RESEARCH ARTICLE

Differential Effects of Inhibitory and Stimulatory Anti-HER2 Monoclonal Antibodies on AKT/ERK Signaling Pathways

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Abstract

Objective: Homo- and heterodimerization of the receptor tyrosine kinase HER2 hyperactivate several downstream signaling pathways, leading to uncontrolled growth and proliferation of tumor cells. Anti-HER2 monoclonal antibodies (mAbs) may induce different effects on HER2 dimerization and signaling. **Methods:** The effect of two inhibitory (2A8, 1T0) and one stimulatory (1H9) anti-HER2 mAbs either alone or in combination with trastuzumab was investigated on AKT and ERK signaling pathways and HER2 degradation in a human breast cancer cell line (BT-474) by Western blotting. **Result:** While 1H9 mAb had no significant effect on AKT and ERK signaling pathways, 1T0 and 2A8 mAbs inhibited phosphorylation of both pathways. Combination of 1T0 mAb with trastuzumab resulted in significant synergistic inhibition of both pathways and HER2 degradation, much more potently than the combination of trastuzumab and pertuzumab. **Conclusion:** Our data indicate that anti-HER2 mAbs may induce different signaling pathways depending on their effect on tumor cell growth and proliferation. The significant inhibition of ERK and AKT phosphorylation by 1T0 alone or particularly in combination with trastuzumab suggests its potential therapeutic application for targeted immunotherapy of HER2 overexpressing malignancies.

Keywords: Breast cancer- AKT/ERK signaling pathways- HER2- Monoclonal antibody

Introduction

ErbB receptors belong to the superfamily I of the receptor tyrosine kinases (RTKs), which include four members: HER1/ErbB1, HER2/ErbB2, HER3/ErbB3 and HER4/ErbB4 (Olayioye et al., 2000). Controlled expression of ErbB receptors plays important roles in cell proliferation and differentiation of the developing embryo and adult tissues (Cho et al., 2003). Overexpression of these receptors may lead to different cancers through hyperactivation of downstream signaling pathways (Olayioye et al., 2000; Yarden et al., 2001). HER2 a member of this family, is overexpressed in many cancers including breast cancer (Neve et al., 2001; Iqbal, 2014). HER2 overexpression leads to homo- and heterodimerization with other members of ErbB receptors specially, HER1 and HER3 (Roskoski, 2014). Among them, HER2/HER3 heterodimers are the most prevalent and potent complexes for activating of signaling pathways specially, PI3K/AKT (Roskoski, 2004; Van Der Horst et al., 2005; Balz et al,. 2012). Following increased formation of HER2 homo- and heterodimers, several changes such

as conformational changes, kinase domain activation and tyrosine residues auto-trans phosphorylation of ErbB receptors lead to PI3K, MAPK, PLC1 and STATs downstream signaling pathways hyperactivation resulting in increased cell proliferation, adhesion, angiogenesis and cell survival (Roskoski, 2014; Zhou et al., 2013; Maruyama, 2014). HER2 overexpression may also cause resistance to common treatment protocols including: chemotherapy, hormone therapy and radiotherapy (Yu and Hung, 2000). Therefore, HER2 molecule is considered as an important target for therapeutic interventions (Neve et al., 2001; Mahdavi et al., 2012). Signaling pathways inhibition by tyrosine kinase inhibitors (TKIs) and anti-HER2 mAbs is an effective treatment strategy (Yarden, 2001).

In recent years, many mAbs to HER2 extracellular domain were produced (Shawver et al., 1994; Pedersen et al., 2015; Shen et al., 2011; Ceran et al., 2012; Fu et al., 2014; De Lorenzo et al., 2005). Two of them, trastuzumab and pertuzumab, were approved by FDA for treatment of patients with HER2 overexpressed breast cancer (Amiri-Kordestani et al., 2014). Monoclonal

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antibodies targeting HER2 affect cancer cell growth through two major mechanisms: 1) direct mechanism through abrogating cell signaling, cell cycle arrest, preventing receptor dimerization and inducing receptor internalization and degradation; 2) indirect mechanism involving activation of the immune system such as antibody dependent cell cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC) (Szymanska et al., 2016; Mortenson and Fu, 2013). Generally, mAbs to various epitopes of HER2 may have different effect on downstream signaling pathways (Yip et al., 2003) and inhibit cell growth more potently than individual mAbs (Meng et al., 2016; Nahta et al., 2004). Among many signaling pathways which are activated by ErbB receptors, Ras, Raf, MEK, ERK1/2, PI3K and PLC1 pathways play important roles in cell proliferation (Yarden et al., 2001; Zhou et al., 2013; Nahta et al., 2004; Appert-Collin et al., 2015).

In our previous studies, we produced and characterized a number of anti-HER2 mAbs including two inhibitory (1T0 and 2A8) mAbs which displayed superior anti-tumor activity in combination with trastuzumab (Tahmasebi et al., 2013; Kazemi et al., 2011). In this study, we investigated the effect of these inhibitory mAbs as well as one stimulatory mAb (1H9) alone and in combination with trastuzumab on two major intracellular downstream signaling pathways of HER2. In addition, we evaluated the effect of mAbs on HER2 degradation which is another mechanism for cell growth inhibition (Mortenson and Fu, 2013).

Materials and Methods

Cell culture, antibody treatment and cell lysate preparation

To determine the effect of mAbs treatment on AKT and ERK phosphorylation, 3×10^6 BT-474 cells (National Cell Bank of Iran, Pasteur Institute, Tehran, Iran) were seeded in T-25 culture flask and fed with RPMI-1640 culture medium (Gibco, California, USA) containing 20% FBS fetal bovine serum (Gibco) and 10 µg/ml insulin (Sigma Aldrich, St Louis, MO, USA), for 48 h at 37°C in a 5% CO_2 humidified atmosphere. Then, cells were treated with 50µg/ml of 1T0, 2A8, 1H9 (produced in our previous works) (Tahmasebi et al., 2013; Kazemi et al., 2011), trastuzumab (Genentech/Roche) and pertuzumab mAbs alone and 25µg/ml of each mAb in combination with 25 µg trastuzumab for 24 h. Trastuzumab, pertuzumab (Genentech/Roche) and their combination were used as controls. After incubation, cells were washed with ice-cold PBS, trypsinized and lysates were prepared using mammalian protein extraction reagent (M-PER, Thermo Fisher Scientific, California, USA). Halt™ protease and phosphatase inhibitor (Thermo Fisher Scientific) was added immediately before preparation of cell lysates according to the manufacturer's instruction. Protein concentration of cell lysates was determined using BCA protein assay kit (Thermo Fisher Scientific).

Analysis of AKT and ERK signaling by Western blot

For detection of AKT and ERK proteins, 12µg/ml of cell lysates were resuspended with 5x sample loading buffer and boiled. Solubilized proteins were resolved by 10% SDS-PAGE under non-reducing condition. PVDF membrane (Roche, Basel, Switzerland) was used for transferring of proteins and blocked with 5% non-fat dried milk (Merck, Darmstad, Germany) in TBS without Tween 20 for 1 hour at room temperature (RT). For detection of p-AKT and p-ERK proteins, the blots were incubated with primary antibodies (Cell signaling Technologies, Danvers, Massachuset, USA) overnight at 4°C, followed by 1 h incubation with goat anti-rabbit Ig horseradish peroxidase-conjugated antibody (Cell Signaling Technologies) at RT. The blots were washed three times with TBS without Tween 20. Target proteins were subsequently detected with the select-ECL kit (Amersham, Amersham Pharmacia Biotech, Chalfont, UK). After detection of phosphoproteins, blotted membranes were stripped (Glycin 15g, SDS 1g, Tween 20 10ml, dissolved in 1 lit distilled water, pH to 2.2 (Ceran et al., 2012) and washed with TBS three times and re-blocked. Then, membranes were reprobed with primary and secondary antibodies for detection of total AKT and ERK (Cell Signaling Technologies) as described for p-AKT and p-ERK. β-actin protein was used as an internal control. After electro-blotting onto PVDF, membrane was blocked in 5% BSA (Sigma Aldrich) for 1h and the membrane was subsequently incubated with rabbit anti human β-actin antibody (Cell Signaling Technologies) at 4°C overnight, followed by incubation with HRP-conjugated goat anti-rabbit Ig antibody (Cell Signaling Technologies) for 1h at RT ultimately, protein bands were scanned and analyzed with ImageJ software (version 1.42q; NIH, Bethesda, MD).

For HER2 degradation analysis, 50µg/ml lysates were resolved by 8% SDS-PAGE under reducing conditions. After electro-blotting onto PVDF, the membranes were blocked with 5% non-fat dry milk in PBS-Tween 20 0.05%, and incubated with mouse anti-HER2 primary mAb (1F2, prepared in our laboratory) for 2 h at RT and then incubated with sheep anti-mouse Ig HRP-conjugated (Sina Biotech, Tehran, Iran) secondary antibody for 1 h at RT. The bands were finally developed and analyzed as described above.

For investigation of mAbs effects on AKT and ERK pathways, the ratio of phosphorylated AKT and ERK was calculated to their total forms. However, the relative density of HER2 protein was calculated to β-actin.

Assessment of tumor cells proliferation inhibition by radioactive thymidine incorporation assay

Twenty four hours before mAbs treatment, 5×10^4 BT-474 cells were seeded in 96-well flat-bottom tissue culture plates in complete culture medium. After adding 12 µg/ml or 6µg/ml of each mAb alone or in combination with trastuzumab, respectively, cultures were incubated for 72 h at 37°C in a humidified atmosphere of 5% CO_2 and then 3H–thymidine (PerkinElmer, MA, USA) was added at 0.5 µCi per well for 18 h. Cells were then harvested and transferred to scintillation buffer for measurement of 3H–thymidine incorporation by a β-counter (Wallac 1410 Liquid Scintillation Counter, Pharmacia, Sweden). The following formula was used to

calculate the proliferation inhibition rate:

Proliferation inhibition (%) = ([Counts per minute [CPM] without antibody-CPM with antibody]/CPM without antibody) × 100

Statistics

The results were analyzed with One-Way ANOVA test using SPSS software (version 20, IBM SPSS statistics data editor). Differences between groups were significant at p values less than 0.05 (p<0.05). The results are presented as mean ± standard deviation. All experiments were performed independently in triplicate.

Results

Impact of 1T0 and 2A8 mAbs on ERK and AKT phosphorylation

1T0 mAb significantly inhibited phosphorylation of AKT and ERK proteins in comparison to untreated cells. The inhibitory effect of this mAb on ERK (P<0.0001) was significantly higher than AKT (P<0.01) phosphorylation.

2A8 mAb, on the other hand, induced significant inhibition only on AKT phosphorylation (p < 0.0001). The effect of this mAb on ERK phosphorylation was marginal similar to trastuzumab. The combination of 2A8 and trastuzumab was not tested due to shortage of materials. The combination of 1T0 and trastuzumab induced inhibition on both AKT (p< 0.000001) and ERK (p< 00001) phosphorylation much more potent than the combination of trastuzumab and pertuzumab (p=0.0001, P-AKT and p= 0.42, P-ERK) (Figure 1 and 4B Tables 1).

The effect of 1H9 mAb on AKT and ERK phosphorylation

While 1H9 mAb alone inhibited neither AKT nor ERK phosphorylation, combination of 1H9 with trastuzumab moderately inhibited both AKT and ERK phosphorylation (p < 0.05) (Figure 2).

HER2 degradation by anti-HER2 mAbs

Analysis of the level of degradation of total HER2 protein relative to the house keeping gene β-actin, reveals that none of the mAbs induced significant effect on HER2 degradation. However, combination of 1T0
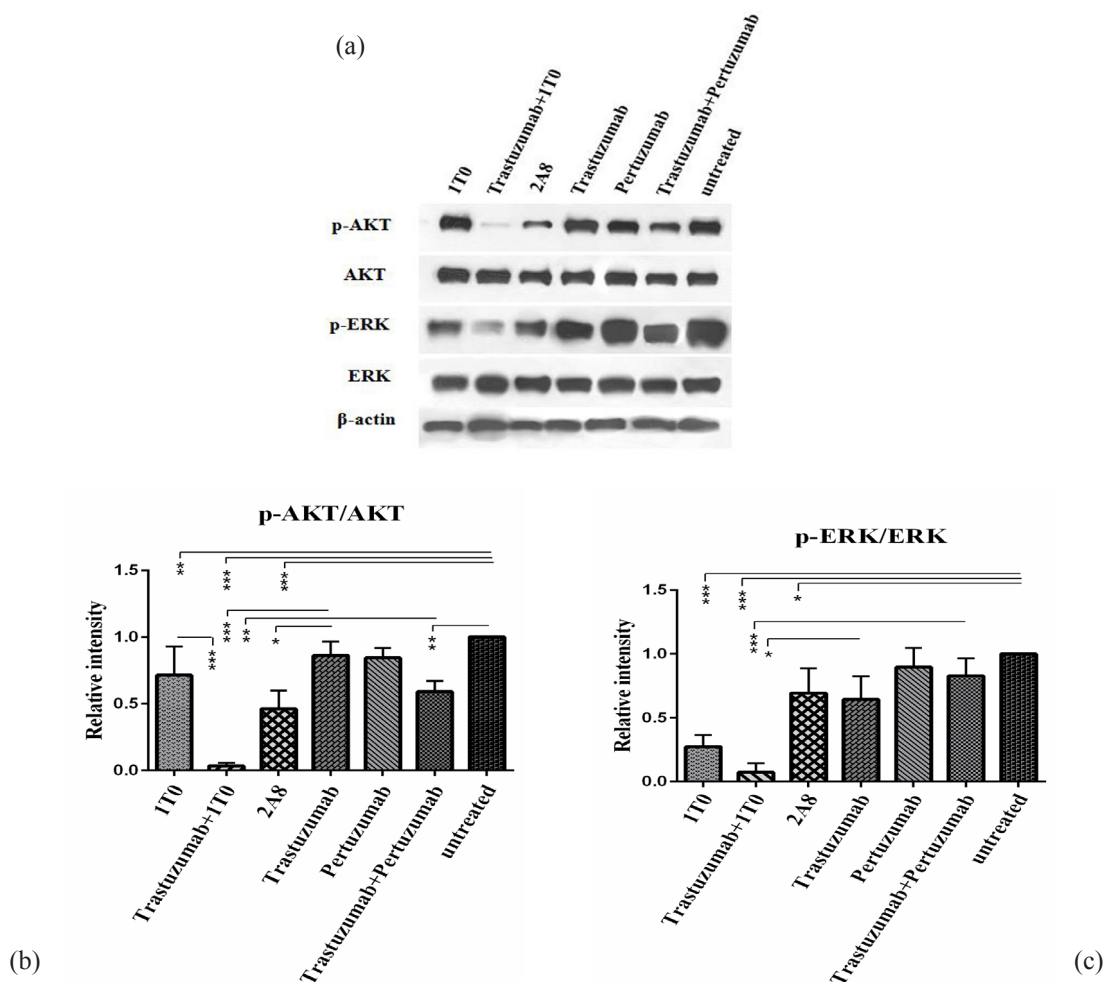

Figure 1. The Effect of Inhibitory Anti-HER2 mAbs on AKT and ERK Phosphorylation. BT-474 cells were treated with inhibitory mAbs alone or in combination with trastuzumab for 24 hours. The combination of trastuzumab and pertuzumab was used as a positive control. (A) Cell lysates were resolved by 10% SDS-PAGE under non-reducing condition and analyzed by immunoblotting. A representative result of three independent experiments is shown. Relative band intensities of p-AKT/AKT (B) and p-ERK/ERK (C) are demonstrated. Statistically significant differences between mAbs are displayed; * p < 0.05, ** p < 0.01 and *** p < 0.001. Results represent the mean ± SD of three independent experiments.
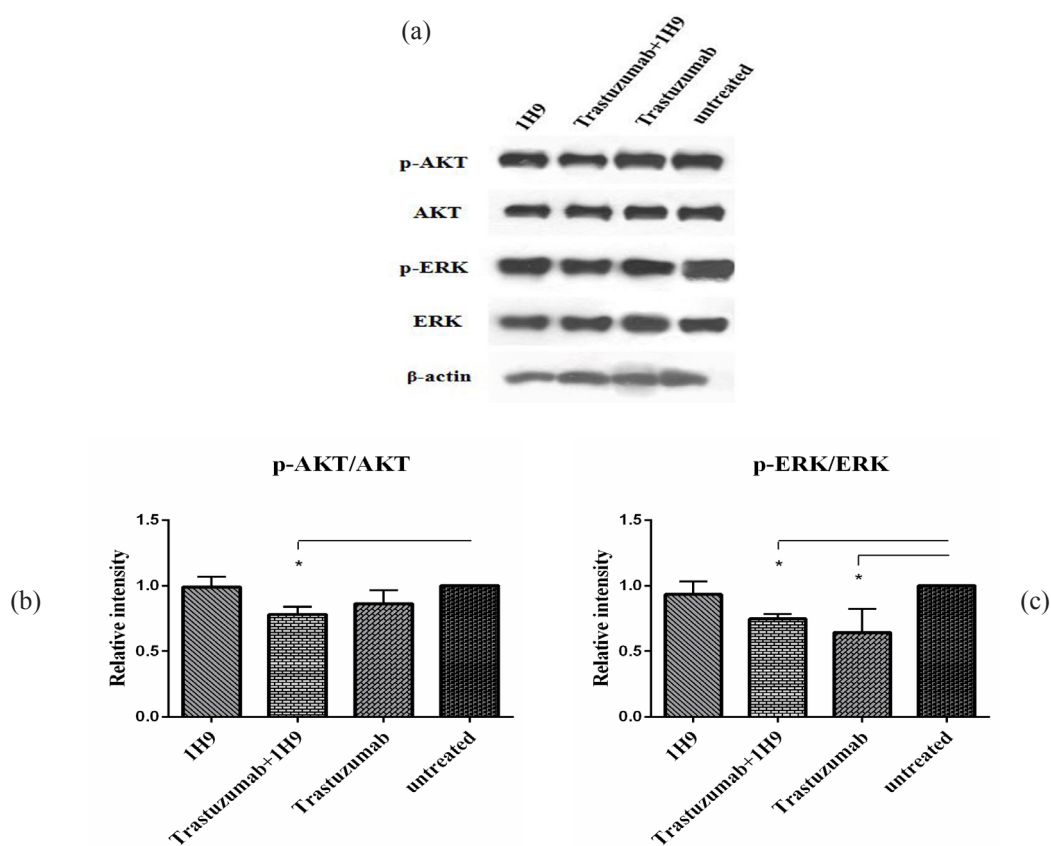
(a)

Figure 2. The Effect of Stimulatory Anti-HER2 mAb on AKT and ERK Phosphorylation. BT-474 cells were treated with 1H9 and trastuzumab plus 1H9. Total cell lysates were obtained after 24 hours of mAbs exposure. (A) Cell lysates were resolved by 10% SDS-PAGE under non-reducing condition and analyzed by immunoblotting. Representative results of three independent experiments are shown. Relative band intensities of p-AKT/AKT (B) and p-ERK/ERK (C) are shown. Statistically significant differences between mAbs are depicted; * p < 0.05, ** p < 0.01 and *** p < 0.001. Results represent the mean ± SD of three independent experiments.

and trastuzumab resulted in significant degradation of HER2 expression (p< 0.01), more potently than the combination of trastuzumab and pertuzumab (p< 0.05) (Figure 3).

Tumor cell growth inhibition by anti-HER2 mAbs

3H-thymidine incorporation assay was performed to determine the effect of mAbs alone and in combination with trastuzumab on growth of BT-474 cells. The results in figure 4 showed that although all inhibitory mAbs (1T0, 2A8, trastuzumab and pertuzumab) could inhibit the proliferation of tumor cells, the stimulatory 1H9 mAb not only did not inhibit proliferation of tumor cells, it induced stimulation of the tumor cell. Moreover,
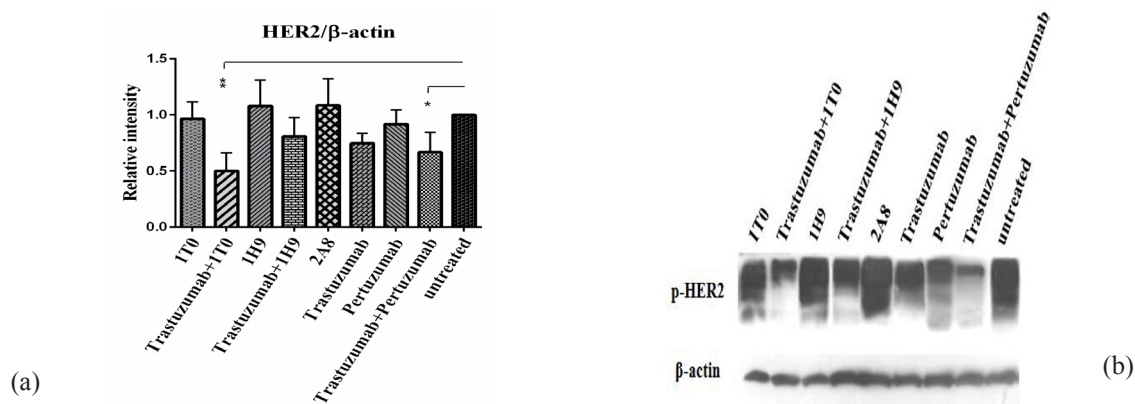

Figure 3. The Effect of Anti-HER2 mAbs on HER2 Degradation. BT-474 cells were treated with inhibitory and stimulatory mAbs alone or in combination with trastuzumab for 24 hours. The combination of trastuzumab and pertuzumab was used as a positive control (A) Cell lysates were resolved by 10% SDS-PAGE under non-reducing condition and analyzed by immunoblotting. A representative result of three independent experiments is shown. (B) Relative band intensities of HER2/β-actin are demonstrated. Statistically significant differences between mAbs are presented; * p < 0.05, ** p < 0.01 and *** p < 0.001. Results represent the mean ± SD of three independent experiments.
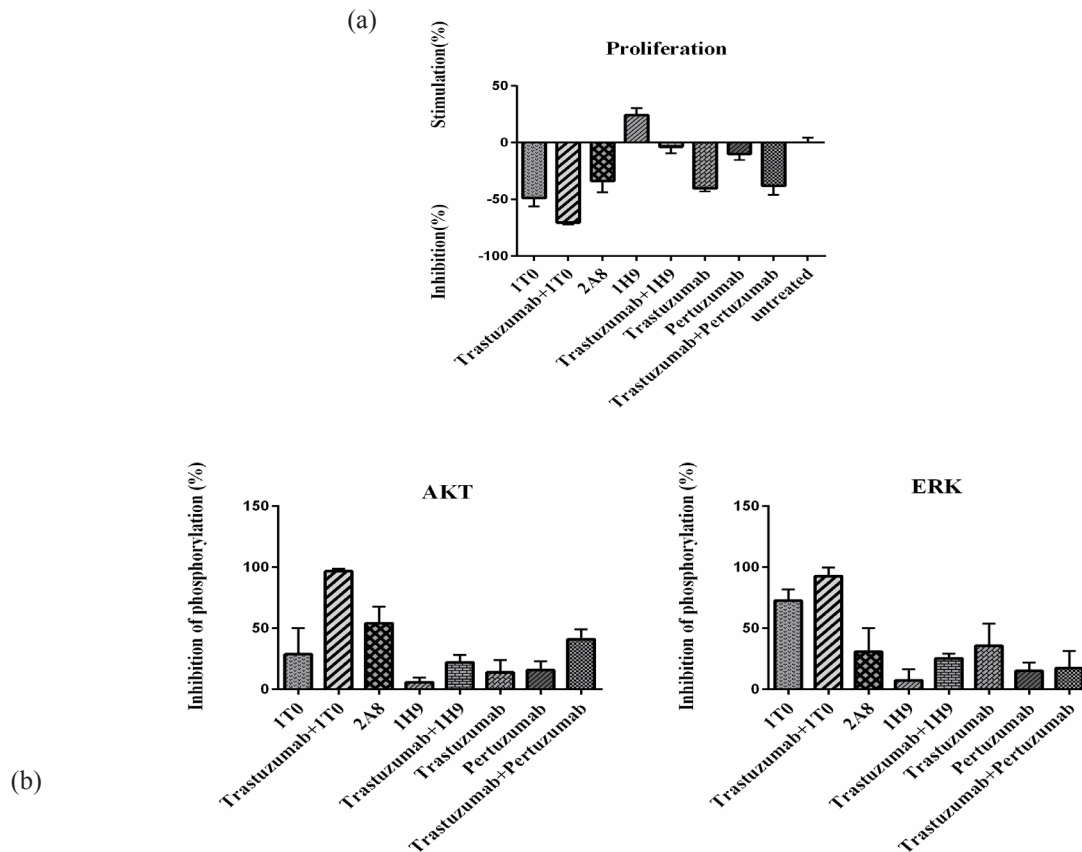
Figure 4. Influence of Anti-HER2 mAbs on Stimulation or Inhibition of Tumor Cells Proliferation and AKT/ERK Phosphorylation. (A) BT-474 cells were treated with a total of 12μg/ml concentration of each mAb alone or a combination of 6μg/ml of two mAbs and after 72 hours, the uptake of 3H-thymidine was evaluated by β-counter and the percent of inhibition or stimulation was calculated as described in the Materials and Methods. Error bars represent standard deviations calculated from triplicate cultures. (B) Percent inhibition of AKT/ERK phosphorylation. Results represent the mean ± SD of three independent experiments.

the combination of 1T0 and trastuzumab showed more potent inhibitory effects on tumor cell growth than combination of trastuzumab and pertuzumab.

These results are in accordance with our previous findings that suggested some of anti-HER2 mAbs induce inhibitory and some are inert or induce stimulatory effects

Table 1. Statistical Significance of AKT and ERK Phosphorylation Results Obtained with Different Anti-HER2 Monoclonal Antibodies

mAbs	1T0	2A8	1H9	Trast	Pertz	Trast+1T0	Trast+1H9	Trast+Pertz	untreated
1T0	— —	NS (NS)	P<0.05 (P<0.01)	NS (NS)	NS (P<0.01)	P<0.0001 (NS)	NS (NS)	NS (P<0.05)	P<0.01 (P<0.001)
2A8	NS (NS)	— —	P<0.001 (NS)	P<0.05 (NS)	P<0.05 (NS)	P<0.01 (P<0.01)	NS (NS)	NS (NS)	P<0.0001 (NS)
1H9	P<0.05 (P<0.01)	P<0.001 (NS)	— —	NS (NS)	NS (NS)	P<00001 (P<0.001)	NS (NS)	P<0.01 (NS)	NS (NS)
Trast	NS (NS)	P<0.05 (NS)	NS (NS)	— —	NS (NS)	P<0.00001 (P<0.01)	NS (NS)	NS (NS)	NS (P<0.05)
Pertz	NS (P<0.01)	P<0.05 (NS)	NS (NS)	NS (NS)	— —	P<0.0001 (P<0.001)	NS (NS)	NS (NS)	NS (NS)
Trast +1T0	P<0.0001 (NS)	P<0.01 (P<0.01)	P<0.00001 (P<0.001)	P<0.00001 (P<0.01)	P<0.00001 (P<0.001)	— —	P<0.0001 (P<0.01)	P<0.001 (P<0.001)	P<0.00001 (P<0.0001)
Trast +1H9	NS (NS)	NS (NS)	NS (NS)	NS (NS)	NS (NS)	P<0.0001 (P<0.01)	— —	NS (NS)	P<0.05 (P<0.05)
Trast+ Pertz	NS (P<0.05)	NS (NS)	P<0.01 (NS)	NS (NS)	NS (NS)	P<0.001 (P<0.001)	NS (NS)	— —	P<0.001 (NS)
Untreated	P<0.01 (P<0.001)	P<0.00001 (NS)	NS (NS)	NS (P<0.05)	NS (NS)	P<0.00001 (P<0001)	P<0.05 (P<0.05)	P<0.001 (NS)	— —

The data represent p values obtained for AKT and ERK (within parenthesis) phosphorylation. NS, not significant, Trast, Trastuzumab, Pertz, Pertuzumab

on tumor cell growth and also the combination of two mAbs could have more inhibitory effect than each mAb alone (Tahmasebi et al, 2013).

Discussion

Anti-HER2 mAbs influence tumor growth and proliferation through different mechanisms, including abrogation of signaling pathways, preventing receptor dimerization through receptor internalization and degradation, as well as immune-mediated mechanisms (Szymanska et al., 2016; Mortenson and Fu, 2013). In our previous studies, a panel of anti-HER2 mAbs were produced which recognize epitopes different from those recognized by trastuzumab and pertuzumab (Tahmasebi et al., 2013; Kazemi et al., 2011). In a recent study, we characterized binding sites of these mAbs on HER2 by producing recombinant HER2-subdomains in CHO-K1 cells (Hosseini-Ghatar et al., 2017). The impact of these mAbs was also investigated on tumor cell proliferation by H3-thymidine incorporation assay and the results were in agreement with our previous works (Tahmasebi et al., 2013) which showed that two of the mAbs (1T0 and 2A8) induced anti-proliferative activity while other mAbs, including 1H9, displayed stimulatory effect on HER2-overexpressing BT-474 cell line (Figure 4). The mechanism of action of these mAbs on HER2 downstream signaling molecules including AKT and ERK has not yet been investigated. PI3K/AKT and MAPK/ERK are recognized as the two major HER2 signalling pathways (Nahta et al., 2004), which play important roles in cell survival and proliferation (Balmanno and Cook, 2009).

In this study, we evaluated the effect of 1T0, 2A8 and 1H9 mAbs individually and in combination with trastuzumab on AKT and ERK signaling pathways. Our results showed that 1T0 mAb, as opposed to trastuzumab, significantly inhibits both AKT and particularly ERK phosphorylation (Figure1, Table 1). The second inhibitory mAb (2A8) could induce inhibition only on AKT ($P<0.001$), but not ERK phosphorylation. No significant effect, however, was found for the stimulatory mAb 1H9 on either AKT or ERK phosphorylation. The two commercial therapeutic mAbs, trastuzumab and pertuzumab, individually either failed or induced marginal effect on these two signaling pathways, with the exception of ERK phosphorylation which was moderately inhibited ($p < 0.05$) by trastuzumab. The differential effects induced by different anti-HER2 mAbs might be associated to their fine specificity (Yip et al., 2003). Trastuzumab and pertuzumab recognize epitopes located on subdomain IV and II of the HER2 extracellular domain, respectively (Cho et al., 2003; Franklin et al., 2004). We have recently shown that our mAbs recognize distinct epitopes within subdomains I+II (1T0), III+IV (2A8) and IV (1H9) of HER2-ECD (Hosseini-Ghatar et al., 2013). Interference of these mAbs with HER2 dimerization, which is mediated by sequences within subdomain II (Rockberg et al., 2009), may partly explain their impact on ERK and AKT phosphorylation.

Trastuzumab has already been reported to inhibit AKT and ERK phosphorylation (Pedersen et al., 2015;

Dubská et al., 2005). Pertuzumab, on the other hand, was found to inhibit AKT phosphorylation in BT-474 cell line, with no substantial effect on ERK signaling pathway (Nahta et al., 2004). Yip et al., (2003) investigated the effects of stimulatory and inhibitory antibodies on ERK pathway and phospho-HER2 expression in BT-474 cell line. They demonstrated that the stimulatory antibody in contrast to the inhibitory mAb increased ERK phosphorylation. However, in our study, 1H9 as a stimulatory antibody, displayed no effect on AKT or ERK phosphorylation. Moreover, in combination with trastuzumab it moderately inhibited AKT, but not ERK phosphorylation, which indicates the dominant role of trastuzumab on ERK signaling. Such controversial results might be due to epitope specificity or duration of treatment of cells with different mAbs.

Interestingly, the combination of 1T0 with trastuzumab almost completely abrogated both AKT and ERK phosphorylation. This effect was much more significant than the combination of trastuzumab and pertuzumab. The latter two mAbs did not significantly affect ERK phosphorylation. Enhancement of the inhibitory effect on signaling pathways by a combination of inhibitory mAbs, as compared to individual mAb, has already been reported (Pedersen et al., 2015; Nahta et al., 2004; Ko et al., 2015). Engagement of multiple epitopes and domains may lead to more efficient blockade of HER2 dimerization and degradation. Different studies have shown that some inhibitory mAbs failed to downregulate HER2 expression (Pedersen et al., 2015; Harwerth et al., 1999). However, combination of two or three mAbs with non-competitive binding sites result in HER2 degradation receptor more efficiently than each mAb individually (Pedersen et al., 2015; Szymanska et al., 2016; Nahta et al., 2004; Kasprzyk et al., 1992). These findings are in parallel with our results, regarding synergistic degradation of HER2 by combination of 1T0 or pertuzumab with trastuzumab, as compared to each mAb alone.

These findings might have important clinical implications. Enhancement of the tumor growth inhibition by trastuzumab in combination with pertuzumab resulted in improved survival of breast cancer patients leading to approval of pertuzumab by FDA for treatment of HER2 positive breast cancer patients in combination with trastuzumab (Amiri-Kordestani et al., 2014; De Mattos-Arruda and Cortes, 2013; Metzger-Filho et al., 2013). Our inhibitory mAbs, particularly 1T0, displayed superior inhibitory effect both at tumor cell proliferation and AKT/ERK phosphorylation levels over trastuzumab and pertuzumab, individually. This together with its significantly higher inhibitory effect in combination with trastuzumab, as compared to the combination of trastuzumab and pertuzumab, suggest its therapeutic potential in HER2 overexpressing tumors either individually or as a better alternative to pertuzumab in combination with trastuzumab. This mAb has recently been chimerized (Amiri et al., 2013) and humanized (Amiri et al., 2017) and is currently being investigated in preclinical studies.

Acknowledgements

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