1 **Locally misfolded HER2 expressed on cancer cells is a promising target for development of**
2 **cancer-specific antibodies**
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20 **Summary**
21 Overexpression of human epidermal growth factor receptor 2 (HER2) in breast and gastric
22 cancers is known to be associated with poor prognosis, making it an attractive therapeutic target. Here,
23 we established a novel cancer-specific anti-HER2 antibody, H_2Mab-214. H_2Mab-214 reacted with
24 HER2 on cancer cells, but unlike the therapeutic antibody trastuzumab, did not react with HER2 on
25 normal cells in flow cytometry. Using X-ray crystallography, we revealed that H_2Mab-214 recognizes
26 a structurally disrupted portion in the HER2 domain IV, which normally forms a β-sheet. This
27 misfolding was shown to be inducible by site-directed mutagenesis mimicking the disulfide bond
28 defects that may occur in cancer cells, indicating that the local misfolding in the Cys-rich domain IV
29 governs the cancer-specificity of H_2Mab-214. Furthermore, we showed that H_2Mab-214 effectively
30 suppresses tumor growth in xenograft mouse models. Our findings offer a potential strategy for
31 developing cancer-specific therapeutic antibodies that target partially misfolded cell surface receptors.
32

33 **Introduction**
34 Human epidermal growth factor receptor (HER) family of receptor tyrosine kinases consists of
35 four members: EGFR (HER1), HER2, HER3, and HER4. They play important roles in regulating cell
36 proliferation, differentiation, and migration, while being implicated in many cancers. More than ten

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37 ligands for HER members have been identified, including epidermal growth factor, transforming
38 growth factor alpha, and neuregulins (NRGs) 1-4, yet HER2 has no known direct activating ligand.[1]
39 The extracellular region of all HER family members is commonly composed of four domains (I-IV).
40 In the absence of ligands, those receptors other than HER2 are stabilized in an inactive "tethered"
41 conformation where a protruding loop (also called dimerization arm) in the domain II interacts with
42 the domain IV.[2-4] Upon ligand binding, the receptors undergo a large conformational change from
43 "tethered" to "extended" and form homo- or heterodimers via the dimerization arm, resulting in the
44 autophosphorylation of the cytoplasmic tyrosine kinase domain.[5-7] HER2 is unique in that it always
45 adopts extended conformation even in the monomeric state.[8] It has recently been shown that the
46 structure of HER2 forming a heterodimer with HER3 is very similar to that of the monomeric state.[9]
47 Hence, HER2 is always ready to be dimerized at cell surface, making it a preferable dimerization
48 partner for other HER family members.[10] In addition, overexpression of HER2 promotes the formation
49 of HER2 homodimers, leading to ligand-independent signaling.[11] HER2 overexpression is found in
50 ~20% of breast cancers and is associated with poor prognosis, higher rates of recurrence, and shorter
51 overall survival.[12] HER2 overexpression is also observed in ~20% of gastric cancers.[13]

52 Trastuzumab, a monoclonal antibody (mAb) against HER2, recognizes the domain IV[8] and
53 exhibits an anti-proliferating effect *in vitro* and a potent antitumor effect *in vivo*.[14] The addition of
54 trastuzumab treatment to a standard chemotherapy improves objective response rates, progression-free
55 survival, and overall survival in HER2-positive breast cancer patients with metastasis.[15] Trastuzumab
56 has been the standard treatment for HER2-positive breast cancers[16] and HER2-positive gastric
57 cancers.[17] Clinically, the efficacy of trastuzumab involves immunologic engagement.[14] The Fc domain
58 of trastuzumab mediates the engagement with Fcγ receptors (FcγRs) on various immune cells.
59 Trastuzumab-FcγR binding allows for phagocytosis of antibody-bound tumor cells, which is called
60 antibody-dependent cellular phagocytosis. The FcγR engagement also activates macrophages,
61 dendritic cells, and neutrophils, which can change adaptive immune responses through chemotaxis,
62 cytokine production, and antigen presentation. Furthermore, the FcγR engagement mediates the
63 activation of natural killer (NK) cells which attack and lyse the target tumor cells, termed antibody-
64 dependent cellular cytotoxicity (ADCC).[18] Margetuximab was developed by introducing several
65 mutations in trastuzumab to improve the FcγRIIIA engagement (and thus ADCC activity),[19] and was
66 approved by U.S. Food and Drug Administration (FDA) and showed significant improvement in
67 progression-free survival in heavily pretreated patients.[20,21] In addition to ADCC, the Fc domain of
68 those mAbs can exert complement-dependent cytotoxicity (CDC).[22,23]

69 Although trastuzumab exhibits potent antitumor effect, some patients develop resistance against
70 trastuzumab treatment, which can be attributed to the hyperactivation caused by the heterodimerization
71 with HER3.[24] Pertuzumab, another clinically approved HER2-targeting mAb, recognizes the domain
72 II and prevents NRG1-induced heterodimerization with HER3 and intracellular signaling.[25] Thus,

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73   pertuzumab is considered to have a complementary mechanism of trastuzumab,[26] and the double anti-
74   HER2 blockade has become the standard therapy in the initial management of metastatic HER2-
75   positive breast cancer.[15] Furthermore, combination therapy using trastuzumab, pertuzumab, and
76   chemotherapy has been evaluated and found to show even higher clinical benefit.[27]

77   Despite the proven success of HER2-targeted immunotherapies described above, they still suffer
78   from several adverse effects, including cardiotoxicity.[28] Because of this side effect, routine cardiac
79   monitoring is required in clinic during the anti-HER2 antibody treatment.[28] Interestingly, mice lacking
80   *ErbB2* (ortholog of HER2) display embryonic lethality due to the dysfunctions associated with a lack
81   of cardiac trabeculae,[29] and ventricular-restricted *ErbB2* deficiency in mice shows features of dilated
82   cardiomyopathy.[30] These results indicate that HER2 plays important role(s) in normal heart
83   development and homeostasis. As a proteomic study of healthy human tissues revealed a high level of
84   broad expression of HER2,[31] anti-HER2 mAb therapies may attack normal tissues not limited to the
85   heart, which may lead to other side effects including ones that are not clearly recognized at present.
86   Therefore, it is highly desirable to use anti-HER2 mAbs that can exclusively recognize HER2
87   expressed on cancer cells, although such reagents have not been available to this day.

88   In this study, we succeeded in establishing a novel mAb highly specific against HER2 expressed
89   on cancer cells but not on normal cells. This antibody, H_2Mab-214, was established through standard
90   immunization and hybridoma technology, but incorporating a special screening strategy called cancer-
91   specific mAb or CasMab method we have developed previously.[32-35] The cancer-specificity of H_2Mab-
92   214 was extensively investigated by flow cytometry analysis using various cell lines of normal or
93   cancer origins, and its antitumor potential was confirmed by *in vivo* xenograft model. Most importantly,
94   we clarified the molecular basis of H_2Mab-214's cancer specificity by determining the crystal structure
95   of its complex with HER2-derived peptide. Combined with the structure-guided mutagenesis at the
96   epitope region, we show evidence that the local misfolding of HER2 domain IV Cys-rich region
97   governs the cancer-specificity of H_2Mab-214, pointing toward a new way of searching cancer-specific
98   antibodies against various cell surface receptors.

99

100   **Results**

101   ***Cancer-specific HER2 recognition by H_2Mab-214.***

102   We have previously established a mouse anti-HER2 mAb H_2Mab-119 (IgG_1, kappa) by
103   immunizing mice with HER2 ectodomain (HER2ec) produced in glioblastoma LN229 cells.[36] This
104   antibody is judged as a pan-HER2 mAb that reacts with HER2 regardless of the cell type, because its
105   FACS staining pattern is indistinguishable from that of trastuzumab (Figures 1A and 1B). In flow
106   cytometric analysis using saturating concentrations of each antibody, H_2Mab-119 and trastuzumab
107   brightly stained CHO-K1 cells expressing exogenously introduced HER2 (CHO/HER2) (Figure 1A)
108   as well as BT-474 and SK-BR-3 breast cancer cell lines that are known to express endogenous HER2

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109 (Figure 1B).[37] They also recognized immortalized normal cell lines, including HEK293T (embryonic

110 kidney), HaCaT (keratinocyte), and MCF 10A (mammary gland epithelial cell), but not triple-negative

111 breast cancer (TNBC) cell line, MDA-MB-468 cells known to be HER2-negative (Figure 1B).[38]

112   Next we aimed at obtaining anti-HER2 mAbs that recognize HER2 only when expressed on

113 cancer cells. We incorporated differential screening protocol in the hybridoma selection step[32] and

114 succeeded in establishing a few mAbs that recognized HER2-positive cancer cell lines, but not HER2-

115 expressing normal cells. As shown in Figure 1B, one of the clones, H_2Mab-214 (IgG$_1$, kappa), reacted

116 with BT-474 and SK-BR-3 cells, but not with HEK293T, HaCaT, and MCF 10A cells. H_2Mab-214

117 also recognized CHO/HER2 (Figure 1A), but the reactivity was relatively low compared to that of

118 trastuzumab and H_2Mab-119. These results suggest that H_2Mab-214 exhibits specificity to HER2-

119 positive breast cancer cells.

120

121 *Epitope mapping of H_2Mab-214.*

122   Puzzled by the apparent cancer cell specificity of H_2Mab-214 in its HER2 recognition, we next

123 explored its binding epitope. To this end, we generated a series of N-terminal deletion mutants of

124 HER2 by successively removing domain I (ΔN218), domain II (ΔN342), and domain III (ΔN511)

125 (Figure 2A). The comparable expression levels of these mutants on CHO-K1 cells were confirmed by

126 the staining with NZ-1 antibody against the 16-residue PA tag attached to the N-terminus of the

127 truncation mutants. H_2Mab-214 exhibited full reactivity toward all mutants (Figure 2B), indicating

128 that its epitope lies exclusively within the domain IV. In contrast, the reactivity of H_2Mab-119 was

129 completely lost upon the deletion of the domain I alone, suggesting the critical involvement of domain

130 I in its HER2 recognition. To narrow down further the location of H_2Mab-214 epitope, we synthesized

131 overlapping 20-mer peptides covering the domain IV and tested their reactivity by ELISA. As shown

132 in Figure 2C, H_2Mab-214 showed strong reactivity with the peptide corresponding to residues 603-

133 622. Subsequent analysis using the second set of shorter peptides derived from this region revealed

134 that the linear segment Pro612-Asp618 of HER2 confers the minimally required binding epitope for

135 H_2Mab-214 (Figure 2D). Finally, binding toward alanine-substituted HER2 peptides were evaluated

136 to see the contribution of individual amino acid. H_2Mab-214 showed significantly reduced reactivity

137 toward K615A and F616A peptides, indicating that Lys615 and Phe616 have major contribution to

138 the recognition by H_2Mab-214 (Figure 2E).

139

140 *Crystallographic analysis of H_2Mab-214 in complex with the epitope peptide.*

141   The epitope mapping of H_2Mab-214 revealed that it recognized linear 7-residue peptide $_{612}$-

142 PIWKFPD-$_{618}$. This segment and the regions surrounding it are present in all major variants of HER2,

143 and they do not contain sequence motifs suspected to undergo chemical modifications including

144 glycosylation, phosphorylation, and deamidation (Figure S1A). Also, this segment is partially

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145 overlapped with the binding footprint of pan-HER2 reactive trastuzumab (residues 579-625) (Figure

146 S1).[9] Therefore, it is difficult to explain the cancer specificity of H_2Mab-214 simply by cancer-specific

147 chemical alterations or large domain-wise conformational change to expose its epitope. To understand

148 how H_2Mab-214 distinguishes HER2 expressed on cancer cells from that on normal cells, we

149 performed a crystallographic analysis of H_2Mab-214 in complex with its epitope peptide. To this end,

150 antigen-binding domain of H_2Mab-214 was expressed as a small and hyper-crystallizable antibody

151 fragment, Fv-clasp,[39] and crystallized in the presence of the excess amount of HER2 (611-618) peptide,

152 and the complex structure was solved at 1.75-Å resolution (Table S1, Figure S2A). At the antigen

153 binding site of H_2Mab-214, a clear electron density corresponding to the epitope peptide was observed,

154 allowing us to build models for all eight residues of the peptide (Figure S2B). The bound peptide is in

155 a compact U-shaped conformation (Figure 3A), but unlike the typical turn conformation found in

156 protein loops, it is not maintained via intramolecular main chain hydrogen bonds. Rather, the

157 conformation is stabilized by intermolecular hydrogen bonding interactions formed between the

158 residues from the peptide and Gly31B (CDR-H1), Arg58, Arg95 (CDR-H3), Ala98 (CDR-H3), and

159 Trp100A (CDR-H3) of H_2Mab-214 (Figure 3B). In addition, numerous inter- and intramolecular van

160 der Waals contacts formed throughout the peptide reinforce highly complementary recognition

161 interface that warrant high affinity for such a short stretch of amino acids (Figure 3A). Among the

162 peptide residues, Lys615 and Phe616 appear to form particularly important interactions: the side chain

163 of Lys615 forms salt bridges with two aspartic acid residues (Asp54 and Asp56) in CDR-H2 (Figure

164 3B), while the side chain of Phe616 is inserted into a deep pocket at the center of the antigen binding

165 site and in contact with the surrounding residues (Figure 3A). These observations are in good

166 agreement with the results of the alanine scanning (Figure 2E).

167 We also conducted a crystallographic analysis for the pan-HER2 antibody H_2Mab-119. As

168 H_2Mab-119 was found to retain high binding affinity for the HER2 domain I fragment (Figure S2C),

169 the H_2Mab-119 Fab was co-crystallized with the domain I fragment. In the complex structure solved

170 at 1.69-Å resolution, H_2Mab-119 is in contact with two β-strands and multiple loops of the domain I,

171 indicating that H_2Mab-119 recognizes three-dimensional (i.e., non-linear) epitope, which is in contrast

172 to H_2Mab-214 (Figures 3C and S2D).

173

174 ***Structural comparison of the epitope peptide bound to H_2Mab-214 and the corresponding region in***

175 ***published HER2 structures.***

176 HER2 domain IV has a stalk-like structure consisting of multiple short β-sheets stabilized by 10

177 disulfide bonds, and the H_2Mab-214 epitope sequence (Pro612 to Asp618) is located near its C-

178 terminus (Figure 3D). So far, ten HER2 ectodomain structures containing atomic models of the

179 Pro612-Asp618 region have been reported (i.e. PDB IDs: 1n8z, 3be1, 3n85, 6bgt, 6j71, 6oge, 7mn5,

180 7mn6, 7mn8, and 8ffj), of which 3n85 and 6j71 are relatively well modeled with no disordered regions

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181 in the domain IV. In all of these structures, the Pro612-Asp618 region assumes an extended
182 conformation, with its central segment (Ile613 to Phe616) assuming a β-strand sandwiched by two
183 strands to form a β-sheet (Figure 3D, inset). This "canonical" conformation of the 612-618 segment
184 (Figure 3E) is drastically different from the U-shaped conformation seen in the same peptide bound to
185 H_2Mab-214 (Figures 3F). Analysis of the residue-wise main chain dihedral angles for the H_2Mab-214-
186 captured peptide revealed large divergence from the values obtained for the published ectodomain
187 structures, especially at Trp614, Lys615, and Phe616 (Table 1). Such fundamental difference in the
188 conformation tells us that the 612-618 segment needs to be dislodged or "pulled-out" from the β-sheet
189 to be recognized by H_2Mab-214, suggesting that the HER2 molecules reactive with H_2Mab-214 must
190 undergo local unfolding near its epitope region (Figures 3G and 3H).

191

192 ***Effect of forced unfolding of HER2 by DTT treatment on the reactivity of anti-HER2 mAbs.***
193   Structural studies suggested that H_2Mab-214 preferentially recognizes HER2 domain IV when it
194 is structurally compromised. To validate this hypothesis, we sought to artificially disrupt the tertiary
195 structure of HER2 on normal cells and investigated antibody bindings to these cells. HER2 has many
196 disulfide bonds in its extracellular region contributing to the overall structural stabilization at varying
197 degree. To break the disulfide bonds of HER2 present on HEK293T cells, cells were cultured in the
198 media containing 1 mM DTT for 1, 5, and 24 hours, and reactivities of the antibody were investigated
199 by flow cytometry (Figure 4A). Remarkably, binding of H_2Mab-214 was increased more than 2-fold
200 after the 1-hour treatment with DTT, indicating that the number of epitope-bearing species increased
201 due to the partial reduction of disulfide bonds. Furthermore, this increase was transient because the
202 binding became less pronounced after 5 hours and returned to the basal level (without DTT treatment)
203 after 24 hours. In sharp contrast, H_2Mab-119 exhibited completely opposite behavior to the DTT-
204 treatment; the binding was reduced by >95% after 1-hour DTT treatment, followed by the gradual
205 recovery over time, returning to the basal level after 24 hours (Figure 4B). These observations are
206 consistent with the idea that brief incubation with DTT induces partial disruption of the tertiary
207 structure of HER2, which is favored by H_2Mab-214 but disfavored by H_2Mab-119, while the normal
208 structure is restored as the effective concentration of DTT goes down by oxidation during the cell
209 culture at 37°C.
210   To generalize the above notion, reactivities of four regular anti-HER2 mAbs, H_2Mab-19 (epitope:
211 domain III),[40] H_2Mab-181(epitope: domain III),[41] H_2Mab-41 (epitope: domain IV),[42] and trastuzumab
212 (epitope: domain IV), with HEK293T cells were investigated 1 and 24 hours after the addition of 1
213 mM DTT. As in the case of H_2Mab-119, all antibodies showed a markedly reduced reactivity with the
214 cells at 1 hour, and that was nearly recovered at 24 hours (Figure S3). The decreased binding of non-
215 cancer-specific mAbs with epitopes in different domains upon addition of DTT suggests that structural
216 disruption occurred throughout the HER2 molecule. These results again highlight the unique character

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217   of H_2Mab-214 in that it recognizes structurally disrupted HER2.

218   If the reactivity of H_2Mab-214 is governed by the partially unfolded nature of HER2 molecules

219   on cell surface, the DTT-induced binding upregulation should be seen in any cell types. In fact, DTT

220   treatment of normal mammary gland epithelial cell line MCF 10A resulted in small but statistically

221   significant ($p<0.05$) increase in the reactivity of H_2Mab-214 (Figure 4C). Furthermore, the same

222   treatment also upregulated H_2Mab-214 reactivity toward SK-BR-3 cells which were already positive

223   for H_2Mab-214 binding before the DTT treatment (Figure 4D), indicating that there is a pool of

224   H_2Mab-214-negative HER2 molecules expressed on breast cancer cells which can be converted to

225   H_2Mab-214-positive ones upon DTT treatment.

226

227   ***Effects of targeted disruption of domain IV disulfide bond on the epitope exposure.***

228   Although the DTT treatment facilitated the conversion of HER2 domain IV to the alternative

229   conformation reactive with H_2Mab-214, it severely reduced the binding of all other mAbs, indicating

230   that the DTT-treated HER2 has globally disturbed structure that does not accurately capture the

231   character of H_2Mab-214-reactive species on cancer cells. Considering the fact that most anti-HER2

232   mAbs do not show cell-type specificity like H_2Mab-214, cancer-specific HER2 structural alteration

233   may be restricted to domain IV, particularly the region encompassing residues 612-618. We therefore

234   set out to investigate whether targeted local disruption of this region via removing individual disulfide

235   linkage can mimic cancer-specific presentation of the H_2Mab-214 epitope. As the H_2Mab-214 epitope

236   is located between the 8th and 9th disulfide bonds in the domain IV (Figure 4E), we chose to remove

237   the 6th to 10th disulfide bonds (C567:C584, C587:C596, C600:C623, C626:C634, and C630:C642),

238   which are relatively close to the epitope. As a control, the first disulfide bond (C511:C520) which is

239   ~55 Å away from the epitope was also removed. To remove individual disulfide bond, the pairing

240   cysteine residues were simultaneously replaced with alanine in the context of full-length HER2. Upon

241   the transient expression in CHO-K1 cells, their reactivities against the anti-HER2 antibodies were

242   evaluated by flow cytometry.

243   As expected, H_2Mab-119 reacted with all mutants at levels nearly equivalent to the wild-type,

244   confirming the structural integrity at the domain I in these domain IV-mutants. Trastuzumab showed

245   greatly reduced reactivity with the C567A/C584A and C587A/C596A mutants and moderately

246   reduced reactivity with the C600A/C623A mutant, but binding to other mutants remained intact

247   (Figure 4F). The epitope for trastuzumab is composed of three loops, Pro579-Gln583 (loop 1),

248   Asp592-Phe595 (loop 2), and Lys615-Pro625 (loop 3) (Figure S1B). Structurally, the C567:C584 and

249   C587:C596 disulfide bonds are located behind the loop 1 and 2, respectively, and appear to support

250   the conformation of these loops, and the C600:C623 is located in a linker connecting the loop 2 and

251   3. Thus, the reduced binding of trastuzumab in their removal correlates well with the structural

252   observations. When the same set of HER2 mutants were evaluated for the binding by H_2Mab-214,

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253 however, they showed increased binding at varying degrees (from 30 to 90%), except for the pair
254 located away from the epitope (i.e., C511:C520) (Figure 4F). This result indicates that the exposure
255 of H_2Mab-214 epitope does not require global misfolding of HER2 but can be facilitated by any local
256 destabilization of the structure near the Pro612-Asp618 segment, which serves as a "weak ankle" of
257 HER2 that readily changes its conformation into an alternative one.
258

259 ***ADCC and CDC by H_2Mab-214-mG_{2a}-f against BT-474 and MDA-MB-468 cells.***

260   The high selectivity of H_2Mab-214 for cancer cells could be a major advantage in its
261 pharmaceutical application. Therefore, we evaluated the potential application of H_2Mab-214 in cancer
262 therapy. We have previously demonstrated that class-switched (from IgG_1 to IgG_{2a}) and defucosylated
263 mAbs exert potent antitumor effects in several mouse xenograft models.[43-50] To evaluate the antitumor
264 activity of H_2Mab-214 and H_2Mab-119, class-switched and defucosylated mAbs (H_2Mab-214-mG_{2a}-
265 f and H_2Mab-119-mG_{2a}-f) were produced by combining their V_H with C_H of mouse IgG_{2a} (Figure S4A).
266 We confirmed that H_2Mab-214-mG_{2a}-f and H_2Mab-119-mG_{2a}-f recognized BT-474, but not MDA-
267 MB-468 cells (Figure S4B). As a defucosylated control mouse IgG_{2a} mAb, we used 281-mG_{2a}-f (anti-
268 hamster podoplanin [PDPN] mAb), which never recognized BT-474 nor MDA-MB-468 cells (Figure
269 S4B).

270   We first investigated whether H_2Mab-214-mG_{2a}-f and H_2Mab-119-mG_{2a}-f could exert ADCC
271 against BT-474 cells. As shown in Figure 5A, H_2Mab-214-mG_{2a}-f showed ADCC activity (48.0%
272 cytotoxicity) against BT-474 cells more potently than did the control mouse IgG_{2a} (4.4% cytotoxicity;
273 P<0.01) and 281-mG_{2a}-f (4.8% cytotoxicity; P<0.01), and the activity was comparable to that induced
274 by H_2Mab-119-mG_{2a}-f (43.6% cytotoxicity). We also investigated CDC activities of H_2Mab-214-
275 mG_{2a}-f and H_2Mab-119-mG_{2a}-f against BT-474 cells. As shown in Figure 5B, both antibodies
276 exhibited similar levels of CDC in BT-474 cells (H_2Mab-214-mG_{2a}-f: 76.5% cytotoxicity, H_2Mab-
277 119-mG_{2a}-f: 72.1% cytotoxicity), which were significantly higher than that induced by control mouse
278 IgG_{2a} (9.5% cytotoxicity; P<0.01) and 281-mG_{2a}-f (17.3% cytotoxicity; P<0.01). There were no
279 differences between H_2Mab-119-mG_{2a}-f or H_2Mab-214-mG_{2a}-f and control mouse IgG_{2a} or 281-mG_{2a}-
280 f in ADCC (Figure 5C) and CDC (Figure 5D) against MDA-MB-468 cells. These results demonstrated
281 that H_2Mab-214-mG_{2a}-f exhibited ADCC and CDC comparable to H_2Mab-119-mG_{2a}-f against HER2-
282 positive BT-474 cells, despite its reduced reactivity in flow cytometry (Figure 1).
283

284 ***Antitumor effects of H_2Mab-214-mG_{2a}-f in BT-474 xenografts.***

285   We next investigated antitumor effects of the antibodies in BT-474 xenograft mouse models.
286 H_2Mab-119-mG_{2a}-f, H_2Mab-214-mG_{2a}-f, control normal mouse IgG, or 281-mG_{2a}-f were injected
287 intraperitoneally on days 7, 14, and 19, after the inoculation of BT-474. The tumor volume was
288 measured on days 7, 12, 14, 19, 22, and 26 after the inoculation. Administration of H_2Mab-119-mG_{2a}-f

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289   and H_2Mab-214-mG_{2a}-f significantly reduced tumor volume compared to that of control normal mouse

290   IgG in the BT-474 xenograft models after days 14 and 19, respectively (Figure 6A). The reduction in

291   tumor volume on day 26 in mice treated with H_2Mab-119-mG_{2a}-f and H_2Mab-214-mG_{2a}-f was 41%

292   and 35%, respectively, compared to mice treated with the control normal mouse IgG. In addition, as

293   shown in Figure 6B, tumors from the H_2Mab-119-mG_{2a}-f and H_2Mab-214-mG_{2a}-f-treated mice

294   weighed significantly less than those from control normal mouse IgG-treated mice (60% reduction;

295    $P<0.01$ and 72% reduction, respectively; $P<0.01$). The reduction in tumor volume and weight in

296   H_2Mab-119-mG_{2a}-f- and H_2Mab-214-mG_{2a}-f-treated mice was also significant compared to 281-

297   mG2a-f-treated mice (Figures 6A and 6B). As a control experiment, the antitumor effects were also

298   investigated in the MDA-MB-468 xenograft models. As a result, no difference was observed between

299   H_2Mab-119-mG_{2a}-f or H_2Mab-214-mG_{2a}-f and control normal mouse IgG or 281-mG_{2a}-f in both tumor

300   volume (Figure S5A) and weight (Figure S5B), confirming that the antitumor effects observed in the

301   BT-474 xenograft models were HER2-dependent.

302   Tumors that were resected from mice on day 26 are demonstrated in Figures 6C and S5C. The

303   body weight loss was not observed in both H_2Mab-119-mG_{2a}-f and H_2Mab-214-mG_{2a}-f treated mice

304   (Figures 6D and S5D). The mice on day 26 about BT-474 xenograft and MDA-MB-468 xenograft

305   were demonstrated in Figures S6A and S6B, respectively.

306

307   **Discussion**

308   Trastuzumab is a pioneer in molecular targeted therapy and has been the most effective treatment

309   for HER2-positive breast cancer for more than 20 years since its FDA approval in 1998.[51] The success

310   of trastuzumab has demonstrated the utility of antibody drugs in cancer therapy, and mAbs have

311   become one of the leading modalities in this field. As of 2018 (i.e., before COVID-19), half of the top

312   10 best-selling antibody drugs were reported to be against cancer.[52] More recently, attention has

313   focused on the development of more innovative mAb-based therapeutic strategies, such as antibody-

314   drug conjugates (ADCs), chimeric antigen receptor (CAR)-T cell therapy, and bispecific antibodies.

315   For HER2-positive metastatic breast cancer, the ADCs, trastuzumab emtansine and trastuzumab

316   deruxtecan, have already been developed. With the trend shifting toward such therapies with high cell-

317   killing activity, antibodies with high specificity for cancer cells are becoming increasingly important.

318   In the development of therapeutic antibodies against cancers, efforts are being made to identify unique

319   characteristics of cancer cells. A major strategy is to identify molecules that are highly expressed on

320   the surface of certain types of cancer cells and have a high cancer cell/normal cell ratio as HER2.

321   However, many of the molecules that are abnormally expressed on cancer cells have already been

322   identified by extensive transcriptome and proteome analyses, making exploration for new cancer

323   antigens very challenging. In addition, in this strategy, the target molecules are common autoantigens

324   that are also expressed on normal cells, raising concerns about adverse effects caused by antibody

9

325   drugs.

326   In this study, we succeeded in developing the highly cancer-specific mAb targeting HER2. The

327 reactivity of H_2Mab-214 against cancer cells was clearly observed, whereas that against normal cells

328 was not detectable even by flow cytometry, which is a highly sensitive detection system (Figure 1).

329 The cancer specificity of H_2Mab-214 was found to be due to the recognition of a structural feature that

330 only HER2 on cancer cells has, namely the misfolded region. Previously, cancer-specific antibodies

331 that appear to recognize structural features of target molecules, anti-EGFR antibody mAb806 and anti-

332 α4β7 integrin antibody MMG49, have been reported.[53,54] Their cancer specificity is, however, thought

333 to be linked to the global conformation of their target molecules rather than to local structural features.

334 More specifically, their epitopes are expected to be hidden on normal cells but exposed on cancer cells

335 for some reason. For mAb806, it has also been demonstrated by crystallographic analysis that the

336 antigen peptide bound to the antibody has almost the same conformation as the corresponding region

337 in the EGFR.[55] By contrast, H_2Mab-214 is a new type of cancer-specific mAb that directly recognizes

338 the unique structural feature of the target. In the current crystal structure, the epitope peptide (Met611-

339 Asp618) assumes U-shaped conformation nestled in the binding pocket of H_2Mab-214, which is not

340 compatible with the canonical conformation seen in the native HER2 reported so far. As the segment

341 is part of a rigid 3-strand β-sheet in the native structure, the HER2 species reactive with H_2Mab-214

342 must have undergone local misfolding at this region to allow the access of the antibody. Therefore,

343 H_2Mab-214-reactive HER2 on cancer cells and H_2Mab-214-non-reactive HER2 on normal cells are

344 chemically identical molecules having a subtle conformational difference present in a restricted region

345 near the Pro612-Asp618 (Figures 5G and 5H).

346   Proteins are inherently prone to misfold. Homeostasis of membrane and secretory proteins is

347 normally maintained by quality control system in the endoplasmic reticulum (ER), but cancer cells are

348 constantly exposed to a variety of stressors such as oxidative stress, hypoxia, and decreased energy

349 supply, which are known to promote production and accumulation of misfolded proteins in the ER

350 (called ER stress).[56] We speculate that in such an abnormal condition of the ER in cancer cells, proteins

351 with minor structural defects (such as a misformation of a disulfide bond that does not impair overall

352 domain stability or function) may escape the quality control system and are transported normally,

353 presenting a cancer-specific "mark" to the immune system. Since our study showed that the

354 conformational integrity of HER2 at residues 612 to 618 is susceptible to the lack of surrounding

355 disulfide bonds, the local misfolding of HER2 may have stemmed from the disregulated redox system

356 in the ER of cancer cells. In breast cancer cells, it has been reported that the expression of protein

357 disulfide isomerases (PDI), PDIA1, PDIA3, PDIA4, and PDIA6, is elevated, suggesting an abnormal

358 state of the redox system in the ER.[57] Disruption of disulfide bond formation in cancer cells has also

359 been proposed for EGFR.[55]

360   As far as we are aware, only H_2Mab-214 shows cancer specificity among the many anti-HER2

10

361 antibodies developed to date, including trastuzumab that recognizes a region very close to the H$_2$Mab-
362 214 epitope. Therefore, the H$_2$Mab-214 epitope (Pro612-Asp618 region) may be very special in that
363 it undergoes spatially confined misfolding to expose highly antigenic peptide in cancer cells.
364 Surprisingly, when we immunized mice with a synthetic peptide containing the H$_2$Mab-214 epitope
365 sequence, all mAbs obtained were found to show cancer specificity (data not shown). This suggests
366 that prior knowledge about the misfold-dependent cancer neoantigen sequence may be leveraged to
367 obtain useful cancer-specific mAbs. By using such strategy, it may be possible to establish H$_2$Mab-
368 214-like antibodies that are more suitable for therapeutic applications, such as fully human mAbs or
369 high-affinity mAbs. As evidenced by the performance of trastuzumab, the lower stalk region of HER2
370 should also be a promising target in terms of the therapeutic efficacy. In fact, even though not all
371 HER2 molecules on cancer cells are H$_2$Mab-214 reactive, H$_2$Mab-214 showed anti-tumor activity
372 comparable to the pan-HER2 reactive H$_2$Mab-119 (Figures 4 and 6). More importantly, the high
373 cancer specificity is of great benefit in applications where very high therapeutic efficacy is expected,
374 such as CAR-T cell therapy and bispecific antibodies.

375   HER2 has a long history as a successful cancer therapeutic target and has been well studied, yet
376 its unique and cancer-specific structural defect that can be targeted by mAb had not been discovered
377 until now. Our results indicate that some molecules that had not been considered as good cancer-
378 specific therapeutic target due to the widespread expression in many tissues may in fact become viable
379 drug target if they are found to undergo cancer-specific local misfolding. It is hoped that the expansion
380 of potential targets will facilitate the development of mAb therapies for cancers for which effective
381 treatments have not yet been found.

382

392

393 **Author contributions**
394   T.A. designed and performed experiments, analyzed the data, and wrote the manuscript. E.M.,
395 H.S., T.O., T.T., and M.K.K. performed experiments and analyzed the data. J.T. and Y.K. conceived
396 the experimental design, analyzed the data, and wrote the manuscript. All authors contributed to the

11

397   preparation of the manuscript.

398

399   **Declaration of interests**

400   Mika K. Kaneko and Yukinari Kato submitted a patent application related to this work: H_2Mab-

401   214. Patent application number WO2022114163. (2021). The remaining authors declare no competing

402   interests.

403

404   **Figure legends**

405

406   **Figure 1. Flow cytometry using trastuzumab, H_2Mab-119, and H_2Mab-214.** (A) CHO-K1 and

407   CHO/HER2 cells were treated with 10 µg/ml of trastuzumab, H_2Mab-119, and H_2Mab-214 or buffer

408   control. (B) BT-474, MDA-MB-468, SK-BR-3, HEK293T, HaCaT, and MCF 10A cells were treated

409   with 10 µg/ml of trastuzumab, H_2Mab-119, H_2Mab-214 or buffer control. Then, cells were stained

410   with Alexa Fluor 488-conjugated anti-mouse IgG (for H_2Mab-119 and H_2Mab-214) or FITC-

411   conjugated anti-human IgG (for trastuzumab). The black line represents the negative control (blocking

412   buffer).

413

414   **Figure 2. Epitope mapping of H_2Mab-214.** (A) Domain organization of HER2 and design of a series

415   of N-terminal domain deletion mutants used for the flow cytometry analysis. (B) Binding of H_2Mab-

416   214 and H_2Mab-119 to the deletion mutants of HER2 transiently expressed on CHO-K1 cells. CHO-

417   K1 cells expressing each mutant were treated with NZ-1, H_2Mab-214, H_2Mab-119, or buffer control

418   (black line), stained with Alexa Fluor 488-conjugated secondary antibodies, and analyzed in flow

419   cytometry. (C-E) Evaluation of reactivity of H_2Mab-214 toward various HER2-derived synthetic

420   peptides by ELISA. 10 µg/ml of H_2Mab-214 was incubated with wells coated with the synthetic

421   peptides, followed by incubation with peroxidase-conjugated anti-mouse antibodies. The optical

422   density was measured at 655 nm. Data are from one experiment (C) or from one triplicate experiment

423   (D and E; mean ± SEM). N.C., negative control.

424

425   **Figure 3. Structural analysis of H_2Mab-214.** (A) Close-up view of the antigen binding site in the

426   crystal structure of H_2Mab-214 complexed with the epitope peptide. H_2Mab-214 is shown as a surface

427   model, and the epitope peptide is shown as a stick model with a transparent sphere model. (B)

428   Hydrogen bonding interactions observed between H_2Mab-214 and the epitope peptide. Hydrogen

429   bonds are denoted by dashed lines. Water molecules are shown as sphere models. (C) Crystal structure

430   of H_2Mab-119 complexed with the HER2 domain I fragment. The domain I is shown as a cyan cartoon

431   model, and the H_2Mab-119 is shown as a cartoon model with a transparent surface model. HER2

432   residues in contact with the H_2Mab-119 (within 4 Å) are indicated in magenta. (D) Overall structure

12

433 of the HER2 ectodomain (PDB ID: 3n85). Met611-Asp618 region is colored in yellow. An expanded

434 view of the region indicated by the black box is shown on the right. The Cα atoms of the residues 611-

435 618 are shown as spheres and disulfide bonds are shown as red stick models. (E-H) Structural

436 comparison of the Met611-Asp618 region in the HER2 ectodomain (E; crystal structure and G;

437 schematic diagram) and the peptide bound to H$_2$Mab-214 (F; crystal structure and H; schematic

438 diagram).

439

440 **Figure 4. Analysis of antibody binding upon disulfide bond breakage in HER2.** (A,B) Flow

441 cytometry analysis of DTT-treated HEK293T cells. HEK293T cells were pre-treated with 1 mM DTT

442 for 1, 5, or 24 hours and then stained with H$_2$Mab-214 (A), H$_2$Mab-119 (B), or buffer control, followed

443 by flow cytometry analysis. Line graphs of the median florescence intensity (MFI) values are provided

444 on the right side of the histograms. (C, D) Effect of DTT treatment of MCF 10A (C) and SK-BR-3

445 (D) cells on antibody binding. MCF 10A and SK-BR-3 cells were either untreated or pre-treated with

446 1 mM DTT for 1 hour and then stained with H$_2$Mab-214, H$_2$Mab-119, or buffer control, followed by

447 flow cytometry analysis. Results are reported as fold change in MFI compared with the buffer control.

448 Data are mean + SEM of four independent experiments. *P* values were calculated using unpaired one-

449 tailed t-test. *P<0.05, **P<0.01. ***P<0.001. (E) HER2 domain IV structure extracted from 3n85.

450 The disulfide bonds are indicated by red sticks. (F) Binding of antibodies to the disulfide bond removal

451 HER2 mutants. CHO-K1 cells transiently expressing each mutant were stained with H$_2$Mab-119,

452 trastuzumab, or H$_2$Mab-214 and analyzed in flow cytometry. Relative impact of each mutation on

453 binding is expressed as the fold change in the % positive cells from the wild-type after normalizing

454 the expression level of each mutant using the NZ-1-stained cells. Signals from cells transfected with

455 empty vectors were used to define antibody-bound cell populations (regions including <1% of cells

456 transfected with empty vectors were used for the calculation of % positive cells). Data are mean ±

457 SEM of three independent experiments.

458

459 **Figure 5. H$_2$Mab-119-mG$_{2a}$-f- and H$_2$Mab-214-mG$_{2a}$-f-mediated ADCC and CDC against BT-**

460 **474 (HER2-positive) and MDA-MB-468 (TNBC) cells.** (A ,C) ADCC induced by H$_2$Mab-119-

461 mG$_{2a}$-f, H$_2$Mab-214-mG$_{2a}$-f, control mouse IgG$_{2a}$ or 281-mG$_{2a}$-f against BT-474 (A) and MDA-MB-

462 468 (C) cells. (B,D) CDC induced by H$_2$Mab-119-mG$_{2a}$-f, H$_2$Mab-214-mG$_{2a}$-f, control mouse IgG$_{2a}$

463 or 281-mG$_{2a}$-f against BT-474 (B) and MDA-MB-468 (D) cells. Values are shown as mean ± SEM.

464 Asterisks indicate statistical significance (**p<0.01; Welch's *t*-test). ADCC, antibody-dependent

465 cellular cytotoxicity; CDC, complement-dependent cytotoxicity.

466

467 **Figure 6. Antitumor activity of H$_2$Mab-119-mG$_{2a}$-f and H$_2$Mab-214-mG$_{2a}$-f against BT-474**

468 **xenograft.** (A) BT-474 cells (5 × 10^6 cells) were subcutaneously injected into BALB/c nude mice. On

13

day 7, 100 μg of H$_2$Mab-119-mG$_{2a}$-f, H$_2$Mab-214-mG$_{2a}$-f, control normal mouse IgG or 281-mG$_{2a}$-f were injected intraperitoneally into mice. Additional antibodies were injected on days 14 and 19. The tumor volume was measured on days 7, 12, 14, 19, 22, and 26 after the inoculation of BT-474. Values are presented as the mean ± SEM. *P<0.05, **P<0.01 (ANOVA and Tukey's multiple comparisons test). (B) Tumor weight (day 26) was measured from excised BT-474 xenograft tumors. Values are presented as the mean ± SEM. **P<0.01 (Welch's *t* test). (C) Appearance of BT-474 xenograft tumors from the H$_2$Mab-119-mG$_{2a}$-f, H$_2$Mab-214-mG$_{2a}$-f, control normal mouse IgG and 281-mG$_{2a}$-f-treated mice on day 26 (scale bar, 1 cm). (D) Mice weight in H$_2$Mab-119-mG$_{2a}$-f, H$_2$Mab-214-mG$_{2a}$-f, control normal mouse IgG and 281-mG$_{2a}$-f-treated groups. n.s., not significant.

Table 1 The main chain dihedral angles.

residue	Phi/Psi angles (º) in the HER2 ectodomain structures		Phi/Psi angles (º) in the H$_2$Mab-214 Fv-clasp/epitope peptide complex
	(PDB: 3n85)	(PDB: 6j71)	
Pro612	-60.9/143.0	-86.6/138.7	-62.7/149.5
Ile613	-104.1/113.8	-91.2/117.5	-106.6/102.1
Trp614	-107.3/128.4	-114.7/112.1	-76.7/-38.3
Lys615	-138.3/166.5	-133.8/170.7	-109.5/-112.4
Phe616	-141.6/152.4	-147.6/157.1	-110.6/161.9
Pro617	-78.1/160.6	-66.4/159.1	-53.0/125.4

STAR Methods

Resource availability

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Takao Arimori (arimori@protein.osaka-u.ac.jp).

Materials availability

H$_2$Mab-214 is available from Tohoku University Graduate School of Medicine.

Method details

Cell lines

Chinese hamster ovary (CHO)-K1, BT-474, SK-BR-3, MDA-MB-468, HEK293T, and MCF 10A cell lines were obtained from the American Type Culture Collection (ATCC). HaCaT cell line

14

497   was obtained from Cell Lines Service GmbH (Eppelheim). CHO/HER2 were generated by transfecting

498   pCAG/PA-HER2-RAP-MAP into CHO-K1 cells using Lipofectamine LTX (Thermo Fisher Scientific,

499   Inc.). A few days after transfection, PA tag-positive cells were sorted by a cell sorter (SH800; Sony

500   Corp.) using NZ-1, which was originally developed as an anti-human PDPN mAb.[58]

501   CHO-K1 and CHO/HER2 were cultured in RPMI-1640 medium (Nacalai Tesque, Inc.) or in

502   Ham's F-12 medium (FUJIFILM Wako Pure Chemical Corporation), and BT-474, SK-BR-3, MDA-

503   MB-468, HEK293T, and HaCaT were cultured in DMEM medium (Nacalai Tesque, Inc.),

504   supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific Inc.), 100

505   units/ml of penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Nacalai Tesque, Inc.).

506   MCF 10A was cultured in Mammary Epithelial Cell Basal Medium BulletKit™ (CC-3150, Lonza)

507   supplemented with 100 ng/ml cholera toxin (Sigma-Aldrich). All cell lines were cultured at 37°C in a

508   humidified atmosphere with 5% CO_2 and 95% air.

509

510   *Antibodies*

511   Anti-HER2 mAb, H$_2$Mab-119 (IgG$_1$, kappa), was established as previously described.[36] H$_2$Mab-

512   214 (IgG$_1$, kappa) was established by the same strategy. In brief, BALB/c mice were immunized with

513   recombinant HER2-extracellular domain produced by LN229 cells together with Imject Alum

514   (Thermo Fisher Scientific, Inc.). After several additional immunizations, spleen cells were fused with

515   P3U1 cells. The culture supernatants of hybridomas were screened using enzyme-linked

516   immunosorbent assay with recombinant HER2-extracellular domain and flow cytometry.

517   Variable (V$_H$) and constant (C$_H$) regions of heavy chain cDNAs of H$_2$Mab-119 and H$_2$Mab-214

518   were subcloned into the pCAG-Neo vector (FUJIFILM Wako Pure Chemical Corporation). Variable

519   (V$_L$) and constant (C$_L$) regions of light chain cDNAs of H$_2$Mab-119 and H$_2$Mab-214 were subcloned

520   into the pCAG-Ble vector (FUJIFILM Wako Pure Chemical Corporation). To produce recombinant

521   H$_2$Mab-119 and H$_2$Mab-214, the vectors of heavy and light chains were transfected into ExpiCHO-S

522   cells using the ExpiCHO Expression System (Thermo Fisher Scientific Inc.). H$_2$Mab-119 and H$_2$Mab-

523   214 were purified using Ab-Capcher (ProteNova Co., Ltd.).

524   To generate H$_2$Mab-119-mG$_{2a}$-f and H$_2$Mab-214-mG$_{2a}$-f, we subcloned these V$_H$ cDNA and C$_H$

525   of mouse IgG$_{2a}$ into the pCAG-Ble vector. These V$_L$ cDNA and C$_L$ cDNA of mouse kappa light chain

526   were also subcloned into the pCAG-Neo vector. The vectors for the H$_2$Mab-119-mG$_{2a}$ or H$_2$Mab-214-

527   mG$_{2a}$ were transduced into BINDS-09 (FUT8-knockout ExpiCHO-S) cells, and recombinant mAbs

528   were produced using the ExpiCHO Expression System. H$_2$Mab-119-mG$_{2a}$-f and H$_2$Mab-214-mG$_{2a}$-f

529   were purified using Ab-Capcher. Preparation of 281-mG$_{2a}$-f (defucosylated anti-hamster PDPN mAb,

530   control defucosylated mouse IgG$_{2a}$) was previously described.[43] Trastuzumab (Herceptin) was

531   obtained from Chugai Pharmaceutical Co., Ltd (Tokyo, Japan).

532

15

533 *Animal Experiments*

534   The animal experiment for generation of anti-HER2 mAbs was approved by the Animal Care and

535 Use Committee of Tohoku University (Permit number: 2019NiA-001). Animal experiments for

536 ADCC and antitumor activity were approved by the Institutional Committee for Experiments of the

537 Institute of Microbial Chemistry (approval no. 2023-001 and 2023-018). Mice were maintained and

538 monitored as described previously.[59] The loss of original body weight was determined to a point >25%

539 and/or a maximum tumor size >3,000 mm^3 as humane endpoints for euthanasia.

540

541 *Flow cytometry*

542   CHO-K1, CHO/HER2, BT-474, SK-BR-3, MDA-MB-468, HEK293T, HaCaT, and MCF 10A

543 cells were obtained using 0.25% trypsin and 1 mM ethylenediamine tetraacetic acid (EDTA; Nacalai

544 Tesque, Inc.). The cells were treated with primary mAbs (H$_2$Mab-119, H$_2$Mab-214, H$_2$Mab-119-mG$_{2a}$-

545 f, H$_2$Mab-214-mG$_{2a}$-f, or trastuzumab) or blocking buffer (control; 0.1% BSA in PBS) for 30 min at

546 4˚C. Then, the cells were treated with Alexa Fluor 488-conjugated anti-mouse IgG (1:1,000; Cell

547 Signaling Technology, Inc.) or FITC-conjugated anti-human IgG (1:1,000; Sigma-Aldrich for

548 trastuzumab) for 30 min at 4˚C.

549   To analyze the antibody binding to DTT-treated cells, HEK293T, SK-BR-3, and MCF 10A cells

550 were cultured in the media containing 1 mM DTT for an arbitrary time. The cells were then washed

551 repeatedly with PBS to remove DTT and treated with 0.5 μg/ml of trastuzumab or 10 μg/ml of H$_2$Mab-

552 119, H$_2$Mab-214, H$_2$Mab-19, H$_2$Mab-181, or H$_2$Mab-41 for 1 hour on ice, followed by treatment with

553 5 μg/ml of Alexa Fluor 488-conjugated anti-human IgG or anti-mouse IgG for 45 min on ice.

554   For the assessment of antibody binding to HER2 mutants, CHO-K1 cells were transiently

555 transfected with vectors encoding various HER2 mutants or empty vector using X-tremeGENE HP

556 DNA Transfection Reagent (Merck KGaA). The cells were stained with antibodies using the same

557 procedure as for the DTT-treated cells.

558   All fluorescence data were collected using SA3800 Cell Analyzer (Sony Corp.) and analyzed

559 using FlowJo (BD Biosciences).

560

561 *Enzyme-Linked Immunosorbent Assay (ELISA)*

562   NUNC Maxisorp 96-well plates (Thermo Fisher Scientific, Inc.) were coated with synthetic

563 peptides (10 μg/ml; PepScreen by Sigma-Aldrich) or HER2ec (10 μg/ml) for 30 min at 37°C and

564 blocked with Superblock reagent (Thermo Fisher Scientific, Inc.) or 1% bovine serum albumin

565 (Nacalai Tesque, Inc.) in 0.05% Tween 20 containing PBS (1% BSA/PBST) for 30 min at 37°C.

566 H$_2$Mab-214 (10 μg/ml) was then added to the plate and incubated for 30 min at 37°C. To detect bound

567 antibodies, horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody (Agilent Technologies)

568 diluted to 1:2,000 in 1% BSA/PBST was added and incubated for 30 min at 37°C. We used ELISA

16

569 POD Substrate TMB Kit (Nacalai Tesque, Inc.) for the enzymatic reactions. The optical density was

570 measured at 655 nm using an iMark microplate reader (Bio-Rad Laboratories, Inc.).

571

572 *ADCC*

573   Spleens were aseptically removed from six female BALB/c nude mice (five-week-old, Charles

574 River Laboratories, Inc.) Single-cell suspensions were obtained using a cell strainer (352360, BD

575 Biosciences). Erythrocytes were removed with the treatment of ice-cold distilled water. The

576 splenocytes were used as effector cells.

577   H_2Mab-119-mG_{2a}-f and H_2Mab-214-mG_{2a}-f-mediated ADCC was assayed as follows. Target

578 cells (BT-474 and MDA-MB-468) were labeled with 10 μg/ml Calcein AM (Thermo Fisher Scientific,

579 Inc.). The target cells (2×10^4 cells) were plated in 96-well plates and mixed with effector cells

580 (effector/target cells ratio, 50), 100 μg/ml of H_2Mab-119-mG_{2a}-f, H_2Mab-214-mG_{2a}-f, 281-mG_{2a}-f

581 (control defucosylated mouse IgG_{2a}) or control mouse IgG_{2a} (Sigma-Aldrich). Following incubation

582 for 4.5 h at 37˚C, the Calcein release into the medium was analyzed using a microplate reader (Power

583 Scan HT; BioTek Instruments, Inc.,) with an excitation wavelength (485 nm) and an emission

584 wavelength (538 nm).

585   Cytotoxicity (% lysis) was calculated as follows: % lysis = (E - S)/(M - S) x 100, where "E" is

586 the fluorescence in cultures of both effector and target cells, "S" is the spontaneous fluorescence of

587 only target cells, and "M" is the maximum fluorescence following the treatment with a lysis buffer

588 (10 mM Tris-HCl [pH 7.4], 10 mM EDTA, and 0.5% Triton X-100).

589

590 *CDC*

591   The Calcein AM- labeled target cells (BT-474 and MDA-MB-468) were plated in 96-well plates

592 and mixed with rabbit complement (final dilution 1:10, Low-Tox-M Rabbit Complement; Cedarlane

593 Laboratories) and 100 μg/ml of H_2Mab-119-mG_{2a}-f, H_2Mab-214-mG_{2a}-f, 281-mG_{2a}-f or control

594 mouse IgG_{2a}. Following incubation for 4.5 h at 37˚C, Calcein release into the medium was measured

595 as indicated above.

596

597 *Antitumor activity of H_2Mab-119-mG2a-f and H_2Mab-214-mG_{2a}-f in xenografts of BT-474 and MDA-*

598 *MB-468 cells.*

599   BT-474 and MDA-MB-468 (5×10^6 cells) resuspended in DMEM and mixed with BD Matrigel

600 Matrix Growth Factor Reduced (BD Biosciences) were subcutaneously injected into the left flank of

601 BALB/c nude mice (Charles River Laboratories, Inc). On day 7 post-inoculation, 100 μg of H_2Mab-

602 119-mG_{2a}-f (n=8), H_2Mab-214-mG_{2a}-f (n=8), 281-mG_{2a}-f (n=8) or control mouse IgG (FUJIFILM

603 Wako Pure Chemical Corporation) (n=8) in 100 μl PBS were intraperitoneally injected. On days 14

604 and 19, additional antibody injections were performed. The tumor volume was measured on days 7,

17

605 12, 14, 19, 22, and 26 after the inoculation of cells. Tumor volumes were determined as previously

606 described.[59]

607

608 *Statistical analyses*

609 　　All data are expressed as mean ± standard error of the mean (SEM). Welch's t test was used for

610 the statistical analyses in ADCC, CDC, and tumor weight. ANOVA with Tukey's post hoc test were

611 used for the statistical analyses in tumor volume and mouse weight. GraphPad Prism 8 (GraphPad

612 Software, Inc.) was utilized for the calculations. $P<0.05$ was considered to indicate a statistically

613 significant difference.

614

615 *Crystallization*

616 　　To generate H$_2$Mab-214 Fv-clasp, V$_H$ (residues 1-113) and V$_L$ (residues 1-108) regions of

617 H$_2$Mab-214 were appended to SARAH domain derived from human Mst1.[39] Hinge-less Fc (residues

618 234-447 of human IgG1) was subsequently appended to the V$_L$-SARAH. The DNA fragments were

619 subcloned into the pcDNA3.1 vector (Thermo Fisher Scientific, Inc.). V$_H$-SARAH and V$_L$-SARAH-

620 Fc were co-expressed in Expi293F cells (Thermo Fisher Scientific, Inc.) and purified with rProtein A

621 Sepharose Fast Flow (Cytiva). The Fc portion was digested by treatment with a His-tagged IdeS

622 protease for 2 hours at 37°C, followed by removal of His-IdeS by Ni-NTA Agarose (QIAGEN). The

623 sample was further purified by anion-exchange chromatography on a MonoQ 5/50 GL column

624 (Cytiva) equilibrated with 20 mM Tris, pH 8.0, and the resultant sample was concentrated by

625 ultrafiltration using Amicon Ultra (Merck Millipore) and mixed with synthetic epitope peptide

626 (residues 611-618 of HER2, MPIWKFPD) to be the final concentration of 5 mg/ml H$_2$Mab-214 Fv-

627 clasp and 0.6 mM peptide. To prepare the recombinant H$_2$Mab-119 Fab, the V$_H$ to C$_H$1 region of the

628 H$_2$Mab-119 heavy chain was subcloned into the pCAG-Neo vector and co-expressed with the H$_2$Mab-

629 119 light chain in ExpiCHO-S cells. H$_2$Mab-119 Fab was purified with Capto L (Cytiva) followed by

630 Protein G Sepharose 4 Fast Flow (Cytiva).

631 　　The HER2 domain I (residues 1-216) with a C-terminal His-tag was subcloned into pcDNA3.4

632 (Thermo Fisher Scientific, Inc.) and expressed in Expi293F GnT1- cells (Thermo Fisher Scientific,

633 Inc.), followed by purification with Ni-NTA Agarose. For the crystallization, H$_2$Mab-119 Fab was

634 mixed with HER2 domain I at 1.5-fold molar excess and subjected to size-exclusion chromatography

635 (SEC) on a Superdex 200 Increase 10/300 GL column equilibrated with 20 mM Tris, 150 mM NaCl,

636 pH 8.0 (Figure S2C). The purified sample was concentrated to 5 mg/ml by ultrafiltration using Amicon

637 ultra (Merck Millipore).

638 　　Crystallization screening was carried out using The Classic Suite (QIAGEN), Wizard Classic 1 &

639 2 (Rigaku), JCSG-plus (Molecular Dimensions), and ProPlex (Molecular Dimensions) crystallization

640 reagents by using the sitting-drop vapor diffusion method at 20°C.

18

641

Data collection, structure determination, and refinement

642

643   Crystals of the H$_2$Mab-214 Fv-clasp/epitope peptide complex obtained under the condition of 0.1

644   M CHES pH 9.5, 20 % w/v polyethylene glycol (PEG) 8000 (JCSG plus, tube 1-7) and the H$_2$Mab-

645   119 Fab/HER2 domain I complex obtained under the condition of 0.1M Sodium citrate pH5.5, 20%

646   w/v PEG3000 (JCSG plus, tube 1-2) were cryo-protected by well solution containing 15% PEG200

647   and 30% PEG3000, respectively.

648   Diffraction data were collected at 100 K at beamline BL44XU of SPring-8 (Harima, Japan). The

649   data were processed and scaled using X-ray Detector Software.[60] Initial phases were determined by

650   molecular replacement with PHAER[61] in the CCP4 package[62] using the crystal structures deposited in

651   Protein Data Bank (PDB) with IDs of 7cea (for H$_2$Mab-214 V$_H$), 7bsc (for H$_2$Mab-214 V$_L$), 7cea (for

652   SARAH domain), 1f3d (for H$_2$Mab-119 V$_H$-C$_H$1), 3wkm (for H$_2$Mab-119 V$_L$-C$_L$), and 4hrl (for HER2

653   domain I) as search models. The structural models were modified with COOT,[63] refined with

654   PHENIX,[64] and validated with MolProbity.[65] Data collection statistics and refinement parameters are

655   summarized in Table S1.

656

657   **Data and code availability**

658   Coordinates for the structural models of H$_2$Mab-214 Fv-clasp/epitope peptide complex and

659   H$_2$Mab-119 Fab/HER2 domain I complex have been deposited to the Protein Data Bank under PDB

660   accession numbers 8jyq and 8jyr, respectively. All deposited data is publicly available as of the date

661   of publication.

662   Any additional information required to reanalyze the data reported in this paper is available from

663   the lead contact upon request.

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665 **Supplemental information**

666

667 **Figure S1. Epitopes for H₂Mab-214 and trastuzumab.** (A) Amino-acid sequence of the HER2
668 domain IV. H₂Mab-214 epitope region is shown in red. Cysteines with the disulfide linkages are
669 connected by black lines. Residues forming a 3_{10} helix and β strands are highlighted in pink and blue,
670 respectively. *N*-glycosylation motifs are underlined, and residues in contact with trastuzumab (in
671 7mn8) are indicated by asterisks. (B) Structure of the trastuzumab-bound HER2 ectodomain (extracted
672 from 7mn8). An expanded view of the trastuzumab binding site is provided in the inset. HER2 residues
673 in contact with the trastuzumab (within 4 Å) are shown as brown stick models. The H₂Mab-214 epitope
674 and disulfide bonds are colored in yellow and red, respectively.

675

676 **Figure S2. Structure determination of H₂Mab-214 and H₂Mab-119.** (A) Overall structure of
677 H₂Mab-214 Fv-clasp in complex with the epitope peptide. (B) Simulated-annealing F_o-F_c omit map
678 for the H₂Mab-214 antigen peptide (blue mesh, contoured at 3.0 σ level). The assigned peptide model
679 is shown as orange sticks. (C) Size-exclusion chromatography (SEC) analysis. HER2 domain I
680 (orange), H₂Mab-119 Fab (cyan), and their complex after mixing at 1.5:1 molar ratio (magenta) were
681 subjected to SEC analysis on Superdex 200 Increase 10/300 GL column. (D) Amino-acid sequence of
682 the HER2 domain I. Residues in contact with the H₂Mab-119 (within 4 Å) in the crystal structure
683 (shown in Figure 3C) are colored in magenta.

684

685 **Figure S3. Flow cytometric analysis of DTT-treated HEK293T cells stained with various anti-**
686 **HER2 antibodies.** HEK293T cells were cultured in DMEM/FBS containing 1 mM DTT for 1 or 24
687 hours and then stained with H₂Mab-19, H₂Mab-181, H₂Mab-41, trastuzumab, or buffer control (gray
688 histogram), followed by flow cytometry analysis using Alexa Fluor 488-labeled secondary antibodies.
689 The epitope of each antibody is given below the antibody name.

690

691 **Figure S4. Flow cytometry using H₂Mab-214-mG₂ₐ-f and H₂Mab-119-mG₂ₐ-f.** (A) A core-fucose-
692 deficient mouse IgG₂ₐ mAbs, H₂Mab-214-mG₂ₐ-f and H₂Mab-119-mG₂ₐ-f were produced from
693 H₂Mab-214 (mouse IgG₁) and H₂Mab-119 (mouse IgG₁). (B) BT-474 and MDA-MB-468 cells were
694 treated with 10 μg/ml of H₂Mab-214-mG₂ₐ-f, H₂Mab-119-mG₂ₐ-f, 281-mG₂ₐ-f, or buffer control,
695 followed by Alexa Fluor 488-conjugated anti-mouse IgG. The black line represents the negative
696 control (blocking buffer).

697

698 **Figure S5. Antitumor activity of H₂Mab-119-mG₂ₐ-f and H₂Mab-214-mG₂ₐ-f against MDA-MB-**
699 **468 xenograft.** (A) MDA-MB-468 cells (5×10^6 cells) were subcutaneously injected into BALB/c
700 nude mice. On day 7, 100 μg of H₂Mab-119-mG₂ₐ-f, H₂Mab-214-mG₂ₐ-f, control normal mouse IgG

20

701   or 281-mG$_{2a}$-f were injected intraperitoneally into mice. Additional antibodies were injected on days

702   14 and 19. The tumor volume was measured on days 7, 12, 14, 19, 22, and 26 after the inoculation of

703   MDA-MB-468. Values are presented as the mean ± SEM. n.s., not significant (B) Tumor weight (day

704   26) was measured from excised MDA-MB-468 xenograft tumors. Values are presented as the mean ±

705   SEM. n.s., not significant. (C) Appearance of MDA-MB-468 xenograft tumors from the H$_2$Mab-119-

706   mG$_{2a}$-f, H$_2$Mab-214-mG$_{2a}$-f, control normal mouse IgG and 281-mG$_{2a}$-f-treated mice on day 26 (scale

707   bar, 1 cm). (D) Mice weight in H$_2$Mab-119-mG$_{2a}$-f, H$_2$Mab-214-mG$_{2a}$-f, control normal mouse IgG

708   and 281-mG$_{2a}$-f-treated groups. n.s., not significant.

709

710   **Figure S6. Body appearance of mice.** Body appearance of BT-474 (A) and MDA-MB-468 (B)

711   xenograft-bearing mice on day 26. Scale bar, 1 cm.

712

21

713 **Table S1 Data collection and refinement statistics**

	H$_2$Mab-214 Fv-clasp/epitope peptide complex (PDB: 8jyq)	H$_2$Mab-119 Fab/HER2 domain I complex (PDB: 8jyr)
Data collection		
Space group	$P2_1$	$P2_1$
Cell dimensions		
a, b, c (Å)	73.14, 62.79, 78.12	53.98, 112.29, 60.54
β (°)	94.42	90.76
Resolution (Å)	47.58 - 1.75 (1.85 - 1.75)[a]	48.65 - 1.69 (1.80 - 1.69)
R_{sym} (%)	5.2 (96.1)	6.6 (91.2)
$I / \sigma I$	10.95 (1.05)	9.46 (1.35)
CC1/2 (%)	99.8 (70.8)	99.7 (69.4)
Completeness (%)	98.6 (97.2)	99.1 (97.9)
Redundancy	3.9 (3.8)	3.5 (3.6)
Refinement		
Resolution (Å)	36.46 – 1.75	40.02 – 1.69
No. reflections	70,773	79,290
R_{work} / R_{free} (%)	19.6 / 23.3	17.3 / 20.9
No. atoms		
Protein	5,261	4,743
Peptide	138	14
Water	193	475
B-factors		
Protein	54.2	37.7
Peptide	52.7	-
Carbohydrate	-	68.1
Water	47.8	42.5
R.m.s. deviations		
Bond lengths (Å)	0.006	0.015
Bond angles (°)	0.881	1.324

714 [a]Values in parentheses are statistics of the highest-resolution shell.

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925

Figure 1

Figure 2

Figure 3

A H₂Mab-214

B H₂Mab-119

C MCF 10A

D SK-BR-3

E
1. C511:C520
2. C515:C528
3. C531:C540
4. C544:C560
5. C563:C576
6. C567:C584
7. C587:C596
8. C600:C623
H₂Mab-214 epitope
9. C626:C634
10. C630:C642

F

Figure 4

A ADCC

B CDC

C ADCC

D CDC

Figure 5

A

Tumor volume (mm³)

- Normal mouse IgG
- H₂Mab-119-mG₂ₐ-f
- H₂Mab-214-mG₂ₐ-f
- 281-mG₂ₐ-f

Days after inoculation

*	**	**	** : H₂Mab-119-mG₂ₐ-f vs normal mouse IgG
n.s.	*	**	** : H₂Mab-119-mG₂ₐ-f vs 281-mG₂ₐ-f
n.s.	*	**	** : H₂Mab-214-mG₂ₐ-f vs normal mouse IgG
n.s.	n.s.	**	** : H₂Mab-214-mG₂ₐ-f vs 281-mG₂ₐ-f

B

Tumor weight (mg)

C

D

Mouse weight (g)

- Normal mouse IgG
- H₂Mab-119-mG₂ₐ-f
- H₂Mab-214-mG₂ₐ-f
- 281-mG₂ₐ-f

Days after inoculation

Figure 6

A

511:520   515:528   531:540   544:560   563:576

511 CHQL**CARGH**CWGPGPTQCV<u>NCS</u>Q**FLRGQ**Q**ECV**EECRVLQGLP**REYV**NARH**CLP**CHPECQPQ<u>NGS</u>VTCF

567:584

587:596   600:623   626:634   630:642

GPEADQCVACAH**YKD**PP**FCV**ARCP**SGV**KPDLSYMPIWKFPDEEGAC**QP**CPI<u>NCT</u>HSCVDLDDKGCPA
     *   *   * *   *   * *   *   *   *   * *

EQRASPLT 652

B

Figure S1

A

Epitope peptide

V_H V_L

SARAH domain

B

M611
D618
I613
P617
P612
F616
W614
K615

C

Complex

H₂Mab-119 Fab

HER2-domain I

— HER2 domain I
— H₂Mab-119 Fab
— HER2 domain I + H₂Mab-119 Fab

D

23 TQVCTGTDMKLRLPASPETHLDMLRHLYQGCQVVQGNLELTYLPTNASLSFLQDI

QEVQGYVLIAHNQVRQVPLQRLRIVRGTQLFEDNYALAVLDNGDPLNNTTPVTGA

SPGGLRELQLRSLTEILKGGVLIQRNPQLCYQDTILWKDIFHKNNQLALTLIDTN

RSRACHPCSPMCKGSRCWGESSEDCQSLT 216

Figure S2

Figure S3

A

H₂Mab-214
H₂Mab-119
(mouse IgG₁)

H₂Mab-214-mG₂ₐ
H₂Mab-119-mG₂ₐ
(mouse IgG₂ₐ)

H₂Mab-214-mG₂ₐ-f
H₂Mab-119-mG₂ₐ-f
(mouse IgG₂ₐ)

class switch

core-fucose
deletion

⤴ : N-glycan   🔵 : fucose   — : disulfide bond

B

H₂Mab-214-mG₂ₐ-f

BT-474

MDA-MB-468

H₂Mab-119-mG₂ₐ-f

BT-474

MDA-MB-468

281-mG₂ₐ-f

BT-474

MDA-MB-468

Figure S4

A

Tumor volume (mm³) vs **Days after inoculation**

- ● Normal mouse IgG
- ■ H₂Mab-119-mG₂ₐ-f
- ▲ H₂Mab-214-mG₂ₐ-f
- ▼ 281-mG₂ₐ-f

n.s.

B

Tumor weight (mg)

n.s.

Normal mouse IgG, H₂Mab-119-mG₂ₐ-f, H₂Mab-214-mG₂ₐ-f, 281-mG₂ₐ-f

C

Normal mouse IgG
H₂Mab-119-mG₂ₐ-f
H₂Mab-214-mG₂ₐ-f
281-mG₂ₐ-f

D

Mouse weight (g) vs **Days after inoculation**

- ● Normal mouse IgG
- ■ H₂Mab-119-mG₂ₐ-f
- ▲ H₂Mab-214-mG₂ₐ-f
- ▼ 281-mG₂ₐ-f

n.s.

Figure S5

A

Normal mouse IgG

H₂Mab-119-mG₂ₐ-f

H₂Mab-214-mG₂ₐ-f

281-mG₂ₐ-f

B

Normal mouse IgG

H₂Mab-119-mG₂ₐ-f

H₂Mab-214-mG₂ₐ-f

281-mG₂ₐ-f

Figure S6