Germline humanization of a murine A β antibody and crystal structure of the humanized recombinant Fab fragment

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Abstract: Alzheimer's disease is the most common form of dementia, affecting 26 million people worldwide. The A β peptide (39–43 amino acids) derived from the proteolytic cleavage of the amyloid precursor protein is one of the main constituents of amyloid plaques associated with disease pathogenesis and therefore a validated target for therapy. Recently, we characterized antibody fragments (Fab and scFvs) derived from the murine monoclonal antibody WO-2, which bind the immunodominant epitope ($_3$ EFRH $_6$) in the A β peptide at the N-terminus. *In vitro*, these fragments are able to inhibit fibril formation, disaggregate preformed amyloid fibrils, and protect neuroblastoma cells against oligomer-mediated toxicity. In this study, we describe the humanization of WO-2 using complementary determining region loop grafting onto the human germline gene and the determination of the three-dimensional structure by X-ray crystallography. This humanized version retains a high affinity for the A β peptide and therefore is a potential candidate for passive immunotherapy of Alzheimer's disease.

Keywords: antibody engineering; humanization; SPR; crystal structure; Alzheimer's disease

Introduction

Alzheimer's disease (AD) is the most common form of dementia and is characterized by a progressive loss of memory and cognitive functions in affected individuals.¹ The A β peptide, derived from the proteolytic cleavage of the amyloid precursor protein (APP), is one of the main constituents of amyloid plaques associated with this disease.² This 4-kDa peptide can self-associate and adopt different structures including oligomers, protofibrils, and fibrils.³ Importantly, all

these forms are detrimental to neurons^{4,5} with soluble oligomers exhibiting the highest level of toxicity.⁶

Currently, immunotherapy remains a promising therapeutic approach for AD.⁷ Active immunization, in which the immune system is exposed to the antigen and responds by producing antibodies, has thus far produced promising results.⁸ Initial studies in AD mice immunized with fibrillar A β peptide decreased the amyloid burden in the brain and improved cognitive function.⁸ These data were rapidly translated into the clinic to evaluate the efficiency of the AN1792 A β vaccine in humans. However, these trials were stopped because of brain inflammation in 6% of the patients.⁹ Consequently, efforts were redirected toward passive immunotherapy by administering monoclonal antibodies instead of A β itself, in the hope that this strategy might be safer and more efficacious. Further studies using passive immunotherapy demonstrated that this approach was equally as effective as active immunization in terms of reducing amyloid plaque and improving cognition in an AD mouse model.^{10,11} Despite these

Abbreviations: A β , beta-amyloid peptide; AD, Alzheimer's disease; ADDL, amyloid β -derived diffusible ligand; CDR, complementary determining region; Fab, fragment antigen binding; FR, framework region; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; scFv, single-chain variable fragment; SPR, surface plasmon resonance.

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successes, reports suggesting that passive immunization may trigger side effects such as cerebral amyloid angiopathy-related microhemorrhages¹² in AD mice have emerged. Importantly, Tamura *et al.*¹³ and Wilcock *et al.*¹⁴ showed that these side effects can be attenuated or avoided by using deglycosylated whole IgG antibody or antibody fragments, both F(ab')₂ and scFv. Hence, it seems that the effector function, which is either reduced or absent in these molecules, is not directly responsible for the amyloid reduction but plays a role in eliciting other immune responses causing undesirable side effects. These results further support a future role of engineered antibody fragments as a safer therapeutic option for treating neurodegenerative diseases such as AD.

Previously, we described antibody fragments (Fab and scFvs) derived from the murine monoclonal antibody WO-2,¹⁵ which bind with high affinity to the N-terminal region (E₃FRH₆) of Aβ.¹⁶ *In vitro*, these fragments were able to inhibit fibril formation, disaggregate preformed amyloid fibril structures, and protect human neuroblastoma cells against oligomers-mediated toxicity.¹⁶

The main disadvantage of with using murine antibodies as human therapeutic agents is the human anti-mouse antibody response, which prevents multiple dosages being administered to patients.¹⁷ To circumvent this problem, antibody humanization technologies, which transfer the murine CDRs onto human framework regions, have been developed.¹⁸ The acceptor framework sequences can be derived from either a human consensus sequence, human framework genes known to be well expressed or human germline sequences.¹⁸ As human IgM antibodies predominantly express germline framework sequences, it is postulated that they are better tolerated by the immune system than framework sequences derived from IgG antibodies, which carry more mutations due to intraclonal somatic hypermutation.¹⁹

In this article, we describe the humanization and engineering of the WO-2 antibody using the CDR-grafting method. Both humanized scFv and Fab fragments retained an affinity in the low nanomolar range for the Aβ_{1–16} peptide. Furthermore, *in vitro* the humanized antibody fragments were able to inhibit amyloid fibril formation, oligomer-mediated neurotoxicity, and disaggregating preformed amyloid fibrils. Finally, we determined the structure of the recombinant humanized WO-2 (hWO-2 Fab) by X-ray crystallography and compared this to the previously solved murine structure (mWO-2 Fab).

Results and Discussion

Humanization of the murine WO-2 Fab fragment

Several methods have been described to humanize murine antibodies to develop a therapeutic antibody with

the lowest *in vivo* immunogenicity. In this work, we have grafted the CDRs of the murine antibody WO-2 onto the human germline sequence. Germline sequences offer a major advantage over consensus sequences or human IgG sequences, as they do not carry somatic hypermutations, which can be potentially recognized as immunogenic. As a first step in the humanization process, the VH and VL sequences of the murine WO-2 were compared with the functional human germline V and J gene repertoires using IMGT/V-QUEST and IMGT/Junctions analysis tools. In the case of the heavy chain, the human germline V and J genes, IGHV2-5*08 and IGHJ4*01, exhibited the highest homology with their murine counterparts sharing 79 and 85% identity, respectively. For the light chain, human IGKV2-28*01 and IGKJ4*02 genes displayed the highest homologies (80 and 81%, respectively) with their murine equivalent sequences. These human genes were selected as acceptor sequences for the grafting of the murine CDRs [Fig. 1(A)]. However, as Foote and Winter²⁰ demonstrated, direct transplantation of the murine CDRs onto the human framework acceptor sequence often results in a loss of affinity and specificity for the target antigen. To minimize this effect, residues in the framework that are involved in the presentation of the CDR loops must be conserved. These residues are in the "Vernier zone"²¹ and support the structure of the CDR loops [Fig. 1(B)]. Among these 30 residues (H2, H29, H30, H31, H32, H52, H53, H54, H76, H78, H80, H82, H87, H105, H106, H118, L2, L4, L41, L42, L52, L53, L54, L55, L78, L80, L84, L85, L87, and L118 according to the IMGT unique numbering), only three differed between the WO-2 murine sequence and their closest human germline genes (H31, H106, and L2). The final humanized VL and VH genes were synthesized and cloned into expression vectors designed specifically to express either a scFv or a recombinant Fab fragment.¹⁶ For the hWO-2 Fab construct, the humanized VH and VL were fused to the IGHG1*01 and IGKC*01 human constant regions, respectively. Both constructs were expressed in the periplasmic space of *E. coli* cells. The proteins were purified stepwise using four different chromatography techniques: immobilized metal affinity chromatography, desalting chromatography, anion exchange chromatography, and size exclusion chromatography. The initial purification process was monitored by analyzing eluants on a nonreducing SDS-PAGE gel stained with Coomassie Blue [Fig. 1(C)]. After the pilot studies, the four-step purification procedure was fully automated on the ÄKTAexpressTM system, which is suitable for unattended multistep chromatography. The final yield of purified protein was ~0.2 mg/L of culture.

Kinetics measurement by SPR

Humanized scFv and Fab fragments were characterized for binding affinity using an surface plasmon resonance (SPR)-based assay on a ProteOn XPR36

Figure 1. (A) Amino acid sequence alignment of murine WO-2 (mWO-2), humanized WO-2 (hWO-2), and the closest human germline VH (IGHV2-5*09) and VL (IGKV2-28*01). The CDRs according to Kabat's nomenclature are in red between parentheses, the CDRs according the IMGT nomenclature are between square brackets. Amino acids are numbered according to the IMGT unique numbering. The dots represent common residues between mWO-2, hWO-2, and the corresponding germline. (B) Ribbon diagram representation of mWO-2 Fv structure. The Framework residues are represented in black, the CDRs are shown in red, and the Vernier zone residues are shown in blue. (C) SDS-PAGE analysis representing the three purification steps of the hWO-2 Fab. Lane 1: IMAC elution fraction; Lane 2: cation exchange elution fraction; Lane 3: size exclusion elution fraction. Approximate MW standards (in kDa) are shown to the left.

biosensor instrument. The recombinant chimeric Fab fragment (cWO-2 Fab), previously synthesized in *E. coli*¹⁶ as well as murine WO-2 Fab fragment prepared by papain cleavage of the parental WO-2 IgG,¹⁵ was included in this assay for comparative purposes [Fig. 2(A–D)]. Data shown in Table I demonstrated that the humanized scFv (hWO-2 scFv) and Fab fragments bound to immobilized A β peptide with $\sim$ 2-fold lower affinity than its recombinant chimeric Fab counterpart and with 5- to 6-fold lower affinity than the papain-cleaved parental Fab fragment. However, both humanized constructs have affinity in the low nanomolar range, which is typically required for therapeutic applications.

Structure of the hWO-2 Fab and comparison with the mWO-2 Fab

We solved the structure of the humanized hWO-2 anti-A β monoclonal Fab to an atomic resolution of 2.2 Å (Table II). The structure of hWO-2 Fab is well defined with good quality of electron density even in flexible regions such as CDR3 H3 [Fig. 3(A)]. The structure of hWO-2 is a conventional immunoglobulin (Ig) Fab heavy-chain/light-chain heterodimer [Fig. 3(B)]. The two independent hWO-2 molecules found in the crystal unit cell are rotated with respect to each other by 170(1)°. Superimposition (performed using FATCAT²²) of C α atoms from these two molecules showed very little difference between

Figure 2. Kinetic data for various WO-2 antibody fragments using the ProteOn XPR36 instrument. Papain-digested WO-2 Fab (A), recombinant chimeric WO-2 Fab (B), humanized recombinant WO-2 Fab (C), and humanized recombinant WO-2 scFv (D) binding to A β _{1–16}-biotin captured on a NLC chip surface. All WO-2 antibody fragment samples were tested at concentrations of 81, 27, 9, 3, and 1 nM. Binding responses (black lines) were globally fitted to a simple 1:1 interaction model (gray lines) using Scrubber-pro software.

the two copies with a root-mean-square deviation (RMSD) of 0.48 Å for the 228 C α atoms from the heavy chain and 0.34 Å for the 219 C α atoms from the light chain.

Superimposition of each of the four domains [Fig. 3(C)] from the hWO-2 (PDB:3AAZ) and mWO-2 anti-A β monoclonal Fab structures (PDB: 3BAE²³) model [hWO-2: VH (1–126), CH (127–229), VL (1–113), and CL (114–227)] yielded RMS deviations on C α atoms of 0.93 Å (126 atoms), 1.26 Å (99 atoms), 0.93 Å (113 atoms), and 0.69 Å (105 atoms), respectively. These analyses revealed that the largest structural differences are localized in the constant heavy chain (CH) followed by VH and VL chains. The most significant C α deviations were observed in the CDRs of the VH heavy chain, in particular H1 (27–35) and H3 (102–110), whereas H2 displayed only minor deviations [Fig. 3(C)]. Another region of marked difference occurred in a loop (74–78) near to the CDR heavy-chain regions H1 and H2. The VL light chain only showed deviations in region (43–48), which corresponded to a loop between CDR light-chain L1 and L2, in the hinge region of the Fab, away from the ligand binding site.

The most significant differences between hWO-2 and mWO-2 arose from a difference in the elbow angles.²⁴ The elbow angle of 140° in the hWO-2 (PDB:3AAZ) structure was significantly smaller than that of 192° in the mWO-2 (3BAE) structure²³ [Fig. 3(D)]. This difference was much greater than deviations of the elbow angles between two different crystal forms of 3BAE (188°–192°). The small hWO-2 elbow angle was well within the range of the most

Table I. Rate and Equilibrium Constants Determined by SPR

Captured A β _{1–16} -biotin	$k_a \times 10^4$ (M ^{–1} s ^{–1})	$k_d \times 10^{-4}$ (s ^{–1})	K_D (nM)
WO-2 mFab	33.18 ± 0.16	3.85 ± 0.05	1.16 ± 0.01
WO-2 cFab	20.87 ± 0.60	7.03 ± 0.10	3.37 ± 0.11
WO-2 hFab	15.57 ± 0.24	9.57 ± 0.16	6.15 ± 0.14
WO-2 hscFv	14.28 ± 0.91	10.8 ± 0.22	7.58 ± 0.40

Kinetic constants were determined from measurements using a ProteOn XPR36 instrument with biotinylated A β _{1–16} peptide immobilized on an NLC chip surface. Measurements were performed at 25°C. The equilibrium dissociation constant, K_D , is calculated from the ratio of the rate constants, k_d/k_a .

Table II. Diffraction Data and Refinement Statistics

	hWO-2
Beamline	AS MX1
Wavelength (Å)	0.9566
Space group	P3 ₁
Unit cell (<i>a</i> = <i>b</i> , <i>c</i>) (Å)	106.73, 90.87
Resolution range (Å)	46.03–2.20 (2.26–2.20) ^a
Unique reflections	51677
Redundancy	3.7 (3.0)
<i>R</i> _{merge} ^b	0.09 (0.43)
<i>I</i> /σ(<i>I</i>)	16.5 (2.0)
Data completeness (%)	92.6 (64.1)
<i>R</i> _{work} ^c (%)	21.3 (29.8)
<i>R</i> _{free} ^d (%)	27.4 (37.0)
Rmsd bonds (Å), angles (°)	0.017, 1.844

^a Values in parentheses are for the highest shell.

^b $R_{\text{merge}} = \sum hkl \sum j |I_j - \langle I_j \rangle| / \sum hkl \sum j |I_j|$, where *hkl* specifies unique indices, *j* indicates equivalent observations of *hkl*, and $\langle I_j \rangle$ is the mean value.

^c $R = \sum hkl | |F_o| - F_c | / \sum hkl |F_o|$, where $|F_o|$ and $|F_c|$ are the observed and calculated structure factor amplitudes, respectively.

^d Represents 5% of the data.

preferred angles of 135°–145° for κ light chains in Fabs.²⁴ It was also close to the 133° elbow angle observed in the molecular replacement search model of the light-chain constant domains [PDB IUM5]. Although amino acids in the elbow regions of hWO-2 (₁₀₉LEIKRT₁₁₄) and 3BAE (₁₀₄LELKRA₁₀₉) differed at two positions, both were among the most preferred amino acids found in elbow regions of κ light chains.²⁴

Inhibition of Aβ_{1–42} fibril formation and disaggregation of preformed Aβ_{1–42} fibrils by hWO-2 Fab

The activity of the hWO-2 Fab on Aβ_{1–42} fibril formation was examined by a thioflavine T (ThT) fluorescence assay over a period of 5 days. When Aβ_{1–42} was incubated with a control Fab (32G12, an irrelevant antibody), the fluorescence intensity increased over time indicating the formation of cross β-sheets structures in the solution, characteristic of insoluble amyloid protofibril and fibril formation. However,

Figure 3. (A) The electron density map ($2mF_o - DF_c$ at 1σ contour level) in the CDR3 H3 region. (B) The structure of humanized WO-2 recombinant Fab in cartoon representation with rainbow coloring of chains. (C) Superimposition in cartoon representation of each of the four domains from hWO-2 (PDB:3AAZ) in cyan and mWO-2 (PDB:3BAE) in green. CDR regions H1/L1, H2/L2, and H3/L3 are colored in red, blue, and yellow, respectively. (D) Superposition in tube representation of two Fabs: hWO-2 (PDB:3AAZ) in cyan and mWO-2 (PDB:3BAE) in orange with different elbow angles. Fabs have been superimposed on their variable light-chain regions. The deviation of elbow angles is from 140° (hWO-2) to about 192° (mWO-2). Regions of variable and constant domains are marked with V and C, respectively.

Figure 4. The effect of hWO-2 Fab on $A\beta_{1-42}$ fibril formation [Fig. 4(A)] and preformed $A\beta_{1-42}$ fibrils [Fig. 4(B)]. Fibril formation was monitored by ThT fluorescence as a function of time in the presence of the hWO-2 Fab (gray line), cWO-2 Fab (dashed gray line), or 32G12 Fab (black line). $A\beta_{1-42}$ preformed fibrils incubated with hWO-2 Fab (gray line), cWO-2 Fab (dashed gray line), or 32G12 Fab (black line) were analyzed by ThT fluorescence every 2 h over a 24-h incubation period. Experiments were performed in duplicate, and each sample was run in triplicate.

incubation of $A\beta_{1-42}$ with hWO-2 Fab and cWO-2 Fab produced a significantly lower level of fluorescence [Fig. 4(A)].

Next, we examined the ability of hWO-2 Fab to disaggregate preformed fibrils [Fig. 4(B)]. In this case, the $A\beta_{1-42}$ peptide was allowed to form fibrils over a period of 7 days, and the fibrils were then treated with either 32G12, cWO-2 Fab or hWO-2 Fab for 24 h. In the presence of 32G12 Fab, the fluorescence decreased to 82% compared with the maximum fluorescence signal. In contrast, when treated with hWO-2 or cWO-2 Fab, the fluorescence signal decreases to ~14% over the same time period. Together, these data imply that the humanized Fab fragment can inhibit the formation of $A\beta_{1-42}$ amy-

loid fibrils and promote the disaggregation of preformed amyloid fibrils.

Protection of $A\beta_{1-42}$ toxicity by WO-2 recombinant antibodies

Previous studies have identified soluble oligomers (also called amyloid β -derived diffusible ligands or ADDLs) as the most toxic form of $A\beta$.⁶ Such structures can be obtained *in vitro* by incubating $A\beta_{1-42}$ monomers in PBS for 24 h at 4°C.

An MTT assay was used to test whether the hWO-2 Fab fragments could protect human neuroblastoma cells (M17) against the toxic effect of these oligomers (Fig. 5). The cell viability was significantly decreased when incubated for 24 h with $A\beta_{1-42}$ oligomers (34% cell viability) compared with cells without the oligomers (100% cell viability). Likewise, cell viability was affected to the same degree (40% cell viability) if cells were incubated with $A\beta_{1-42}$ oligomers that were preincubated with 32G12 Fab, a control antibody fragment. In contrast, if $A\beta_{1-42}$ oligomers were preincubated with hWO-2 Fab (molar ratio 1:5 of antibody: $A\beta_{1-42}$), cell viability was only reduced to ~78%, suggesting that this antibody fragment can protect the cells from the neurotoxic effect of $A\beta_{1-42}$ oligomers as efficiently as cWO-2 Fab.

Conclusions

Current passive immunotherapy experiments suggest that deleterious side effects (edema or cerebral microhemorrhages) can be avoided by using antibody fragments.²⁵ These results highlight the future role of engineered antibody fragments for a safer treatment of not only AD but also other misfolding diseases including Creutzfeldt-Jakob disease,

Figure 5. M17 neuroblastoma cells were incubated with $A\beta_{1-42}$ oligomers alone or with $A\beta_{1-42}$ oligomers preincubated with 32G12 Fab, cWO-2 Fab, or hWO-2 Fab. The MTT assay was used to measure cell viability. Experiments were performed in duplicate, and each sample was run in triplicate. Data were expressed as a percentage of the control (M17 neuroblastoma cells only) $\pm$ SD.

Parkinson's disease, and Huntington's disease.²⁶ However, the use of murine antibodies in human trials can present a hurdle because of the potential harmful human anti-mouse antibody response.

We describe in this report the humanization of WO-2 by CDR-grafting onto human germline FR genes and the resulting high-resolution X-ray crystal structure. To our knowledge, this is the first crystal structure describing a humanized antibody Fab directed against the immunodominant epitope of the amyloid β peptide. Moreover, the abilities of hWO-2 Fab to inhibit aggregation and oligomer-mediated toxicity as well as promoting amyloid fibrils disaggregation show the value of such fragment for AD therapy.

Material and Methods

Humanization of the WO-2 antibody by CDR-grafting

IMGT/V-QUEST²⁷ and IMGT/Junctions analysis tools²⁸ were used to identify human germline genes in which sequences from the variable regions of both the heavy and light chains were closely aligned with those of murine antibody. Framework sequences of these selected human germline genes were used as acceptor sequences for the mWO-2 CDRs. However, murine residues were retained in the critical "Vernier" zone. The humanized VH and VL genes were synthesized by GENEART AG (Regensburg, Germany).

Expression and purification of the humanized antibody fragments

Synthetic genes were inserted into the expression vector pGC and pGC-Fab as previously described.¹⁶ Transformed *E. coli* TOP10F' cells (Invitrogen, Mount Waverley, VIC) were grown at 30°C in 10 L of 2 YT medium containing glucose (0.1%) and ampicillin (100 μ g/mL). When the OD₆₀₀ = 1 was reached, isopropyl β -D-thiogalactopyranoside was added to a final concentration of 0.1 mM, and the temperature was reduced to 26°C. After 4 h of protein induction, the cells were pelleted and a periplasmic extract was prepared by sequential extraction with ice-cold 1 $\times$ TES buffer (0.2M Tris-HCl, pH 8.0, 0.5 mM EDTA, and 0.5M sucrose) and 0.2 $\times$ TES buffer. The periplasm extract was clarified by centrifugation (20,000g; 30 min) and filtered through 0.45- μ m membrane (Millipore, Bedford, USA).

The hWO-2 Fab was affinity purified on the ÄKTA PurifierTM system (GE Healthcare, Uppsala, Sweden) using the construct's histidine tag. A four-step protocol was used. The cleared periplasmic extract was loaded onto a 5-mL HisTrapTM FF crude column (GE Healthcare) preequilibrated with binding buffer (20 mM sodium phosphate pH 8.0, 0.5M NaCl, and 20 mM imidazole). The hWO-2 Fab was eluted from the IMAC column with 50% elution

buffer (20 mM phosphate pH 8.0, 0.5M NaCl, and 500 mM imidazole) and passed through a HiPrepTM 26/10 desalting column (GE Healthcare) preequilibrated in desalting buffer (20 mM sodium acetate pH = 5.5 and 20 mM NaCl). Subsequently, the hWO-2 Fab was bound to a 1-mL HiTrapTM SP HP (GE Healthcare), washed with low ionic strength desalting buffer, and eluted with a gradient of 0.02–1M NaCl in 20 mM sodium acetate, pH 5.5. The eluate was then loaded onto a HiLoadTM 26/60 Superdex 200 (GE Healthcare) preequilibrated in 1 $\times$ TBS (50 mM Tris-HCl, pH 7.4 and 150 mM NaCl). Subsequently, this method was adapted for automated multistep chromatography on the ÄKTExpressTM system (GE Healthcare). The hWO-2 Fab-containing fractions from the gel-filtration step were combined and concentrated to $\sim$ 4 mg/mL using a Millipore Ultrafree centrifugal concentrator (molecular weight cut off = 10,000 Da; Millipore, Bedford, MA).

Protein crystallization

Crystallization screening was performed at the Bio-21 Collaborative Crystallization Centre (www.csiro.au/c3/). The screening was done in 96-well sitting drop plates, using droplets consisting of 100 nL protein sample and 100 nL of crystallant. Initial screening carried out using the JCSG+ and PACT screens (Qiagen) at two temperatures. Crystals suitable for diffraction analysis of the hWO-2 Fab were grown at 20°C from conditions containing 20% PEG 3350 as the precipitant with either 0.2M sodium sulfate or 0.2M lithium citrate. Crystallization trials of the hWO-2 Fab in complex with the A β _{1–16} peptide are continuing.

Data collection

A single crystal was transferred briefly into a droplet of the reservoir solution supplemented with 10% (v/v) glycerol and 10% (v/v) ethylene glycol and flash cooled in the cold nitrogen stream at beamline MX1 of the Australian Synchrotron. A 2.1 Å data set consisting of 180° (0.5°/frame, 5 s exposures) was collected from a single crystal. The initial data processing was conducted with the HKL2000 package.²⁹ The space group was *P*3₁, with cell parameters *a* = *b* = 106.73 Å, *c* = 90.87 Å, and solvent content of 59.1%, see Table II.

Structure determination and refinement

The initial structure was determined using molecular replacement using the program PHASER³⁰ within CCP4.³¹ The search model consisted of murine Fab WO-2 (PDB: 3BAE)²³ variable light and heavy chains domains and humanized Fab fragments PDB:1UM5 (light chain) and PDB:8FAB (heavy chain). All of the components of the search model shared over 85% sequence identity with the

corresponding regions of the target molecule. Two independent molecules were identified in the asymmetric unit. Iterative crystallographic refinement and model building were conducted using REFMAC³² and XTALVIEW,³³ respectively, and yielded a model encompassing residues hWO-2 1–229 (H chain) and 1–228 (A chain) of the 232 residues of the heavy chain and all 1–277 residues (L and B chains) of the light chain (including the nine residues AAADYKDDD from FLAG tag) and 348 water molecules. Progress of the refinement was monitored using the R_{free} statistic based on a set encompassing 5% of the observed diffraction amplitudes.³⁴ Following the convergence in standard REFMAC5 refinement, further improvement of R factors (over 2%) was achieved by refining the variable and constant domains of light and heavy chains (total eight regions) as separate rigid anisotropic domains with the TLS procedure (transition-libration-screw motion tensors refinement).³⁵ The libration tensor showed significant anisotropy. The final crystallographic refinement statistics is presented in Table II. In total 93% of hWO2 residues are in the most favored regions of the Ramachandran plot, with 5.1% in the allowed regions. The final refinement converged to R/R_{free} values of 0.213/0.274.

Biosensor binding analysis

SPR measurements were performed using Bio-Rad's ProteOn XPR36 array biosensor.³⁶ Various WO-2 antibody fragments were analyzed for binding to the A β_{1-16} -biotin peptide (AUSPEP, Parkville, Australia) captured on a NeutrAvidin-coated chip (NLC, Bio-Rad). Initially, 1 $\mu\text{g/mL}$ solution of the biotinylated peptide was injected in the "ligand" direction in Channel 6 (L6) for 2 min at 30 $\mu\text{L/min}$. This approach resulted in a significant variation (>30%) in capture levels of biotinylated peptide across the channel, ranging from 124 resonance units (RU) in spot L6A6 to 84 RU in spot L6A1. This made the "one-shot" kinetics approach, involving a single delivery of concentration series of antibody fragments in "analyte" direction³⁶ impractical as the binding surface capacity (R_{max} values) varied significantly across each spot. Binding measurements were therefore performed using a "classical kinetics" approach whereby each spot was treated as a separate flowcell with five different concentrations (81, 27, 9, 3, and 1 nM) of antibody fragments plus buffer blank (for double referencing) being injected across each spot one after the other. This approach generated a five-point concentration data set for each spot. Association and dissociation phases were monitored at 30 $\mu\text{L/min}$ for 150 and 300 s, respectively. The A β_{1-16} -coated chip was regenerated between analyte injections with 10 mM glycine pH 1.5 at 100 $\mu\text{L/min}$ for 18 s. To determine the kinetic rate constants of the binding interactions, binding data were

processed, double referenced with responses from buffer blank injections, and fitted globally to a 1:1 interaction model using Scrubber-Pro software package (obtained from D. Myszk, University of Utah). Binding measurements reported in this article represent average and standard deviation values generated from each of the six spots.

Preparation of synthetic A β_{1-42} oligomers

A β_{1-42} synthetic lyophilized peptide (W. M. Keck Laboratory, Yale University, New Haven, CT) was dissolved in 1,1,1,3,3,3-hexafluoro-2-isopropanol (HFIP) (Sigma, St Louis, MO) and aliquoted. HFIP was removed by evaporation under a fume hood, and residual traces of HFIP were removed by drying under vacuum in a SpeedVac (Savant Instrument). The resulting peptide film was stored at -80°C until required. For the A β_{1-42} oligomeric preparation, the peptide film was dissolved in dimethyl sulfoxide (DMSO) at 5 mM and then diluted to 100 μM in ice cold $1\times$ PBS. The solution was left for 24 h at 4°C and centrifuged at 16,000g for 15 min at 4°C . The supernatant containing the soluble oligomers was used for the assays.

Thioflavine T fluorescence assay

The inhibition of A β_{1-42} fibril formation and the disaggregation of preformed A β_{1-42} fibrils by hWO-2 Fab were monitored by ThT fluorescence assay as described previously.^{16,37}

In vitro neuroprotection (MTT) assay against oligomer-mediated toxicity

The human neuroblastoma cell line M17 (ATCC Number CRL-2267) was maintained in serum-free OPTI-MEM medium (Invitrogen, Mount Waverley, VIC, Australia) containing 0.1 mM nonessential amino acids, 50 IU/mL penicillin, 50 $\mu\text{g/mL}$ streptomycin (Gibco, Gaithersburg, MA), 1 mM sodium pyruvate, and 10% FCS in 5% CO_2 at 37°C . Cells were seeded in 96-well tissue culture plates (Nunc, Roskilde, Denmark) at $\sim 10^4$ cells per well and incubated for 24 h. A β_{1-42} oligomers were incubated either alone or with purified hWO-2 Fab, cWO-2 Fab or 32G12 Fab for 3 h at RT. These reactions (10 μL) were added to the cells (final concentration of peptide and antibodies of 15 and 0.5 μM , respectively). Plates were incubated for an additional 24 h at 37°C .

Cell viability was determined using a MTT toxicity assay. Briefly, 10 μL of 5 mg/mL MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma) was added to each well and incubated for a further 24 h at 37°C . Plates were centrifuged, the medium was removed, and 100 μL of DMSO was added to each well to dissolve the crystals. The absorbance was measured at 560 nm.

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