Co-targeting HER2/ErbB2 and insulin-like growth factor-1 receptors causes synergistic inhibition of growth in HER2-overexpressing breast cancer cells

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: The humanized anti-HER2 monoclonal antibody trastuzumab (Herceptin®) is useful in the treatment of ErbB2-overexpressing breast cancers, but its efficiency is limited because development of resistance is common. In order to study the possibility of improving the efficacy of therapies directed against HER2/erbB2, we investigated the effects of co-targeting this receptor and the insulin-like growth factor 1 receptor (IGF-1R), a widely-expressed protein tyrosine kinase with important roles in suppression of apoptosis and stimulation of proliferation.

Material/Methods: The experimental strategy involved combining trastuzumab treatment and reduction of IGF-1R signaling through incremental heat-induced expression of the dominant-negative IGF-1 receptor 486/STOP under the control of the heat-sensitive Drosophila HSP70 promoter, in HER2/erbB2-overexpressing MCF7her18 breast cancer cells.

Results: Isobologram analysis of combinatorial treatment data revealed a strong synergistic interaction between trastuzumab treatment and the induction of the dominant-negative IGF-1R expression, resulting in potentiation of growth inhibition in transfected cancer cells.

Conclusions: These observations support the concept that simultaneously co-targeting tyrosine kinase receptors may be therapeutically useful, and provide a specific rationale for combining IGF-1R and HER2/erbB2 targeting strategies in anti-neoplastic approaches.

key words: **IGF-1R • HER2/ErbB2 • Trastuzumab • Herceptin • co-targeting • synergy**

Abbreviations: EGFR – epidermal growth factor receptor; HER2/ErbB2 – human erbB-related/erythroblastosis virus gene B; IGF-1R – insulin-like growth factor-1 receptor; MAPK – mitogen-activated protein kinase; P1-3K – phosphatidyl-inositol 3-kinase; PTK – protein tyrosine kinase; RT – reverse transcriptase

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BACKGROUND

A promising field of oncological study has been opened by the elucidation of the crucial role of protein tyrosine kinases (PTKs) in signal transduction, and by the discovery that PTK signalling is frequently abnormal in cancer. Many novel approaches in antineoplastic therapy take aim at PTK functions [1], and one receptor in particular, HER2/erbB2 (also known as EGFR2 or *neu*) is an excellent clinical target because its frequent overexpression in several types of cancer is associated with poor prognosis [2]. In contrast to other members of the EGFR family, HER2/erbB2 does not seem to possess its own high-affinity ligand, but forms with other ligand-bound EGFR family members a variety of active heterodimers which send growth signals through the MAPK and PI-3K pathways and lead to increased cell proliferation and malignancy progression [2]. In addition, when overexpressed, HER2/erbB2 self-associates into homodimers and becomes constitutively activated [3]. Cancer cells overexpressing HER2 are often resistant to many cytotoxic drugs and radiation [4] and experimental approaches targeting the HER2 signal pathway have focused on specific inhibitors such as the humanized monoclonal antibody trastuzumab (Herceptin®, Genentech, South San Francisco, CA), which has shown clinical activity against breast tumours overexpressing HER2/erbB2. [5]. The precise mode of action of trastuzumab is not completely understood but involves HER2 protein downregulation, inhibition of heterodimer formation and HER2 cleavage, G1 arrest, p27kip1 induction, prevention of angiogenesis and Fc-receptor-dependent mechanisms [6,7]. Unfortunately, patients respond to trastuzumab for only a limited period of time before resistance develops [8], and recent studies have suggested that a combination of treatments simultaneously targeting more than one PTK receptor may result in antineoplastic activity greater than that seen with single agent therapy [9–14].

A potential PTK receptor candidate for such a co-targeting approach is the insulin-like growth factor receptor (IGF-1R), which is widely expressed in human tissues and has a crucial role in organ development, mitogenesis, and cellular survival [15]. Studies involving IGF-1R-blocking techniques and IGF-1R overexpression point to a link between the receptor and transformation [16]. Experimental neoplasms have been growth-inhibited by anti-IGF strategies [16,17] and extension of this research to patients is provided by a recent report of activity of an IGF-1R antisense oligonucleotide used *ex vivo* [18]. Through a wide variety of cellular factors involving at least the PI-3K and MAPK major cell proliferation routes and probably 14.3.3/Raf as a third pathway [19], IGF-1R signalling leads to suppression of apoptosis and stimulation of proliferation, and can therefore be described as an important cell survival cascade. We reported that an increased level of IGF-1R signaling interferes with inhibition of breast cancer cell proliferation by trastuzumab [10] which raises the possibility that a co-targeting of IGF-1R and HER2 may be of therapeutic value. In this study, we use trastuzumab and a temperature-inducible dominant-negative IGF-1 receptor to study the effects of combined targeting of HER2 and IGF-1R. We provide the first evidence using quantitative isobologram analysis for synergistic interaction between two therapies that target distinct tyrosine kinase receptors.

MATERIAL AND METHODS

Cell cultures and reagents

Human breast cancer MCF7her18 cells overexpressing the HER2/erbB2 receptor (gift from Dr M. Alaoui-Jamali, Lady Davis Institute, McGill University, Montreal) were cultured at 37°C (except where indicated) with 5% CO2 in RPMI 1640 medium containing 25 mM HEPES, L-glutamine, 10% fetal bovine serum, and 0.7 mg/ml Geneticin (G-418 sulfate) (InVitrogen). Trastuzumab (Herceptin®) was purchased as a lyophilized powder from Roche Pharmaceuticals. The pGR228 plasmid [20] was a gift from Dr R. Baserga, Jefferson Cancer Center, Thomas Jefferson University, Philadelphia. The plasmid contains a truncated IGF-1 receptor sequence (the first 486 a.a. of the IGF-1R α-chain) under the control of the *Drosophila* HSP70 heat-

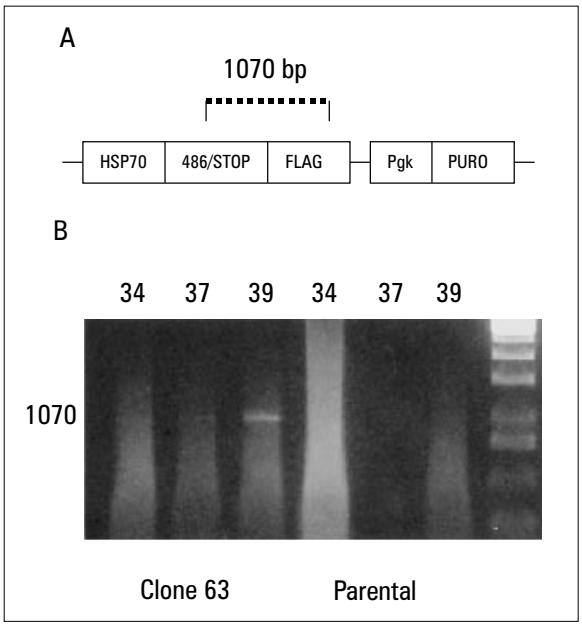

Figure 1. Temperature-dependent expression of 486/STOP dominant-negative. (A): Partial diagram of pGR228 plasmid used for transfection of MCF7her18 cells (Dr. R. Baserga, personal communication). HSP70: *Drosophila* Hsp70 heat-inducible promoter. 486/STOP: 3' truncated α-chain of IGF-1R. FLAG: oligopeptide epitope. Pgk: temperature-independent promoter. Puro: puromycin selectable marker. The dotted line corresponds to the RT-PCR-amplified fragment seen in Fig 1B. (B): RT-PCR amplification of the truncated dominant-negative transcript in cells exposed to various temperatures for 3 days. The PCR reaction used an IGF-1R-specific upstream primer and a FLAG-specific downstream primer. Left: expression pattern for 486/STOP (clone 63) cells at 34°, 37° and 39.5°C. Right, expression pattern for parental MCF7-her18 cells.

inducible promoter. The shortened IGF-1R sequence also has a C-terminus FLAG epitope (Fig 1A). MCF7her18 cells were transfected with pGR228 using a standard $CaCl_2$ protocol; permanent transfectants resistant to both G418 (0.7 mg/ml) and puromycin (1 ug/ml) were isolated and characterised, and clone 58 and 63 chosen as typical MCF7her18-486/STOP lines.

Cell growth estimation

Anchorage-independent growth conditions were achieved by using standard culture plasticware coated with polyHema (Sigma) [21]. Briefly, a 1.2% solution of polyHema in 95% ethanol was uniformly spread inside culture wells under sterile conditions, the plates were allowed to dry, and were re-coated with a final 6% polyHema solution in ethanol. Dried plates were rinsed with sterile phosphate-buffered saline immediately before use. Double-coating ensures complete absence of cell attachment to plastic surfaces. Cell counts were obtained after gentle centrifugation of the non-adherent cultures followed by resuspension of the pellets in trypsin-EDTA with 0.02% trypan blue, then hemacytometer counting. MCF7her18 cells and transfected lines grow in loose clumps and come apart easily in trypsin with gentle pipetting.

RT-PCR

The following oligonucleotides were designed as primers to amplify a 1070-bp which exists only in pGR228 plasmid DNA: an IGF-1R-specific upstream primer (20-mer) 5'GGA TGC GGT GTC CAA TAA CT 3', and a FLAG-specific downstream primer (21-mer) 5'CTT GTC GTC ATC GTC CTT GTA 3'. Parental (MCF7-her18) and 486/STOP transfected (clone 63) cell lines were grown at various temperatures for 24 hours, and RNA was extracted by the RNA-B method (Tel-Test, Friendswood, Texas). Reverse transcriptase (RT) reactions were conducted as per standard protocols using 1 μg total RNA, oligo(dT) primers and M-MLV reverse transcriptase (InVitrogen) for 1 h at 37°C. PCR reactions were conducted on 1/10 volume of the RT reactions with the IGF-1R and FLAG-specific primers described above. PCR conditions were: denaturation at 94°C 30 sec, annealing at 57°C 30 sec, elongation at 72°C 1.5 min, for 35 cycles, followed by one final 10 min elongation at 72°C. Because of the FLAG-specific 3' primer, no wild-type IGF-1R sequence can be amplified, and only transcripts from the truncated 486/STOP will appear at the expected 1070 bp size.

Isobolograms and statistical analysis

Interactions between treatment with trastuzumab and 486/STOP expression were analysed by the method of Berenbaum [22]. Briefly, IC_{50} values were established for the two agents individually, and combination experiments using 6 doses of each agent provided a matrix from which equi-effective dose combinations (isoboles) could be plotted. If there are no interactions between the agents (i.e. the effects show simple additivity), the graphical representation for all combinations giving the same growth effect is a straight line. Deviation from the straight line to the right indicates antagonism between the agents, deviation to the left indicates synergy [22, 23]. Data from cell growth experiments are presented as arithmetic means ± S.D. Statistical analysis was conducted using the two-way ANOVA test. p values smaller than 0.05 are considered significant.

RESULTS

Temperature-dependent expression of 486/STOP dominant-negative

All MCF7her18 breast cancer cell lines transfected with plasmid pGR228 and resistant to both puromycin and G418 (MCF7her18-486/STOP) were shown by PCR to possess the specific truncated IGF-1R-FLAG construct in their genomic DNA (not shown). RT-PCR conducted with a 3' IGF-1R-specific and a 5' FLAG-specific primer on RNA from transfected cells incubated at permissive and non-permissive temperatures demonstrated that expression of the truncated dominant-negative occurs at elevated temperatures only (Fig 1 B). A very low level of expression was observed at 34° and 37°C and is likely due to a serum component-induced activation of the promoter. Parental cells (MCF7her18) present negative patterns.

Proliferation characteristics of cell lines inducibly expressing the 486/STOP dominant-negative IGF-1R

Parental cells and selected transfected lines were tested for growth characteristics under anchorage-independent conditions (polyHema-coated plasticware) [21], and were observed to resist anoikis (suspension-induced cell death) under control conditions over more than 3 days. Anchorage-independent conditions are used here, as the role of IGF-1R in stimulating non-adherent growth conditions is greater that that seen in monolayer proliferation [24,25]. The *Drosophila* HSP70 heat-inducible promoter controlling the expression of the 486/STOP dominant negative IGF-1R was tested by exposing parental and transfected cells to continuous 34°C, 37°C or 39.5°C temperatures over 3 days. While the heat-dependent promoter is reported to be completely inactive at 34°C in cell types that can be maintained at that temperature [20], MCF7her18 and derived cell lines show negligible growth at 34°C and need warmer conditions. Proliferation curves reveal that growth of parental cell lines is not substantially affected (< 10% decrease) by raising the temperature from 37°C to 39.5°C over 3 days. In contrast, all MCF7her18-486/STOP lines tested show complete inhibition of proliferation after 3 days at 39.5°C (Fig. 2).

Synergistic effect on growth inhibition caused by trastuzumab and IGF-1R downregulation

In order to formally quantify the effect of simultaneous HER2 and IGF-1R co-targeting, we first established that the magnitude of the inhibition effect of the IGF-1R dominant-negative could be controlled through increas-

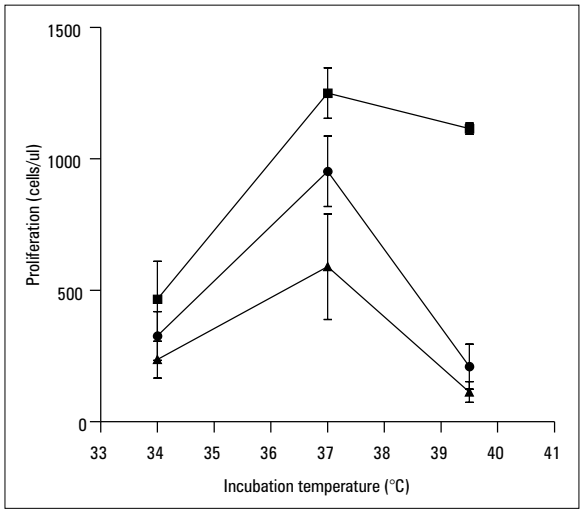

Figure 2. Temperature curve for anchorage-independent growth of parental MCF7her18 (squares), and two typical clones of MCF7her18-486/STOP cell lines, clone 58 (triangles) and clone 63 (circles). Cells were exposed to indicated temperatures for 72 hours in anchorage-independent conditions, in RPMI medium containing 5% FBS without G418 or puromycin. Cell counts were obtained by hemacytometer and proliferation is expressed as numbers per μl above original seeding. Data presented as arithmetic means±s.d. ($p<0.01$).

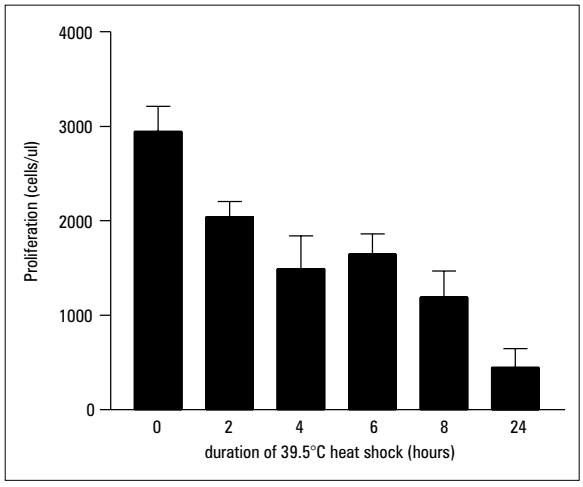

Figure 3. The effect of increasing heat shock duration on MCF7her18-486/STOP clone 63 proliferation. Cells in non-adherent cultures were exposed to 39.5°C for indicated periods of time, then transferred to 37°C for a total of 72 hours prior to counting with hemacytometer. Cell proliferation is expressed as numbers per μl above original seeding. ($p<0.01$). For comparison, parental MCF7her18 cells without the 486/STOP construct presented a less than 10% reduction in cell numbers after 3 days at 39.5°C with respect to 37°C (see fig 2).

ing doses of 39.5°C heat-shock. Figure 3 shows that an incremental series of exposures (ranging from 2 to 24 hours) to the permissive 39.5°C temperature, followed by growth at 37°C for a total of 72 h, caused a differential induction of the heat-shock promoter which is reflected in growth patterns. This property allowed the design of a combinatorial protocol involving increasing doses of trastuzumab and increasing levels of expression of IGF-1R 486/STOP, from which isobologram analysis for the determination of antagonism, additivity or synergy could be conducted [22,23]. MCF7her18-486/STOP clone 63 cells were transferred to polyHema-coated plates containing media with 1% FBS and 0, 2.5, 5, 10, 20 or 50 μg/ml trastuzumab. The suspensions were exposed to 39.5°C heat shocks for periods of 0, 2, 4, 6, 8 or 22 hours then transferred to 37°C incubation conditions for the rest of the 3-day period. For each experiment, 36 combinations were tested in duplicates or triplicates, and cell growth estimated by hemacytometer counting. Results provided a 36-point matrix from which curves could be plotted to derive the isobole for IC_{50} values. The IC_{50} for trastuzumab used as a single agent in cells grown in anchorage-independent conditions for 3 days at 37°C was established to be 48±5 μg/ml (not shown). Isobologram analysis for growth in suspension of MCF7her18-486/STOP cells reveals a strong synergistic interaction between trastuzumab and 486/STOP expression (Fig. 4 shows a representative experiment).

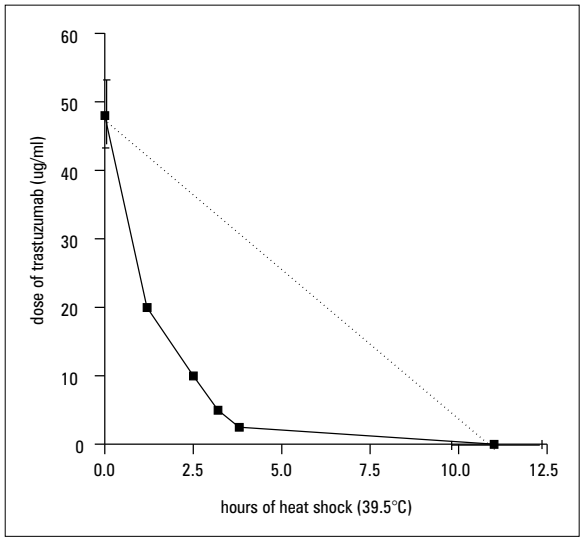

Figure 4. Synergistic inhibition of MCF7her18-486/STOP (clone 63) cell growth by trastuzumab and IGF-1R dominant-negative expression. Clone 63 cells were transferred to polyHema-coated plates containing media with 1% FBS and increasing doses of trastuzumab. The suspensions were exposed to 39.5°C heat shocks for periods of 0, 2, 4, 6, 8 or 22 hours then transferred to 37°C incubation conditions for the rest of the 3-day period. For each experiment, 36 combinations were tested in duplicates or triplicates, and cell proliferation estimated by hemacytometer counting. An isobologram [24,25] was constructed for analysis of the combinatorial treatments and was calculated for IC_{50} of agents individually. The observed deviation of the curve to the left of the line of additivity (dotted line) indicates a synergistic interaction between treatments.

Discussion

A common strategy for blocking the function of a given protein is the use of a sequence encoding an inactive

mutant product capable of inhibiting the wild-type protein function, i.e. a dominant-negative. In this study, a truncated IGF-1R dominant-negative construct (486/STOP) under the control of the *Drosophila* HSP70 heat-sensitive promoter [20] was used for controlled reduction of IGF-1R signalling. Expression of the 486/STOP shortened protein has been shown to decrease autophosphorylation of endogenous IGF-1 receptors in transfected cells, and is known to cause a blockage of cell proliferation [26,27]. Heat-dependent effects on cell growth in the absence of the 486/STOP construct are negligible in the conditions used here as shown by parental cell controls, and the complete growth inhibition observed at 39.5°C in all MCF7her18-486/STOP lines tested is therefore related to IGF-1R dominant-negative interference with cell metabolism. The use of heat shocks of various durations to incrementally induce the expression of genes under the control of heat-sensitive promoters has been reported before [28,29], and this property is used here to allow the design of a combinatorial protocol for isobologram analysis [24,25] which reveals a strong synergistic interaction between trastuzumab treatment and 486/STOP expression. Other synergistic interactions with trastuzumab have been observed for drugs such as cisplatin, docetaxel, thiotepa, and etoposide [30], but this is the first report of synergistic inhibition of growth through interference with the HER2/ErbB2 and IGF-1R signaling pathways. In this type of study, a simple additivity pattern would imply that the agents are acting independently on different metabolic pathways, while synergy (potentiation of one agent on the action of another) suggests the existence of interactions between the pathways targeted. Trastuzumab allows $p27^{kip1}$ expression and facilitates its release from sequestering proteins; it also decreases the levels of cyclin E and of early and mid G1 cyclins, resulting in accumulation of cells in G1 phase [4,5]. IGF-1 has been demonstrated in MCF7 cells to increase cyclin D1 and 5 expression, and to post-transcriptionally decrease $p27^{kip1}$ levels, all of which facilitate passage from G1 to S phase [31]. Signaling pathways for HER2/ErbB2 and IGF-1R have been shown to cross-talk [32], and unpublished results from our laboratory suggest that IGF-1 antagonism to trastuzumab is effected through the targeting of $p27^{kip1}$ to the proteasome degradation machinery (Lu et al, unpublished). It is therefore likely that the synergistic action of trastuzumab and 486/STOP treatments involves potentiation of this cell cycle control step towards the significant growth inhibition observed in this study.

CONCLUSIONS

Our results represent the first report of formal isobologram analysis for evaluating antagonism, additivity or synergy between treatments targeting two tyrosine kinase receptors. The finding of synergy confirms that there are benefits to co-targeting approaches, and more specifically, that the antineoplastic effects of blocking tyrosine kinase receptors of the EGF family may be enhanced by co-targeting IGF-1R.

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