. H.). K. S. was supported by the Research Fellowship for Young Scientists (JSPS, 17J05032) and Advanced Graduate Course on Molecular Systems for Devices (Kyushu University). We thank Dr. S. Kishimoto and Dr. Y. Ito (Kagoshima University) for assistance with SPR experiments. We thank the Edanz Group (https://en-author-services.edanzgroup.com/ac) for editing a draft of this manuscript.

Notes and references

1 G. M. Bartelds, C. A. Wijbrandts, M. T. Nurmohamed, S. Stapel, W. F. Lems, L. Aarden, B. A. Dijkmans, P. P. Tak and G. J. Wolbink, *Ann. Rheum. Dis.*, 2007, **66**, 921–926.
2 T. T. Hansel, H. Kropshofer, T. Singer, J. A. Mitchell and A. J. George, *Nat. Rev. Drug Discovery*, 2010, **9**, 325–338.
3 M. E. Cromwell, E. Hilario and F. Jacobson, *AAPS J.*, 2006, **8**, E572–E579.
4 P. J. McEnaney, C. G. Parker, A. X. Zhang and D. A. Spiegel, *ACS Chem. Biol.*, 2012, **7**, 1139–1151.
5 C. R. Bertozzi and M. D. Bednarski, *J. Am. Chem. Soc.*, 1992, **114**, 2242–2245.
6 J. Li, S. Zacharek, X. Chen, J. Wang, W. Zhang, A. Janczuk and P. G. Wang, *Bioorg. Med. Chem.*, 1999, **7**, 1549–1558.
7 K. P. Naicker, H. Li, A. Heredia, H. Song and L.-X. Wang, *Org. Biomol. Chem.*, 2004, **2**, 660–664.

8 M. F. Perdomo, M. Levi, M. Sällberg and A. Vahlne, *Proc. Natl. Acad. Sci. U. S. A.*, 2008, **105**, 12515–12520.
9 C. G. Parker, R. A. Domaoal, K. S. Anderson and D. A. Spiegel, *J. Am. Chem. Soc.*, 2009, **131**, 16392–16394.
10 Y. Lu and P. S. Low, *Cancer Immunol. Immunother.*, 2002, **51**, 153–162.
11 R. M. Owen, C. B. Carlson, J. Xu, P. Mowery, E. Fasella and L. L. Kiessling, *ChemBioChem*, 2007, **8**, 68–82.
12 C. B. Carlson, P. Mowery, R. M. Owen, E. C. Dykhuizen and L. L. Kiessling, *ACS Chem. Biol.*, 2007, **2**, 119–127.
13 M. K. O'Reilly, B. E. Collins, S. Han, L. Liao, C. Rillahan, P. I. Kitov, D. R. Bundle and J. C. Paulson, *J. Am. Chem. Soc.*, 2008, **130**, 7736–7745.
14 R. P. Murelli, A. X. Zhang, J. Michel, W. L. Jorgensen and D. A. Spiegel, *J. Am. Chem. Soc.*, 2009, **131**, 17090–17092.
15 A. Dubrovska, C. Kim, J. Elliott, W. Shen, T.-H. Kuo, D.-I. Koo, C. Li, T. Tuntland, J. Chang, T. Groessl, X. Wu, V. Gorney, T. Ramirez-Montagut, D. A. Spiegel, C. Y. Cho and P. G. Schultz, *ACS Chem. Biol.*, 2011, **6**, 1223–1231.
16 C. E. Jakobsche, P. J. McEnaney, A. X. Zhang and D. A. Spiegel, *ACS Chem. Biol.*, 2012, **7**, 316–321.
17 K. Sasaki, M. Harada, Y. Miyashita, H. Tagawa, A. Kishimura, T. Mori and Y. Katayama, *Chem. Sci.*, 2020, **11**, 3208–3214.
18 K. Sasaki, M. Harada, T. Yoshikawa, H. Tagawa, Y. Harada, Y. Yonemitsu, T. Ryujin, A. Kishimura, T. Mori and Y. Katayama, *ChemBioChem*, 2020, **21**, 1–6.
19 K. Muguruma, K. Fujita, A. Fukuda, S. Kishimoto, S. Sakamoto, R. Arima, M. Ito, M. Kawasaki, S. Nakano, S. Ito, K. Shimizu, A. Taguchi, K. Takayama, A. Taniguchi, Y. Ito and Y. Hayashi, *ACS Omega*, 2019, **4**, 14390–14397.
20 Y. Gong, L. Zhang, J. Li, S. Feng and H. Deng, *Bioconjugate Chem.*, 2016, **27**, 1569–1573.
21 C. D. Chang and J. Meienhofer, *Int. J. Pept. Protein Res.*, 1978, **11**, 246–249.
22 K. Muguruma, F. Yakushiji, R. Kawamata, D. Akiyama, R. Arima, T. Shirasaka, Y. Kikkawa, A. Taguchi, K. Takayama, T. Fukuhara, T. Watabe, Y. Ito and Y. Hayashi, *Bioconjugate Chem.*, 2016, **27**, 1606–1613.
23 S. Kishimoto, Y. Nakashimada, R. Yokota, T. Hatanaka, M. Adachi and Y. Ito, *Bioconjugate Chem.*, 2019, **30**, 698–702.