



**Karolinska
Institutet**

This is an author produced version of a paper accepted by **Immunotherapy**. This paper has been peer-reviewed but does not include the final publisher proof-corrections or journal pagination.

Construction and characterization of a new chimeric antibody against HER2. Immunotherapy. 2013;5(7): 703–715.

Jeddi-Tehrani, Mahmood; Kazemi, Tohid; Bahadori, Motahhare; Maddah, Mahshid; Hojjat-Farsanghi, Mohammad; Khoshnoodi, Jalal; Rabbani, Hodjatallah; Shokri, Fazel; Hojat Farsangi, Mohammad; Amiri, Mohammad Mehd

DOI: **10.2217/imt.13.67**

Access to the published version may require subscription.
Published with permission from: **Future Medicine**

1 **Construction and characterization of a new chimeric**
2 **antibody against HER2**

3 **Mohammad Mehdi Amiri ¹, Mahmood Jeddi-Tehrani ², Tohid Kazemi ^{1,3},**
4 **Motahhare Bahadori ², Mahshid Maddah ², Mohammad Hojjat-Farsangi ⁴,**
5 **Jalal Khoshnoodi ¹, Hodjatallah Rabbani ² & Fazel Shokri ^{*1,2}**
6
7
8
9

10 **¹ Department of Immunology, School of Public Health, Tehran University of**
11 **Medical Sciences, Tehran, Iran**

12 **² Monoclonal Antibody Research Center, Avicenna Research Institute,**
13 **ACECR, Tehran, Iran**

14 **³ Department of Immunology, Faculty of Medicine, Tabriz University of**
15 **Medical Sciences, Tabriz, Iran**

16 **⁴ Immune & Gene Therapy Laboratory, Cancer Center Karolinska,**
17 **Karolinska Hospital, Stockholm, Sweden**

18
19
20 ***Author for correspondence:**

21 Monoclonal Antibody Research Center,
22 Avicenna Research Institute, ACECR,
23 Tehran, Iran

24 Tel.: +98 21 22432020

25 Fax: +98 21 22432021

26 fshokri@tums.ac.ir

27 **Abstract**

28 **Aims**

29 Immunotherapy with anti-HER2 antibody has shown promising results in patients with
30 HER2-positive breast cancer. We have recently reported characterization of a mouse
31 monoclonal antibody (mAb) against HER2, which binds to an epitope different from that
32 recognized by Trastuzumab and specifically inhibits proliferation of tumor cells
33 overexpressing HER2. In the present study we report chimerization of this antibody.

34 **Materials and Methods**

35 The immunoglobulin variable region heavy (VH) and light (VL) chain genes of 1T0
36 hybridoma cells were amplified and ligated to human gamma-1 and kappa constant
37 region genes using Splice Overlap Extension (SOE) PCR. The chimeric antibody was
38 subsequently expressed and characterized by ELISA, Western blot and flow cytometry.

39 **Results**

40 The purified chimeric antibody specifically binds to recombinant HER2 and HER2
41 overexpressing tumor cells and inhibited proliferation of these cells. The binding affinity
42 of the chimeric mAb was comparable to the parental mouse mAb.

43 **Conclusion**

44 This chimeric anti-HER2 mAb is potentially a valuable tool for targeted immunotherapy.

45

46 **Keywords:** Chimeric antibody, breast cancer, HER2, monoclonal antibody

47

48 **Introduction**

49 The human proto-oncogen HER2, also known as ErbB2 is located on chromosome 17
50 and encodes a 185 kDa transmembrane glycoprotein that belongs to the epidermal
51 growth factor receptor (EGFR) family of receptor tyrosine kinases [1]. HER2 acts as the
52 preferred heterodimerization partner for other members of HER receptors (HER1/
53 EGFR, HER3 and HER4) and triggers several downstream signaling cascades such as
54 MAPK and PI3K/AKT pathways [2]. HER2 gene overexpression is found in a number of
55 human malignancies including breast cancer, pancreatic adenocarcinoma, ovarian and
56 colorectal cancers [3, 4]. Overexpression of HER2 correlates with tumor metastasis and
57 poor prognosis. Approximately 30% of women with breast cancer have HER2 protein
58 overexpression, which is associated with poor prognosis [5]. The oncogenic potential
59 and accessibility of HER2 have made it a suitable target for cancer immunotherapy by
60 monoclonal antibodies (mAbs). Trastuzumab (Herceptin, Genentech Inc., San
61 Francisco Calif, USA) represents the first humanized mAb which was approved by the
62 United States Food and Drug Administration (FDA) in 1998, for therapeutic use in
63 patients with HER2-overexpressing breast cancer [6]. However, many patients do not
64 respond and progress within 1 year of initiating Trastuzumab therapy, which could be
65 due to the inefficiency of Trastuzumab to inhibit HER2 binding to other members of the
66 HER family [7].

67 Combination of two mAbs recognizing two distinct epitopes on HER2 is an effective
68 alternative strategy to overcome this resistance [8]. Pertuzumab is another humanized
69 mAb that binds to an epitope on domain II of the extracellular region of HER2, different
70 from the binding site of Trastuzumab on domain IV. Consequently, it potently blocks

71 ligand-activated signaling transduced from HER-2/HER-1 and HER-2/HER3
72 heterodimers. Antitumoral activity of Pertuzumab has been shown both in vitro and in vivo
73 models [9]. Combination of Pertuzumab and Trastuzumab has recently demonstrated
74 improved survival in patients with breast cancer [10]. Based on these findings, FDA has
75 recently approved Pertuzumab in combination with Trastuzumab for patients with
76 HER2-positive metastatic breast cancer [11]. Development of new HER2 specific mAbs
77 may improve the therapeutic efficacy of the current anti-cancer treatment protocols. In
78 the present study, we present data on chimerization of a new mouse mAb against
79 HER2 [12], which binds to an epitope of HER2 different from that of Trastuzumab.

80 **Materials and Methods**

81

82 **Cell lines**

83 The 1T0 monoclonal antibody producing hybridoma was prepared as described previously [12]. It
84 was grown in RPMI 1640 Medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal
85 bovine serum (Gibco), 100 µg/mL streptomycin, and 100U/mL penicillin (Gibco) at 37°C in a
86 humidified atmosphere of 5% CO₂. HER2-overexpressing human breast cancer cell line BT-474
87 and CHO-K1 were purchased from National Cell Bank of Iran (NCBI, Tehran, Iran) and cultured
88 under similar conditions, with the exception of BT-474 culture medium which was also
89 supplemented with 10 µg/mL insulin (Exir Co., Boroojerd, Iran).

90

91 **Amplification, cloning and sequencing of antibody variable region genes**

92 Variable regions of the heavy chain (VH) and light chain (VL) of 1T0 antibody were amplified by
93 RT (reverse transcriptase)-PCR using RNA isolated from the hybridoma. In brief, total RNA was
94 isolated with RNA Bee-RNA Isolation Reagent (AMS Biotechnology, UK) from 1×10⁷ murine
95 hybridoma cells that secrete 1T0 antibody, and the corresponding cDNA was synthesized with
96 avian myeloblastosis virus (AMV) reverse transcriptase using oligo dT as primer (Fermentas,
97 Thermo Fisher Scientific Inc, USA). The VH gene was amplified using the degenerate primers
98 mUlgVH-S and mUlgGHC-AS and the VL gene was amplified using the degenerate primers
99 mUlgVkL-S and mlgkC-AS (Table 1). PCR reactions were performed in 25 µl volume,
100 containing 1 µl of cDNA, 6 and 1µM of forward and reverse primers, respectively, 2 mM MgSo₄
101 concentration, 1u/µl Pfu DNA polymerase (Fermentas) and 10X reaction buffer. After 3 min
102 denaturation at 94°C, the PCR reaction was followed by 45 cycles of 1 min at 92°C, 1 min at
103 52°C, 1 min at 72°C and a final 72°C for 10 min. To confirm the identity of PCR products, the VH

104 and VL genes of 1T0 were cloned into pGEM-T easy vector system (Promega, Madison, WI,
105 USA) and sequenced.

106

107 **Isolation of human IgG1 and IgCκ constant region genes**

108 Similarlly to VH and VL, Cκ of human kappa chain and CH of human IgG1 were amplified by RT-
109 PCR, using the RNA isolated from human peripheral blood mononuclear cell (PBMC). The Cκ was
110 amplified using the primers Cκ-S and XhoI Cκ-AS and the CH was amplified using the primers CH-S
111 and BamHI CH-AS (Table 1). PCR reactions were performed in 25 µl volume, containing 1 µl of
112 cDNA, 1µM primers, 2 mM MgSO₄, 1u/µl Pfu DNA polymerase (Fermentas) and 10X reaction buffer.
113 After 3 min denaturation at 94°C, the PCR reaction was continued by 30 cycles of 1 min at 92°C, 1
114 min at 58°C, 1.5 min at 72°C and a final 10 min 72°C. To confirm the validity of PCR products, the
115 CH and CL genes were cloned into pGEM-T vector system (Promega) and sequenced.

116

117 **Construction of the mouse-human chimeric antibody expression vector** 118 **using Splice Overlap Extension (SOE) PCR**

119 Splice overlap extension (SOE) PCR allows the fusion of two sequences of DNA without the
120 use of restriction enzymes [13]. PCR products of VH and VL genes were modified to contain
121 restriction sites, Kozak sequences and a leader sequence taken from the original cDNA of
122 1T0 hybridoma in the forward primers and 15bp complementary region of CH and CL in the
123 reverse primers (Table 1), respectively. The VH, CH and VL, CL were amplified, extracted
124 from gel and fused during PCR1, as the overlapping sequences. They were subsequently
125 hybridized and extended to produce full-length chimeric VH-CH and VL-CL sequences. The
126 VH-CH and VL-CL obtained from PCR1 were then amplified by external primers (VH-Sall-T-
127 S, BamHI CH-AS for VH-CH and Vκ-kpn-T-S, Cκ-S for VL-CL) in a second round of PCR
128 (PCR2). The PCR product of VH-CH was inserted into pBudCE4.1 (Invitrogen, Grand

129 Island, NY, USA) at Sall/BamHI restriction sites to generate pBud-VH-CH, which contains
130 the heavy chain sequence of mouse-human chimeric antibody. After sequencing and
131 confirmation of this construct, PCR product of VL-CL was subsequently inserted into pBud-
132 VH-CH at KpnI/XhoI sites to generate pBud-VH-CH-VL-CL (pBud-c-1T0), which contains
133 the heavy and light-chain sequences of mouse-human chimeric antibody (Figure 1).

134 PCR1 reactions were performed in 20 μ l volume, containing 1 μ l of cDNA extracted from VH
135 and CH or VL and CL, 2 mM MgSo₄ concentration, 1u/ μ l Pfu DNA polymerase (Fermentas)
136 and 10X reaction buffer. After 3 min denaturation at 94°C, the PCR reaction was followed by
137 5 cycles of 1 min at 92°C, 1 min at 58°C, 1.5 min at 72°C. The temperature was hold on
138 92°C for 3min and after addition of external primers in 5 μ l volume containing 10X reaction
139 buffer, PCR2 reaction was performed by 35 cycles of 1 min at 92°C, 1 min at 58°C, 1.5 min
140 at 72°C and a final cycle at 72°C for 10 min. To confirm the identity of PCR products, the
141 amplified VH-CH and VL-CL genes were cloned into pGEM-T vector (Promega) and
142 sequenced.

143

144

145 **Table 1: Sequences of PCR primers**

146

| Primer | Amplified genes | Sequence | Amplicon size |
|-------------|-----------------|---|---------------|
| mUlgVH-S | VH | CAGGTSMARCTGCAGSAGTCWGG | 348 bp |
| mUlgGHC-AS | VH | AGGGGCCAGTGGATAGACAGATGG | |
| mUlgVkL-S | Vk | GAHRRTSWGNTSACYCAGWCTCCA | 321 bp |
| mlgkC-AS | Vk | TGGTGGGAAGATGGATACAG | |
| Ck-S | Ck | ACTGTGGCTGCACCATCTGTCTTCATCTTCCC | 318 bp |
| XhoICk-AS | Ck (SOE PCR) | CTCGAGCTAACACTCTCCCCTGTTGAAGCTCTTTGTGACGGGCGA | |
| CH-S | Cy1 | GCCTCCACCAAGGGCCCATCGGTC | 990 bp |
| BamHICH-AS | Cy1 (SOE PCR) | GGATCCTCATTTACCCGGAGACAGGGAGAGGCTCTT | |
| Vk-kpn-T-S | VkT0 (SOE PCR) | GGTACCGCCACCATGGAGTTTCAGACCCAGGTCTTTGTATTCTGTGTT G | 381 bp |
| Jk-T-AS | VkT0 (SOE PCR) | AAGCTTTTTTATTTCCAGCTTGGTCCCCCTCCGAACGTG | |
| VH-Sall-T-S | VHT0 (SOE PCR) | GTCGACGCCACCATGGACTTTGGGTTTCAGCTTG | 405 bp |
| JH-T-AS | VHT0 (SOE PCR) | GCCCTTGGTGGAGGCAAGCTTTGAGGAGACGGTGAG | |

147 (In degenerate primers, R=A or G, S=C or G, K=G or T, M=A or C, Y=C or T, W=A or T, H= A, T

148 or C, N= A, T, C or G).

149

150 **Transfection of chimeric antibody and establishment of stable transfected** 151 **cell lines**

152 To develop a stable transfectant expressing chimeric 1T0 antibody (c-1T0), CHO cells were
153 grown to 80% confluency in 12-well culture plates. pBud-c-1T0 construct was prepared using
154 Plasmid Maxiprep (Qiagen, Stockholm, Sweden) and then, CHO-K1 cells were transfected with
155 6 µg DNA of pBud-c-1T0 construct in combination with 6 µL JetPEI transfection reagent
156 (Polyplus-transfection, New York, NY) according to the manufacturer's recommendations. After
157 48 h culture, transient expression of c-1T0 was assessed by ELISA. To establish stable
158 transfectant, cells were subsequently selected using 1mg/ml of Zeocin (Gibco, Grand Island,
159 NY, USA) within a minimum of two weeks.

160

161 **Screening of chimeric antibody production by ELISA**

162 Chimeric antibody activity was detected using an indirect ELISA method [12]. In brief, a 96-well
163 ELISA plate (Maxisorp, Nunc, Roskilde, Denmark) was coated with 0.5 µg/mL recombinant
164 extracellular part of HER2 (eBioscience Inc., San Diego, USA) in PBS and incubated 1.5 h at
165 37°C and blocked with PBS supplemented with 0.05% Tween (Sigma, St Louis, MO, USA) and
166 3% non-fat skim milk. Fifty microliters of supernatants of transfected CHO cells were added at
167 37°C for 1.5 h. Trastuzumab was used as positive control. After washing with PBS-Tween,
168 horseradish peroxidase (HRP)-conjugated rabbit anti-human Ig (prepared in our lab) was added
169 and plate incubated for 1 h at 37°C. After further washing, the reaction was revealed with
170 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Sigma). Sulfuric acid was added to stop the
171 reaction and the optical density (OD) was measured by a multiscan ELISA reader (Organon
172 Teknika, Turnhout, Belgium) at 450 nm.

173

174 **Structural characterization of chimeric antibody by ELISA and SDS-PAGE**

175 Stable transfected cells producing c-1T0 were adapted to serum free medium (EX-CELL™
176 Sp2/0, Sigma, St Louis, MO, USA). Supernatant of c-1T0 was purified using a 1 ml HiTrap
177 Protein G HP column (Amersham Biosciences, New Jersey, USA). The culture supernatant
178 (1 litre) was passed through the column and the column was subsequently washed
179 thoroughly with PBS. Bound recombinant chimeric antibody was eluted by 50 ml of elution
180 buffer (0.1 M glycine/HCl, pH 2.7; flow rate 1 ml/min). The pH of eluted fraction was
181 immediately normalized using 1 M Tris/HCl, pH 9.0 buffer. For verification of c-1T0, we
182 examined presence of human IgG1 and IgCk in c-1T0 antibody by ELISA method. Briefly, a
183 96-well ELISA plate was coated with 5 µg/mL mouse monoclonal antibody against human
184 IgG (8a4, kindly provided by Professor Roy Jefferis) in PBS. SPG purified c-1T0 in different

185 concentrations were added at 37°C for 1.5 h. Herceptin was used as positive control. After
186 washing, horseradish peroxidase (HRP)-conjugated rabbit anti-human Ig and sheep anti-
187 human IgG₁ was added separately for assessment of human IgG₁ and IgG₁C_κ and the plate
188 was incubated for 1 h at 37°C. After further washings, the reaction was revealed with TMB
189 substrate. Sulfuric acid was added to stop the reaction and ODs were measured as
190 mentioned above.

191 The structure of c-1T0 antibody was analysed by SDS-PAGE [14]. Briefly, 500 ng of SPG
192 purified c-1T0 antibody was separated on 10% SDS-PAGE under reducing and non-
193 reducing conditions and visualized with silver staining. Herceptin was used as a positive
194 control. After electrophoretic separation, the gel was washed three times with distilled water
195 and incubated for 30 min with 50 ml of fixing solution (Methanol 50 ml, acetic acid 10 ml and
196 40 ml H₂O). Three times washing was repeated and the gel was incubated 1 min with 50 ml
197 of 0.2g/lit solution of sodium thiosulphate. After further washings, the gel was incubated 25
198 min with 50 ml of silver nitrate solution (20%). The gel was subsequently washed and developed
199 with a solution containing sodium carbonate (30g/lit), 100μl of formaldehyde (37-41%) and 4 ml
200 sodium thiosulphate (0.2g/lit). Finally, citric acid (3%) was added to stop the reaction.

201

202 **Analysis of specific binding of chimeric antibody to rHER2 by Western blot** 203 **technique**

204 Western blot was employed to compare specific binding of mouse 1T0 and c-1T0 to rHER2.
205 Fifty nanogram of rHER2 was separated on 12% SDS-PAGE and transferred to PVDF
206 membrane (Roche Diagnostics, Mannheim, Germany). After blocking of membrane with
207 blocking buffer (PBS-Tween-20 containing 5% non-fat skim milk) overnight at 4°C and washing
208 three times with washing buffer (PBS-Tween-20) for 15min, mouse 1T0 and c-1T0 antibodies
209 were added at 10 μg/mL in blocking buffer at room temperature for 1.5 h while shaking. Ten

210 $\mu\text{g/mL}$ of Herceptin was used as a positive control. Washing steps were repeated and HRP-
211 conjugated sheep anti-mouse immunoglobulin (prepared in our lab) for mouse 1T0 and HRP-
212 conjugated sheep anti-human Ig (prepared in our lab) for c-1T0 and Herceptin were added at
213 room temperature for 1.5 h on shaker. After washing, PVDF membrane was treated with ECL
214 (Amersham Biosciences, New Jersey, USA) and the bands were visualized on Kodak X-ray
215 film (Eastman Kodak, Rochester, NY, USA).

216

217 **Affinity constant determination by ELISA**

218 An ELISA-based method was used to define the binding affinity of mouse 1T0 and c-1T0 [15].
219 Briefly, wells of a micotiter ELISA plate were coated with several concentrations (2-0.031
220 $\mu\text{g/mL}$) of recombinant extracellular part of HER2. After blocking with 0.05% Tween (Sigma)
221 and 3% non-fat skim milk, serial concentrations of mouse 1T0 (10-0.15 $\mu\text{g/mL}$) and c-1T0 (5-
222 0.07 $\mu\text{g/mL}$) in blocking buffer were added into coated wells and incubated at 37°C for 1.5 h.
223 Washing was repeated and wells incubating with HRP-conjugated sheep anti-mouse Ig and
224 sheep anti-human Ig (prepared in our lab) for 1.5 h at 37°C. After the final wash step, TMB
225 substrate solution was added followed by stopping solution and ODs were measured. Sigmoidal
226 curves of ODs versus the logarithm of antibody concentrations were constructed. The antibody
227 concentration giving 50% of the maximum absorbance value ($[\text{Ab}]_t$) at a particular antigen
228 coating concentration was chosen for the affinity measurement using the formula $K_{\text{aff}} = 1/2(2$
229 $[\text{Abo}]_t - [\text{Ab}]_t)$. $[\text{Abo}]_t$ and $[\text{Ab}]_t$ represent the antibody concentrations resulting in 50% of the
230 maximum absorbance value at two consecutive concentrations of coated antigen where $[\text{Ag}] =$
231 $2[\text{Ago}]$. The mean of such calculations for three non-overlapping antigen concentrations was
232 taken as the final K_{aff} value.

233

234 **Analysis of cell surface binding of chimeric antibody by flow cytometry**

235 Indirect staining at surface membrane level was performed on BT-474 cells (National Cell Bank
236 of Iran, Tehran, Iran). After trypsinization, 10^6 cells were harvested, washed two time with
237 washing buffer (PBS, 0.1% NaN₃), and incubated with 100 μ L of 10 μ g/mL of mouse 1T0 and c-
238 1T0 antibodies as primary antibodies at 4°C for 1 h. Mouse IgG1 mAb and human IgG of
239 irrelevant specificity (produced in our lab) were included as negative controls. After incubation
240 and washing process, cells were incubated with FITC-conjugated sheep anti-mouse Ig and
241 sheep anti-human Ig (prepared in our lab) at 4°C for 1 h. The cells were finally scanned by a
242 flow cytometer (Partec, Nuremberg, Germany). Flomax flow cytometry analysis software
243 (Partec) was used to analyse the data.

244

245 **Assessment of tumor growth inhibition by XTT assay**

246 For tumor growth inhibition experiments, BT-474 cells were seeded in 96-well flat-bottom tissue
247 culture plates (30000 cells/well) in serum-containing RPMI-1640 medium (Gibco, Grand Island,
248 NY, USA). The cells were treated with different concentrations of mouse 1T0, c-1T0 and
249 Herceptin (10, 2 and 0.2 μ g/ml) for 16h at 37°C in a humidified atmosphere of 5% CO₂. After
250 incubation, the RPMI medium was exchanged with serum free medium containing XTT solution
251 (Roche, Indianapolis, IN) for 16h at 37°C as recommended by the manufacturer. After
252 incubation with XTT, microtiter plates were read by an ELISA reader (Organon Teknika,
253 Turnhout, Belgium) at 450 nm with the reference wavelength of 690 nm. Controls included
254 background (cells only) and Herceptin. All experiments were performed in triplicate. The
255 following formula was used to estimate the tumor growth inhibition rate induced by anti-HER2
256 antibodies:

257 Tumor growth inhibition (%) = $[(\text{OD without antibody} - \text{OD with antibody}) / \text{OD without}$
258 $\text{antibody}] \times 100$

259

260 **Assessment of tumor proliferation inhibition by radioactive thymidine**
261 **incorporation assay**

262 The antiproliferative activity of mouse 1T0 and c-1T0 was tested on HER2-overexpressing cell
263 line BT-474. BT-474 cells were seeded in 96-well flat-bottom tissue culture plates and were
264 allowed to recover and adhere overnight. Antibodies were added to wells at different
265 concentrations of mouse 1T0, c-1T0 and Herceptin (10, 2 and 0.2 µg/ml) for 16h at 37°C in a
266 humidified atmosphere of 5% CO₂. After incubation, 3H-thymidine (PerkinElmer, Boston, USA)
267 was added at 0.5 µCi per well for 8 h. Cultures were then harvested and transferred to
268 scintillation fluid for measurement of 3H-thymidine incorporation by a beta counter (Wallac 1410
269 Liquid Scintillation Counter, Pharmacia, Sweden). Controls included background (cells only) and
270 Herceptin. All experiments were performed in triplicate. The following formula was used to
271 estimate the proliferation inhibition rate:

272 Proliferation inhibition (%) = [(CPM without antibody-CPM with antibody)/CPM without
273 antibody]×100

274 **Results**

275 **Amplification of the VH and VL genes of mouse 1T0 mAb**

276 The VH and VL genes were amplified using specific primers designed for the leader
277 sequences of VH and VL genes (Figure 2, A and B). The ORF of the VH region of the mAb
278 1T0 is 405 bp in length, encoding a 135-aa polypeptide and the ORF of VL is 381 bp in
279 length, encoding a 127-aa polypeptide, including the leader peptides. Both VH and VL
280 genes have a signal leader sequence on their N-terminal region, encoding 19- and 20-aa
281 polypeptides, respectively. The Ck and CY1 were amplified with specific primers from cDNA
282 of normal human PBMC encoding 107- and 330-aa polypeptides, respectively (Figure 2, C
283 and D). Finally, the VLT0-Ck and VHT0-Cy1 segments (Figure 2, E and F) were linked to
284 each other by SOE PCR technique as described in the Materials and Methods.

285

286 **Expression of chimeric c-1T0 antibody in CHO cells**

287 The c-1T0 construct was transfected in CHO cells by JetPEI transfection reagent and
288 culture supernatants were collected to assess chimeric antibody production by antigen
289 specific indirect ELISA. After selection in Zeocin and four rounds of subcloning, a stable
290 transfected cell line (c17) that produces high levels of chimeric antibody was selected.
291 Based on the results obtained from the antigen specific ELISA using recombinant
292 extracellular region of HER2 as the coating antigen and different concentrations of
293 Herceptin as the standard protein, 960 ng/ml of chimeric antibody was detected in serum
294 free medium (Figure 3, A and B).

295

296 **Structural characterization of c-1T0 antibody**

297 Transfected CHO cells were maintained in a serum-free culture medium. The chimeric
298 antibody was purified from the culture supernatant by affinity chromatography using SPG
299 column. The purified chimeric antibody was analysed by SDS-PAGE under non-reducing
300 and reducing conditions. Silver staining of SDS-PAGE gel (Figure 4) shows monomeric
301 (~150 kDa) form of the chimeric antibody under non-reducing condition (c-1T0, lane 1). The
302 monomeric light (~25 kDa) and heavy chains (~50 kDa) were detected under reducing
303 conditions (c-1T0, lane 2). The parental mouse 1T0 mAb gave a similar pattern under non-
304 reducing and reducing conditions.

305 Western blot analysis revealed that the chimeric c-1T0 and the parental mouse 1T0 mAbs
306 react with the non-reduced recombinant extracellular HER2 protein (Figure 5). Lack of
307 reactivity with the reduced HER2 protein indicates recognition of a conformational epitope
308 by our mAb. A similar pattern of reactivity was observed for Trastuzumab, which was used
309 as a control.

310

311 **Affinity constant determination**

312 The binding affinity of the chimeric antibody was determined by an ELISA method as
313 described in the Materials and Methods. Based on the binding curves obtained for
314 the chimeric and mouse parental mAbs (Figure 6, A and B), the mean K_{aff} of mouse
315 1T0 and c-1T0 were 0.6×10^9 and 1.3×10^9 , respectively.

316

317 **Assessment of cell binding activity by flow cytometry**

318 In order to determine the binding reactivity of c-1T0 to the HER2 overexpressing
319 cells, we performed flow cytometric analysis using c-1T0 and mouse 1T0 as first
320 layer and sheep-anti human-FITC and sheep-anti mouse-FITC as second layer,

321 respectively. In parallel to Trastuzumab as positive control, c-1T0 showed positive
322 reactivity and detected HER2 on surface of BT-474 cells similar to the parental
323 mouse 1T0 antibody (Figure 7).

324

325 **Tumor cell growth inhibition by c-1T0 chimeric mAb**

326 A colorimetric (XTT) assay was performed to assess the effect of c-1T0 on growth of
327 BT-474 cell line. The growth inhibition rate of triplicate wells was determined and percent
328 of inhibition was calculated according to the formula described in the Materials and
329 Methods. Accordingly, c-1T0 induced a dose dependent growth inhibition, similar to the
330 parental mouse 1T0 mAb in the BT-474 tumor cell line (Figure 8).

331

332 **Tumor proliferation inhibition by c-1T0 chimeric mAb**

333 The ability of c-1T0 to inhibit tumor cell proliferation was assessed in vitro in parallel to
334 the parental mouse 1T0 mAb by radioactive labeled thymidine assay. The stimulation
335 index of triplicate wells was determined and percent of inhibition of cell proliferation was
336 calculated for mouse 1T0 and c-1T0 (Figure 9). Both mAbs induced a similar dose
337 dependent pattern of inhibition.

338

339 **Discussion**

340 Monoclonal antibodies are a part of the biological drugs that represent a growing
341 segment of the pharmaceutical industry. Approximately 26 mAbs have so far been
342 approved by FDA and over 200 mAbs are still awaiting approval [16]. An early
343 success of mouse mAb for therapeutic purposes provoked a response similar to
344 serum sickness of antisera therapy [17]. When a mouse mAb is multiply injected to a
345 patient, the human anti-mouse antibody response (HAMA) is induced [18].
346 Chimerization is one approach to reduce the immunogenicity of therapeutic mouse
347 mAb for human treatment. In 1984, Boulianne et al. [19] and Morrison et al. [20]
348 produced chimeric antibodies by joining the mouse variable domains to human
349 constant domains. Although chimerization reduces HAMA response of murine
350 antibodies, human anti-chimeric antibody (HACA) response could be created
351 because of immunogenic epitopes in the mouse variable regions. Despite their
352 potential immunogenicity, chimeric antibodies have been widely used for
353 immunotherapy of cancers. One of the most widely used therapeutic chimeric
354 antibodies is Rituximab. If these antibodies prove to be effective in vivo then their
355 humanization might be considered later, particularly if they are found to be highly
356 immunogenic in human. Another approach to further reduce the immunogenicity of
357 murine mAb is humanization in which all framework regions (FWR) residues that are
358 not essential for antigen binding are replaced with human FWR counterpart
359 sequences [21].

360 HER2 gene overexpression has been found in a number of human malignancies
361 [22] and is a proven therapeutic target. In 1990, Fendly and coworkers [23]
362 produced mAbs directed against extracellular domain of HER2. Two of these
363 antibodies, 4D5 and 2C4 were shown to inhibit growth of breast cancer cells both in
364 vitro and in vivo [24]. These mouse mAbs were chimerized and subsequently
365 humanized and designated as Trastuzumab and Pertuzumab [25, 26]. Trastuzumab
366 is a humanized mAb that binds to the extracellular domain IV of HER2, and induces
367 down-regulation of the PI3K/Akt pathway. Treatment with Trastuzumab has proven
368 to be effective in management of HER2-amplified/overexpressing tumors [27].
369 Nevertheless, resistance to therapy is a serious challenge [28]. The majority of
370 metastatic breast cancer patients who initially respond to Trastuzumab begin to
371 demonstrate disease progression within one year [29]. Newly generated mAbs with
372 specificity to novel epitopes on extracellular domain of HER2 [9, 12, 30] might be
373 able to enhance anti-cancer activity. Synergistic effect of some mAbs with
374 Trastuzumab has been demonstrated in HER2 overexpressing breast cancer
375 xenograft models [8, 31]. Among these antibodies, FDA has approved Pertuzumab
376 (Perjeta-Genentech) in combination therapy with Trastuzumab [11]. Pertuzumab is
377 another humanized mAb that binds to domain II and efficiently inhibits dimerization
378 of HER2 [32].

379 We have recently generated a panel of mouse mAb directed against HER2 which
380 recognize epitopes distinct from Trastuzumab [12]. Two of these mAbs (1T0 and
381 2A8) were later found to significantly inhibit the proliferation of HER2-expressing
382 tumor cell line, BT-474, dose-dependently (manuscript in preparation). In the present

383 study, we presented data on chimerization of one of these mAbs, 1T0. The VH and
384 VL genes were successfully amplified and integrated to human IgG1 and Ck by SOE
385 PCR. Liu and colleagues [33] generated a mouse/human chimeric mAb against
386 HER2 and assessed its structural and biological activities. They amplified the VH
387 and VL genes of the mouse mAb from genomic DNA of the hybridoma clone. Luo
388 and coworker [34] isolated Fab genes of a mouse mAb from cDNA of hybridoma cell
389 line and constructed a mouse/human chimeric mAb. Either DNA sequencing or
390 ELISA could be used to show that the expression vector of chimeric antibody is
391 constructed successfully [34, 35]. The results of ELISA in this work showed that the
392 transfected CHO cells produce mouse/human chimeric mAb (c-1T0).

393 Using an antigen based indirect ELISA as well as immunoblotting techniques we
394 demonstrated the HER2 binding activity of the chimeric c-1T0 antibody. The
395 immunoblot results showed that similar to the parental mouse 1T0 mAb, c-1T0
396 recognizes a conformational epitope on extracellular domain of HER2 (Figure 5).
397 The results obtained by flow cytometry indicate that c-1T0 binds to native HER2
398 expressed on the surface of tumor cells as efficiently as the mouse counterpart and
399 Trastuzumab (Figure 7). These findings suggest that this antibody could be used to
400 target tumor cells. Interestingly, the affinity constant of the chimeric antibody was
401 slightly higher than the parental mouse 1T0 mAb. The improved binding activity of
402 our chimeric antibody might be due to the higher flexibility of the hinge region of
403 human IgG1 as compared to mouse IgG1. A similar mouse/human chimeric IgG1
404 mAb with specificity for *Cryptococcus neoformans* and a higher binding affinity
405 compared to the parental mouse IgG1 mAb has previously been reported [36].

406 The in vitro biological activity of our chimeric antibody was assessed by
407 incorporation of radioactive thymidine and XTT techniques (Figure 8 and 9). The
408 results indicated that c-1T0 inhibits the proliferation of BT-474 cells dose
409 dependently similar to Trastuzumab and the mouse 1T0. However, despite the
410 overall similarity, c-1T0 displayed a better inhibitory response in the XTT assay, but
411 not the thymidine incorporation assay, which could be due to the differences of the
412 assay systems employed in this study. The XTT assay measures the metabolic
413 activity of the growing cells, whereas the thymidine incorporation assay measures
414 the DNA synthesis status of proliferating cells. Furthermore, Trastuzumab failed to
415 inhibit cell growth and proliferation at low concentration (2.5ug/ml) in both assay
416 systems, implying functional limitation of this mAb at low concentrations.

417 The mechanisms of anticancer activity of c-1T0 are not completely known, but taking
418 into consideration the similar in vitro tumor growth inhibitory activity of c-1T0 and
419 Trastuzumab, several mechanisms could be proposed including: (a) downregulation
420 of total levels of HER2 on the cell surface [37], (b) blocking cleavage of the
421 extracellular domain of HER2 and thereby preventing formation of the constitutively
422 active membrane-bound 95-kDa HER2 protein called p95HER2 [28], (c) induction of
423 cell cycle arrest by p27kip1 and inhibition of cdk2 activity [38] and (d) blocking of the
424 dimerization of HER2 with HER3.

425 Considering the synergistic anti-tumor effect induced by combination of two different
426 mAbs with different epitope specificities, such as Trastuzumab and Pertuzumab [8]
427 and the fact that c-1T0 recognizes an epitope of HER2 different from that recognized
428 by Trastuzumab, our mAb might display a synergistic anti-tumor effect in

429 combination with Trastuzumab or Pertuzumab. We are currently investigating the
430 biological activity of c-1T0 mAb alone and in combination with Trastuzumab antibody
431 in vivo in nude mice implanted with breast tumor cells to assess its potential
432 implication for immunotherapy of HER2-expressing malignancies. Further in vivo
433 investigations are also required to assess functional activities of c-1T0 mAb
434 mediated by the host T lymphocytes and NK cells, such as antibody-dependent cell
435 cytotoxicity.

436

437

438 Executive Summary

439 The oncogenic potential and accessibility of HER2 have made it a suitable target for
440 cancer immunotherapy by monoclonal antibodies.

441 This study describes chimerization and characterization of a new mouse mAb (1T0)
442 against HER2, which binds to an epitope of HER2 different from that of
443 Trastuzumab.

444 The chimeric antibody was expressed in eukaryotic cells and characterized by
445 ELISA, SDS-PAGE and flow cytometry.

446 Tumor cell growth and proliferation inhibition were assessed by XTT and thymidine
447 incorporation assays, respectively.

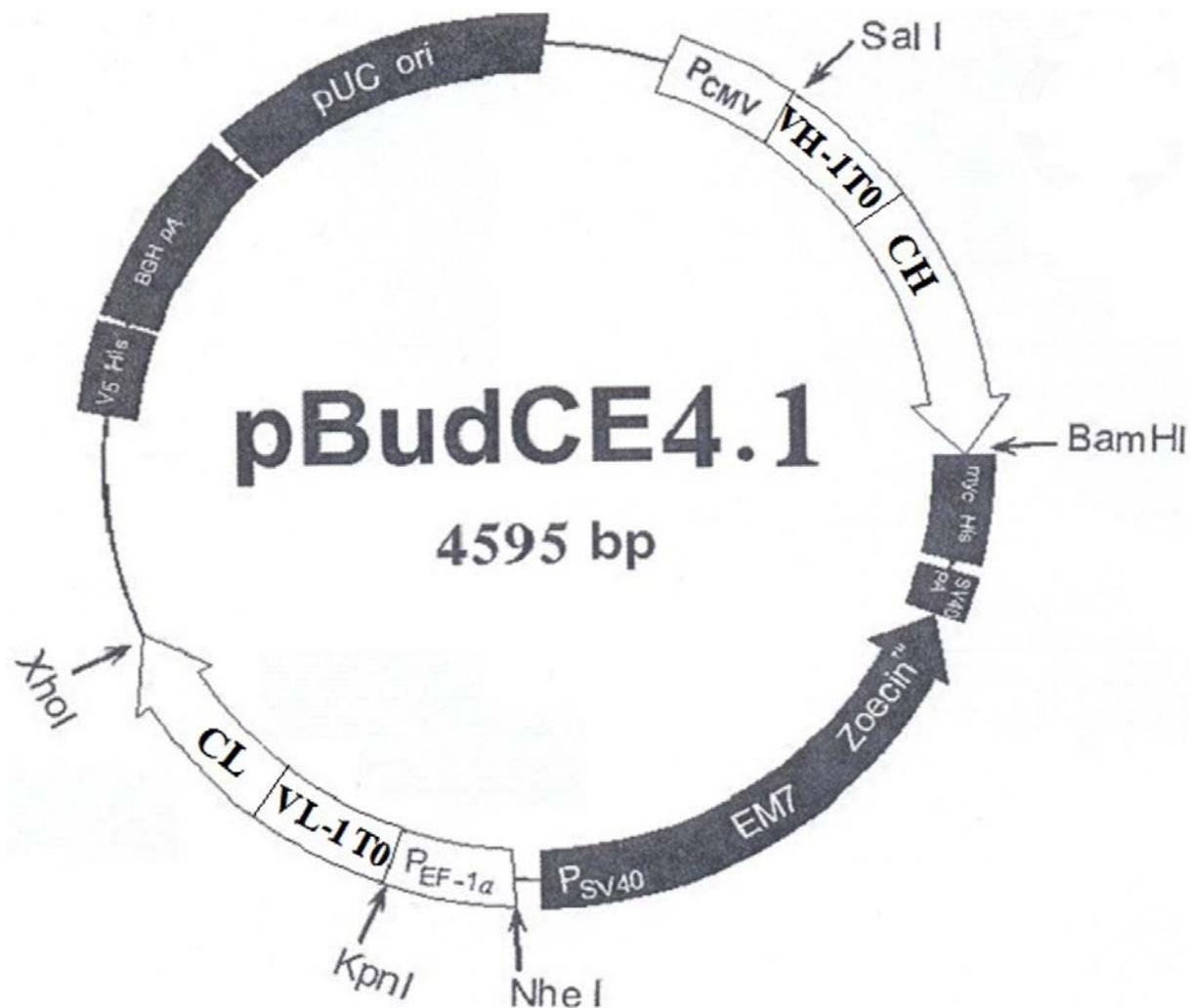
448 c-1T0 recognized a conformational epitope within the extracellular domain of HER2
449 distinct from Trastuzumab and displayed a binding affinity comparable to the
450 parental mouse mAb.

451 The purified chimeric mAb induced a dose dependent cell growth and proliferation
452 inhibition similar to the parental mouse mAb in HER2 overexpressing BT-474 tumor
453 cell line.

454 Our chimeric mAb with specificity to a novel epitope on extracellular domain of
455 HER2 is potentially a suitable tool for targeted immunotherapy of HER2
456 overexpressing malignancies.

457

458 **Figure 1:**

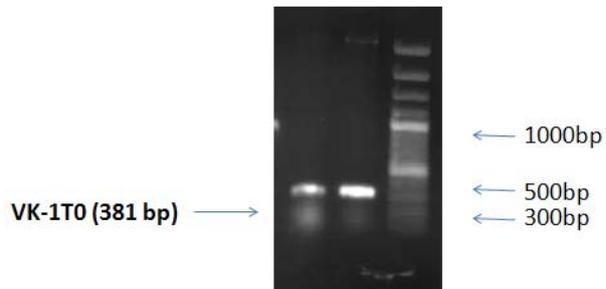


459

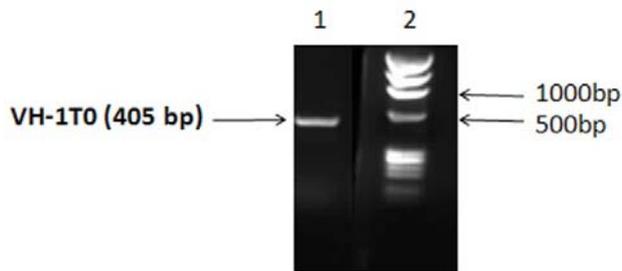
460 Map of pBudCE4.1 expression vector containing the mouse-human chimeric antibody (c-1T0). Light chain
461 sequences were introduced in XhoI and KpnI sites after P_{EF-1α} promoter and heavy chain sequences were
462 introduced in SalI and BamHI sites after P_{CMV} promoter.

463

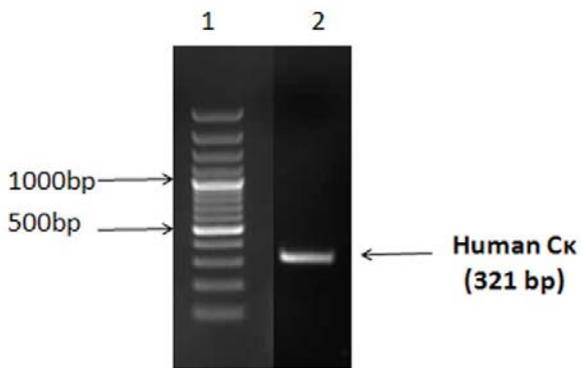
464 **Figure 2:**



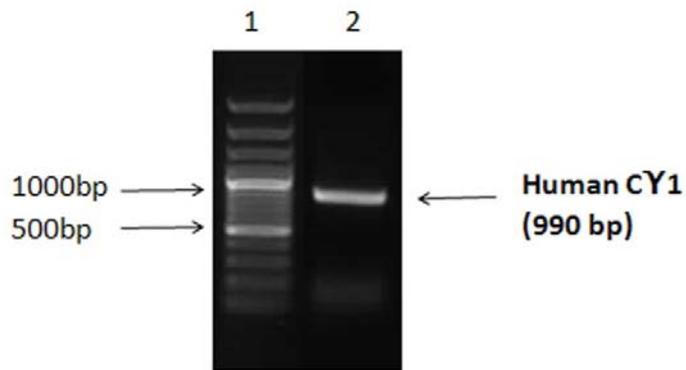
465 **A**



466 **B**

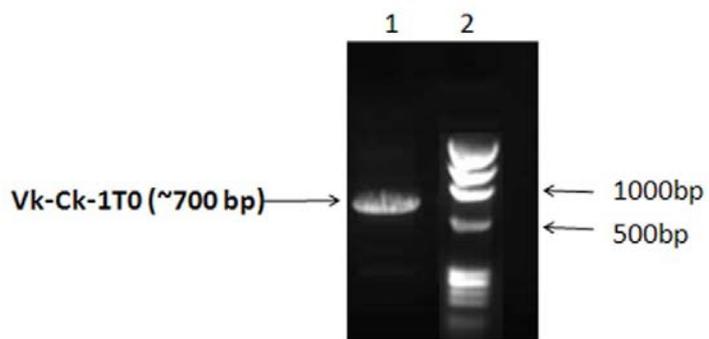


467 **C**



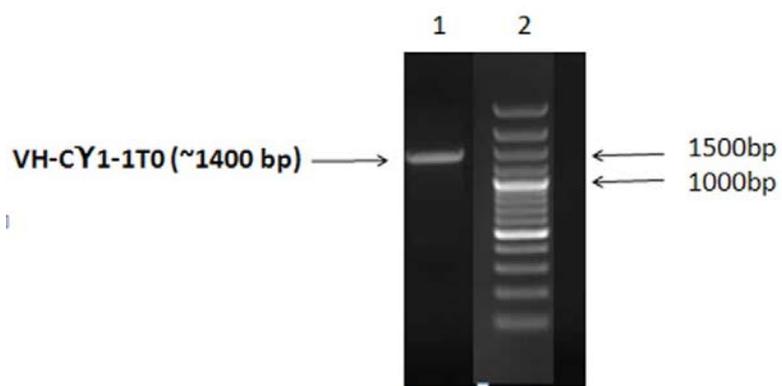
468

D



469

E



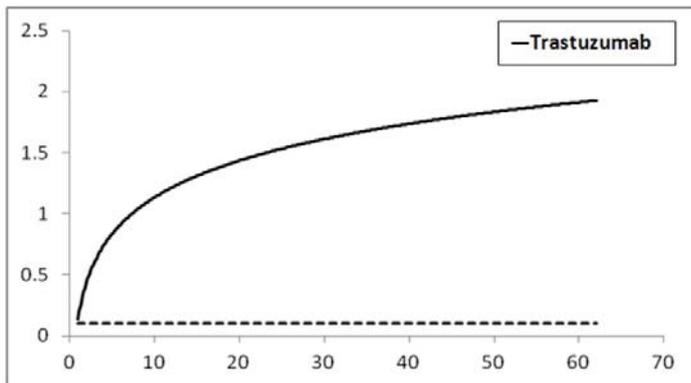
470

F

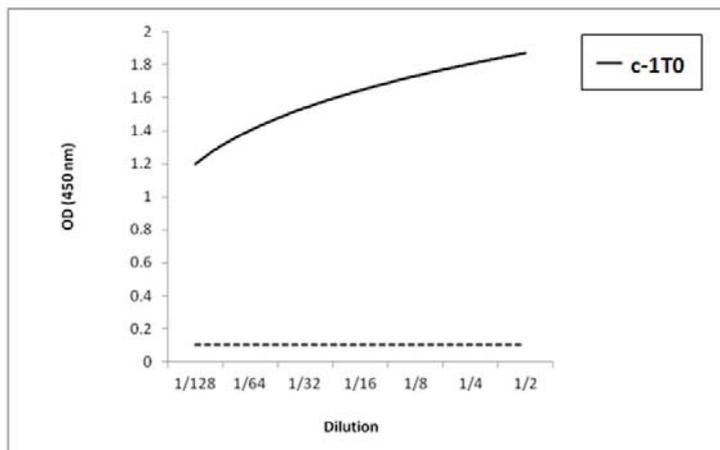
471 PCR amplification of VH-CH and VL-CL genes for construction of the chimeric antibody. Mouse variable region
 472 heavy (VH) and light (VL) chain genes (A and B) and human constant region heavy chain of IgG1 (CY1) and
 473 kappa light chains (Cκ) (C and D) were amplified from cDNA of the mouse hybridoma 1T0 clone and cDNA of
 474 human PBMC, respectively. Vk-Ck (E) and VH-Cy1 (F) fragments were amplified by SOE PCR as described in

475 Materials and Methods. The PCR products were run in 1% agarose gel. A 100bp Plus DNA ladder (A,C,D and
476 F) (Sinaclone, Iran) and DNA molecular weight marker IX (B and E) (Roche, Germany) were used.

477 **Figure 3:**



478 **A**

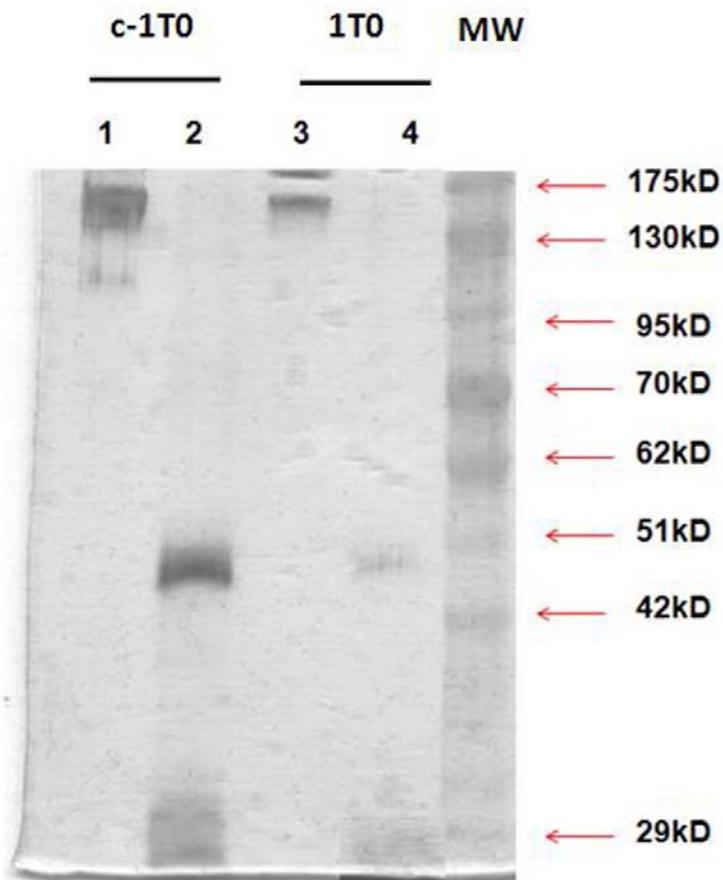


479 **B**

480 Measurement of chimeric anti-HER2 mAb in culture supernatant of transfected cells. Titration of Trastuzumab
481 (A) and c-1T0 culture supernatant (B) on recombinant extracellular region of HER2 is shown. Supernatant of
482 Mock transfected CHO cell line was used as a control (dot line).

483

484 **Figure 4:**

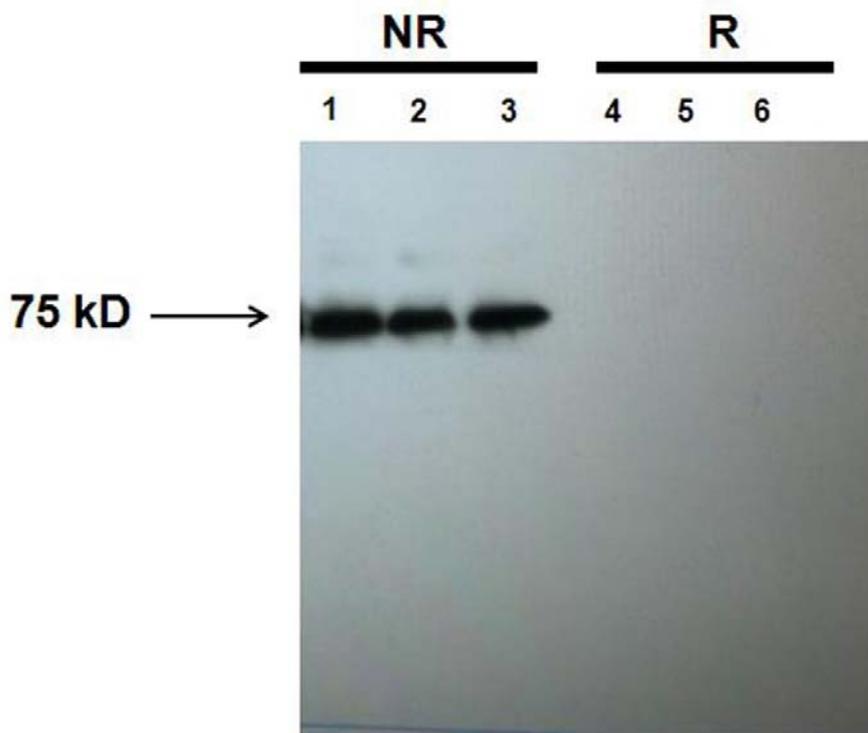


485

486 SDS-PAGE electrophoresis pattern of chimeric antibody. SPG purified c-1T0 and mouse 1T0 IgG
487 preparations were separated on 10% gel in non-reducing (1 and 3) and reducing (2 and 4)
488 conditions. MW: molecular weight ladder (Sinaclon, Iran).

489

490 **Figure 5:**

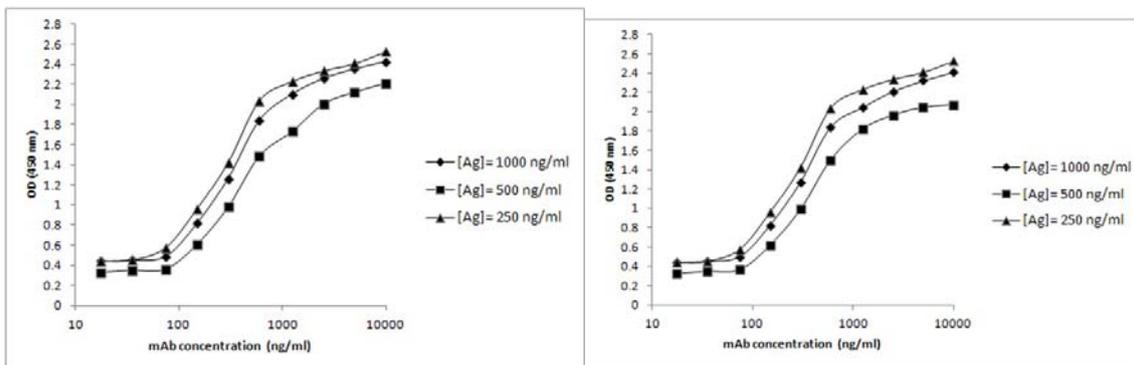


491

492 Western-blot analysis of chimeric antibody. Non-reduced (NR) and reduced (R) forms of rHER2 extracellular
493 protein was separated on 10% SDS-PAGE gel and transferred onto nitrocellulose membrane. The membrane
494 was blotted with c-1T0 (Lanes 3 and 6), mouse 1T0 (Lanes 2 and 5) and Trastuzumab (Lanes 1 and 4) and
495 then visualized by ECL, as described in Materials and Methods.

496

497 **Figure 6:**



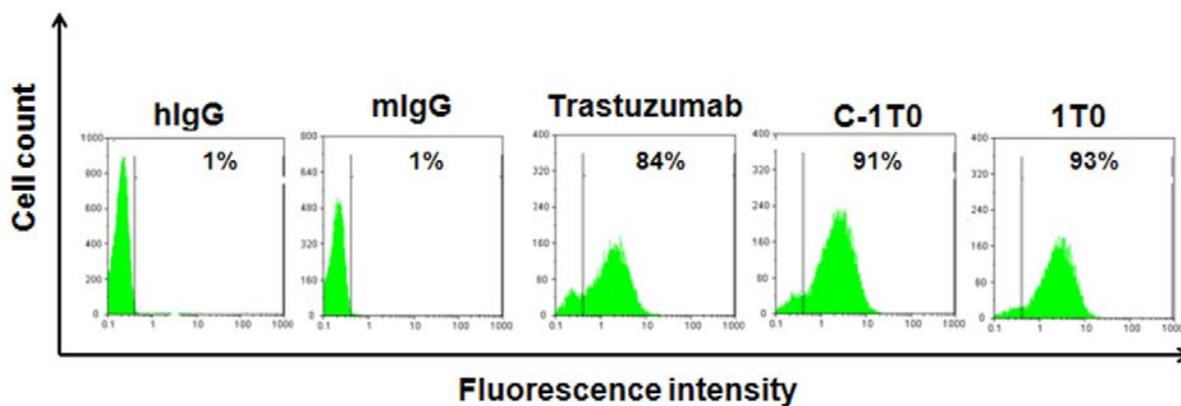
498 **A**

B

499 Experimental dose-response curves for mouse 1T0 (A) and chimeric c-1T0 (B) monoclonal antibodies at three
500 different concentrations of recombinant extracellular HER2 protein.

501

502 **Figure 7:**



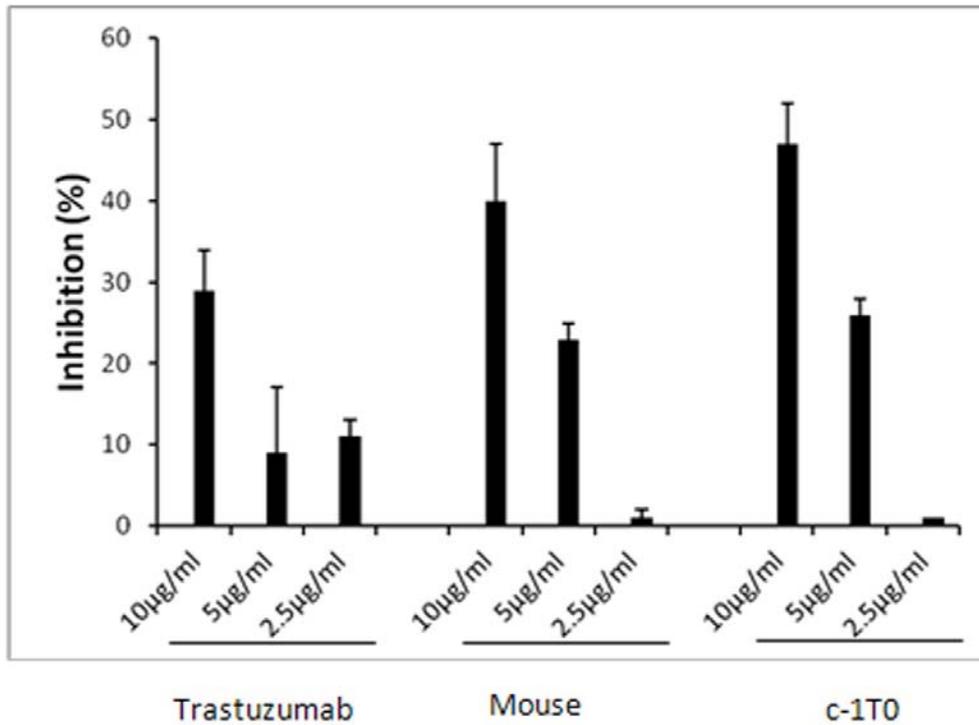
503

504 Detection of binding activity of chimeric c-1T0 antibody to HER2-expressing BT-474 cells by
505 flow cytometry. BT-474 were harvested and stained with mouse 1T0 and chimeric c-1T0.
506 Irrelevant mouse mAb (mlgG), irrelevant human IgG (hlgG) and Trastuzumab were used with

507 the same concentration as negative and positive controls, respectively. Figures represent
508 percent of positive cells.

509

510 **Figure 8:**

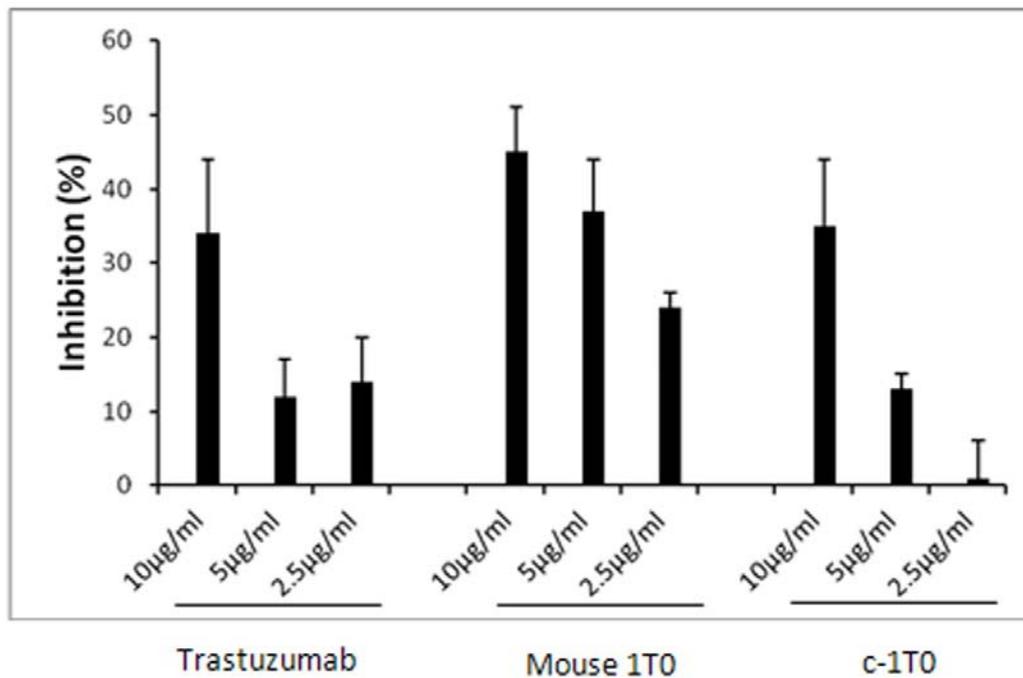


511

512 Assessment of tumor growth inhibition activity of chimeric antibody by XTT assay. Serial
513 concentrations of c-1T0 antibody were added to BT-474 cells. Cells were then incubated with
514 XTT and OD was measured. Percent of inhibition was measured as described in Materials and
515 Methods. Serial concentrations of mouse 1T0 and Trastuzumab were employed as controls.

516

517 **Figure 9:**



518

519 Assessment of tumor proliferation inhibition activity of chimeric antibody by radioactive
520 thymidine incorporation assay. Serial concentrations of antibody were added to BT-474 cells.
521 Cells were then incubated with ³H thymidine and radioactive thymidine incorporation was
522 subsequently measured by a beta-counter. Percent of proliferation inhibition was calculated as
523 described in Materials and Methods.

524

525 **References**

526
527 1. Kruser TJ, Wheeler DL: Mechanisms of resistance to HER family targeting antibodies.
528 *Exp Cell Res* 316(7), 1083-1100 (2010).
529 * An interesting review on the mechanisms contributing to resistance to HER2 and EGFR
530 antibody-based therapies.
531 2. Hynes NE, Macdonald G: ErbB receptors and signaling pathways in cancer. *Curr Opin*
532 *Cell Biol* 21(2), 177-184 (2009).
533 3. Ladjemi MZ, Jacot W, Chardes T, Pelegri A, Navarro-Teulon I: Anti-HER2 vaccines:
534 new prospects for breast cancer therapy. *Cancer Immunol Immunother* 59(9), 1295-
535 1312 (2010).
536 4. Shen G, Huang H, Zhang A *et al.*: In vivo activity of novel anti-ErbB2 antibody chA21
537 alone and with Paclitaxel or Trastuzumab in breast and ovarian cancer xenograft
538 models. *Cancer Immunol Immunother* 60(3), 339-348 (2011).
539 5. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, Mcguire WL: Human breast
540 cancer: correlation of relapse and survival with amplification of the HER-2/neu
541 oncogene. *Science* 235(4785), 177-182 (1987).
542 6. Nahta R, Esteva FJ: HER2 therapy: molecular mechanisms of trastuzumab resistance.
543 *Breast Cancer Res* 8(6), 215 (2006).
544 7. Fizman GL, Jasnis MA: Molecular Mechanisms of Trastuzumab Resistance in HER2
545 Overexpressing Breast Cancer. *Int J Breast Cancer* 2011, 352182 (2011).
546 8. Baselga J, Gelmon KA, Verma S *et al.*: Phase II trial of pertuzumab and trastuzumab in
547 patients with human epidermal growth factor receptor 2-positive metastatic breast
548 cancer that progressed during prior trastuzumab therapy. *Journal of clinical oncology :*
549 *official journal of the American Society of Clinical Oncology* 28(7), 1138-1144 (2010).
550 ** The combination of Pertuzumab and Trastuzumab induces remission in patients who had
551 experienced progression during prior trastuzumab therapy.
552 9. Ceran C, Cokol M, Cingoz S, Tasan I, Ozturk M, Yagci T: Novel anti-HER2 monoclonal
553 antibodies: synergy and antagonism with tumor necrosis factor-alpha. *BMC Cancer*
554 12(1), 450 (2012).
555 10. Baselga J, Cortes J, Kim SB *et al.*: Pertuzumab plus trastuzumab plus docetaxel for
556 metastatic breast cancer. *N Engl J Med* 366(2), 109-119 (2012).
557 ** A cocktail of pertuzumab, trastuzumab and docetaxel significantly prolonged progression-
558 free survival, with no increase in cardiac toxic effects
559 11. Traynor K: FDA approves pertuzumab for breast cancer. *Am J Health Syst Pharm*
560 69(14), 1178 (2012).
561 12. Kazemi T, Tahmasebi F, Bayat AA *et al.*: Characterization of novel murine monoclonal
562 antibodies directed against the extracellular domain of human HER2 tyrosine kinase
563 receptor. *Hybridoma (Larchmt)* 30(4), 347-353 (2011).
564 13. Jones ML, Barnard RT: Chimerization of multiple antibody classes using splice overlap
565 extension PCR. *Biotechniques* 38(2), 181-182 (2005).
566 14. Yan JX, Wait R, Berkelman T *et al.*: A modified silver staining protocol for visualization of
567 proteins compatible with matrix-assisted laser desorption/ionization and electrospray
568 ionization-mass spectrometry. *Electrophoresis* 21(17), 3666-3672 (2000).
569 15. Saboor-Yaraghi AA, Ghods R, Gharagozlou S *et al.*: Identification of cross-reactive and
570 restricted epitopes localized on human chorionic gonadotropin beta-subunit by
571 monoclonal antibodies. *Hybrid Hybridomics* 23(2), 101-107 (2004).
572 16. Caravella JA, Wang D, Glaser SM, Lugovskoy A: Structure-Guided Design of
573 Antibodies. *Curr Comput Aided Drug Des*, (2010).

- 574 17. Goldstein G: Monoclonal antibody specificity: Orthoclone OKT3 T-cell blocker. *Nephron*
575 46 Suppl 1, 5-11 (1987).
- 576 18. Schroff RW, Foon KA, Beatty SM, Oldham RK, Morgan AC, Jr.: Human anti-murine
577 immunoglobulin responses in patients receiving monoclonal antibody therapy. *Cancer*
578 *Res* 45(2), 879-885 (1985).
- 579 19. Boulianne GL, Hozumi N, Shulman MJ: Production of functional chimaeric
580 mouse/human antibody. *Nature* 312(5995), 643-646 (1984).
- 581 20. Morrison SL, Johnson MJ, Herzenberg LA, Oi VT: Chimeric human antibody molecules:
582 mouse antigen-binding domains with human constant region domains. *Proc Natl Acad*
583 *Sci U S A* 81(21), 6851-6855 (1984).
- 584 21. Jones PT, Dear PH, Foote J, Neuberger MS, Winter G: Replacing the complementarity-
585 determining regions in a human antibody with those from a mouse. *Nature* 321(6069),
586 522-525 (1986).
- 587 22. Park DI, Kang MS, Oh SJ *et al.*: HER-2/neu overexpression is an independent
588 prognostic factor in colorectal cancer. *Int J Colorectal Dis* 22(5), 491-497 (2007).
- 589 23. Fendly BM, Winget M, Hudziak RM, Lipari MT, Napier MA, Ullrich A: Characterization of
590 murine monoclonal antibodies reactive to either the human epidermal growth factor
591 receptor or HER2/neu gene product. *Cancer Res* 50(5), 1550-1558 (1990).
- 592 24. Agus DB, Akita RW, Fox WD *et al.*: Targeting ligand-activated ErbB2 signaling inhibits
593 breast and prostate tumor growth. *Cancer Cell* 2(2), 127-137 (2002).
- 594 25. Adams CW, Allison DE, Flagella K *et al.*: Humanization of a recombinant monoclonal
595 antibody to produce a therapeutic HER dimerization inhibitor, pertuzumab. *Cancer*
596 *Immunol Immunother* 55(6), 717-727 (2006).
- 597 26. Carter P, Presta L, Gorman CM *et al.*: Humanization of an anti-p185HER2 antibody for
598 human cancer therapy. *Proc Natl Acad Sci U S A* 89(10), 4285-4289 (1992).
- 599 27. Witton CJ: Structure of HER receptors and intracellular localisation of downstream
600 effector elements gives insight into mechanism of tumour growth promotion. *Breast*
601 *cancer research : BCR* 5(4), 206-207 (2003).
- 602 28. Gajria D, Chandralapaty S: HER2-amplified breast cancer: mechanisms of trastuzumab
603 resistance and novel targeted therapies. *Expert Rev Anticancer Ther* 11(2), 263-275
604 (2011).
- 605 29. Wong AL, Lee SC: Mechanisms of Resistance to Trastuzumab and Novel Therapeutic
606 Strategies in HER2-Positive Breast Cancer. *International journal of breast cancer* 2012,
607 415170 (2012).
- 608 30. Cho WC, Roukos DH: Trastuzumab emtansine for advanced HER2-positive breast
609 cancer and beyond: genome landscape-based targets. *Expert Rev Anticancer Ther*
610 13(1), 5-8 (2013).
- 611 31. Baselga J, Swain SM: Novel anticancer targets: revisiting ERBB2 and discovering
612 ERBB3. *Nat Rev Cancer* 9(7), 463-475 (2009).
- 613 32. Capelan M, Pugliano L, De Azambuja E *et al.*: Pertuzumab: new hope for patients with
614 HER2-positive breast cancer. *Annals of oncology : official journal of the European*
615 *Society for Medical Oncology / ESMO*, (2012).
- 616 33. Liu HL, Parkes DL, Langton BC *et al.*: Construction of a chimeric antibody with
617 therapeutic potential for cancers which overexpress c-erbB-2. *Biochem Biophys Res*
618 *Commun* 211(3), 792-803 (1995).
- 619 34. Luo W, Chen Y, Li L *et al.*: Construction and characterization of the chimeric antibody
620 8C11 to the hepatitis E virus. *FEMS Immunol Med Microbiol* 51(1), 18-25 (2007).
- 621 35. Chang D, Zhang JH, Zhao XM, Liang P: [Vector construction and expression of anti-
622 Abeta human-mouse chimeric antibody against Alzheimer's disease]. *Zhonghua Bing Li*
623 *Xue Za Zhi* 39(8), 542-547 (2010).

- 624 36. Zebedee SL, Koduri RK, Mukherjee J *et al.*: Mouse-human immunoglobulin G1 chimeric
625 antibodies with activities against *Cryptococcus neoformans*. *Antimicrob Agents*
626 *Chemother* 38(7), 1507-1514 (1994).
- 627 37. Nahta R, Yu D, Hung MC, Hortobagyi GN, Esteva FJ: Mechanisms of disease:
628 understanding resistance to HER2-targeted therapy in human breast cancer. *Nat Clin*
629 *Pract Oncol* 3(5), 269-280 (2006).
- 630 38. Le XF, Claret FX, Lammayot A *et al.*: The role of cyclin-dependent kinase inhibitor
631 p27Kip1 in anti-HER2 antibody-induced G1 cell cycle arrest and tumor growth inhibition.
632 *J Biol Chem* 278(26), 23441-23450 (2003).

633

634