Pharmacological blockade of Fatty Acid Synthase (FASN) reverses acquired autoresistance to trastuzumab (Herceptin™) by transcriptionally inhibiting 'HER2 super-expression' occurring in high-dose trastuzumab-conditioned SKBR3/Tzb100 breast cancer cells

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Abstract. Elucidating the mechanisms underlying resistance to the human epidermal growth factor receptor 2 (HER2)-targeted antibody trastuzumab (Tzb; Herceptin™) is a major challenge that is beginning to be addressed. This dilemma is becoming increasingly important as recent studies strongly support a role for Tzb in the adjuvant setting for HER2-overexpressing early-stage breast cancers. We previously reported that pharmacological and RNA interference-induced inhibition of tumor-associated fatty acid synthase (FASN; Oncogenic antigen-519), a key metabolic enzyme catalyzing the synthesis of long-chain saturated fatty acids, drastically down-regulates HER2 expression in human breast cancer cells bearing HER2 gene amplification. Given that FASN blockade was found to suppress HER2 overexpression by attenuating the promoter activity of the HER2 gene, we here envisioned that this mechanism of action may represent a valuable strategy in breast cancers that have progressed while under Tzb. We created a preclinical model of Tzb resistance by continuously growing HER2-overexpressing SKBR3 breast cancer cells in the presence of clinically relevant concentrations of Tzb (20-185 μg/ml Tzb). This pool of Tzb-conditioned SKBR3 cells, which optimally grows now in the presence of 100 μg/ml trastuzumab (SKBR3/Tzb100 cells), exhibited HER2 levels notably higher (~2-fold) than those found in SKBR3 parental cells. Real-time polymerase chain reaction studies showed that up-regulation of HER2 mRNA levels closely correlated with HER2 protein up-regulation in SKBR3/Tzb100 cells, thus suggesting that 'HER2 super-expression' upon acquisition of autoresistance to Tzb resulted, at least in part, from up-regulatory effects in the transcriptional rate of the HER2 gene. SKBR3/Tzb100 cells did not exhibit cross-resistance to C75, a small-compound specifically inhibiting FASN activity. On the contrary, SKBR3/Tzb100 cells showed a remarkably increased sensitivity (~3-fold) to the cytotoxic effects occurring upon C75-induced inhibition of FASN enzymatic activity. Both HER2 mRNA and HER2 protein 'super-expression', which have not been reported in earlier Tzb-resistant breast cancer models, were entirely suppressed following pharmacological blockade of FASN activity. Moreover, while Tzb was still able to reduce HER2 protein expression by ~20% in SKBR/Tzb100 cells, C75 and Tzb co-exposure synergistically down-regulated HER2 protein levels by >85%. The nature of the interaction between Tzb and C75 in Tzb-resistant SKBR3/Tzb100 cells was also found to be strongly synergistic when analyzing the extent of apoptotic cell death using ELISA-based detection of histone-associated DNA fragments. In summary, a) the molecular mechanism(s) contributing to Tzb resistance in our SKBR3/Tzb100 model appear to be clearly different to those previously reported as we found important transcriptional up-regulatory transcriptional changes in HER2 gene expression levels relative to parental cells; b) since FASN inhibition acts on HER2 gene expression via reduction of its transcription rate, Tzb-conditioned HER2-overexpressing breast cancer cells not only retain but further gain sensitivity to FASN inhibition; and c) transcriptional suppression of HER2 expression using FASN blockers may represent a new molecular strategy in the management of Tzb-resistant breast cancer disease.

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Introduction

Experimental studies demonstrating that high levels of HER2 (*erb*B-2) transform cultured cells as well as clinical studies showing poorer long-term survival rates for patients whose tumors overexpress HER2 implied that HER2 should be suitable as a therapeutic target (1-6). While various approaches have been taken to accomplish this goal, the most prominent strategy for the treatment of HER2-overexpressing carcinomas has involved antibody-targeting of the extracellular domain

(ECD) of HER2 (6-11). Trastuzumab (Tzb; Herceptin™), a humanized monoclonal antibody IgG1 binding with high affinity to the ECD of HER2, has likewise demonstrated clinical activity in a subset of HER2-positive breast cancer patients (12-14). Unfortunately, not all HER2-overexpressing human cancer cells respond to treatment with Tzb and its clinical benefit is limited by the fact that resistance develops rapidly in virtually all Tzb-treated patients (15-18). Indeed, the majority of patients who achieve an initial response to Tzb develop resistance within 1 year. As reported by Nahta and Esteva, multiple preclinical studies and some clinical evidence have revealed several molecular mechanisms that could contribute to the development of Tzb resistance (15,17,18). Nonetheless, unraveling the ultimate mechanisms underlying resistance to Tzb-based therapy in human cancer is a major challenge that is beginning to be addressed, and this dilemma is becoming increasingly important as recent studies strongly support a role for Tzb in the adjuvant setting for HER2-overexpressing early-stage breast cancers (19-21).

Previous studies from our group revealed that pharmacological blockade of FASN activity using the natural compound cerulenin, the cerulenin-derived semi-synthetic anti-metabolite C75, or the ß-lactone Orlistat, significantly repressed HER2 protein expression in cancer cells naturally bearing HER2 gene amplification (22-29). Real-time polymerase chain reaction (RT-PCR) studies showed that down-regulation of HER2 mRNA levels closely correlated with FASN blockade-induced reduction of HER2 protein expression, thus suggesting that changes in HER2 oncogene expression upon pharmacological inhibition of FASN activity resulted from effects at the transcriptional level (22). Indeed, FASN inhibition was found to up-regulate the expression of the Ets class transcription factor PEA3, a potent trans-repressor of HER2 promoter activity that specifically reverses the *in vitro* transformed phenotype of HER2-overexpressing cancer cells and inhibits HER2-induced tumorigenesis *in vivo* (30-33). When transient transfection experiments with a Luciferase reporter gene driven by the HER2 promoter were performed in HER2-overexpressing breast cancer cells, treatment with chemical FASN blockers such as C75 profoundly repressed the constitutive hyperactivity of the HER2 gene promoter (25-28). When these anti-HER2 effects of FASN inhibitors and those obtained following suppression of FASN gene expression using the highly specific mechanism of RNA interference (RNAi) were compared for the ability to knock-down the transcriptional activation of the HER2 gene, RNAi-mediated silencing of FASN likewise reduced HER2 promoter activity, decreased HER2 mRNA transcripts and suppressed HER2 protein overexpression, while significantly up-regulating PEA3 expression. Moreover, when the effects of FASN inhibition on the transcriptional activity of the HER2 gene were characterized using an HER2 promoter bearing a mutated PEA3 binding sequence, the Luciferase reporter gene driven by the PEA3 site-mutated sequence was not subject to negative regulation by either chemical FASN blockers or FASN-targeting RNAi (25-28).

The above findings, altogether, provided strong support to the notion that FASN blockade acts on HER2 oncogene expression via regulation of its transcription rate by inducing formation of inhibitory complexes between the PEA3 transcription factor and a positive regulatory PEA3-binding motif at the endogenous HER2 gene promoter. Considering that high levels of FASN expression have recently been positively related to HER2 overexpression in breast cancer specimens (34,35), a promising therapeutic approach against HER2-overexpressing breast carcinomas may combine FASN blockers, which could repress HER2 oncogene expression at the transcriptional level, and monoclonal antibodies to HER2, which target the ecto-domain of HER2 and promote its degradation (29). We here hypothesized that FASN-regulated activity of the endogenous HER2 gene promoter may represent an anti-HER2 strategy that should not be affected by the upstream mechanisms of resistance previously described for Tzb-based anti-HER2 immunotherapy (15-18).

Materials and methods

Drugs. Trastuzumab (Tzb; Herceptin™), kindly provided by Hospital Universitari de Girona Dr. Josep Trueta Pharmacy (Girona, Spain), was solubilized in bacteriostatic water for injection containing 1.1% benzyl alcohol (stock solution at 21 mg/ml), stored at 4˚C and used within one month. C75 was purchased from Alexis Biochemicals (San Diego, CA), dissolved in DMSO, and stored in the dark as stock solution (25 mg/ml) at -20˚C until utilization. For experimental use, Tzb and C75 were prepared freshly from stock solutions and diluted with growth medium. Control cells were cultured in medium containing the same concentration (v/v) as the experimental cultures with treatments. The vehicle solutions had no noticeable influence on the proliferation of experimental cells.

Cell culture. SKBR3 breast cancer cells were obtained from Dr H. Riese [Centro Nacional de Biotecnología (CNB), Madrid, Spain] and they were passaged in McCoy's 5 A medium (Gibco) containing 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, 1% sodium pyruvate, 50 U/ml penicillin and 50 μg/ml streptomycin. Cells were maintained at 37˚C in a humidified atmosphere of 95% air and 5% CO_2. Cells were screened periodically for *Mycoplasma* contamination.

Metabolic status assessment. The ability of C75 to modulate breast cancer cell sensitivity to Tzb was determined using a standard colorimetric MTT (3-4, 5-dimethylthiazol-2-yl-2, 5-diphenyl-tetrazolium bromide) reduction assay. Cells in exponential growth were harvested by trypsinization and seeded at a concentration of ~$2.5x10^3$ cells/200 μl/well into 96-well plates, and allowed an overnight period for attachment. Then the medium was removed and fresh medium along with various concentrations of Tzb, C75 or combinations of compounds, were added to cultures in parallel. Agents were studied in combination concurrently. Control cells without agents were cultured using the same conditions with comparable media changes. Compounds were not renewed during the entire period of cell exposure. Following treatment, the medium was removed and replaced by fresh drug-free medium (100 μl/well), and MTT (5 mg/ml in PBS) was added to each well at a 1/10 volume. After incubation for 2-3 h at 37˚C, the supernatants were carefully aspirated, 100 μl of DMSO was added to each well, and the plates were agitated to dissolve the crystal product. Absorbances were measured at 570 nm using a multi-

well plate reader (Model Anthos Labtec 2010 1.7 reader). The cell viability effects from exposure of cells to each compound alone and their combination were analyzed as percentages of the control cell absorbances, which were obtained from control wells treated with appropriate concentrations of the compound vehicles that were processed simultaneously. For each treatment, cell viability was evaluated as a percentage using the following equation: (A570 of treated sample/A570 of untreated sample) x100.

Tzb sensitivity was expressed in terms of the concentration of drug required to decrease by 30% cell viability (IC_{30}). Since the percentage of control absorbance was considered to be the surviving fraction of cells, the IC_{30} value was defined as the concentration of Tzb that produced a 30% reduction in control absorbance (by interpolation). The degree of sensitization to Tzb by C75 was evaluated by dividing IC_{30} values of control cells by those obtained when cells were simultaneously exposed to C75.

HER2- and EGFR-specific ELISAs. Determination of HER2 and EGFR protein content was performed with commercially available quantitative ELISAs (Oncogene Science, Bayer Diagnostics) according to the manufacturer's protocol. To assess the effects of Tzb and/or C75 on HER2 and EGFR protein concentrations, breast cancer cells, after a 24-h starvation period in media without serum, were incubated with Tzb, C75 or combinations of these compounds as specified. After treatment, cells were washed twice with cold-PBS and then lysed in buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM ß-glycerolphosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonylfluoride) for 30 min on ice. The lysates were cleared by centrifugation in an Eppendorff tube (15 min at 14,000 x g, 4˚C). Protein content was determined against a standardized control using the Pierce protein assay kit (Rockford, IL, USA).

Total cell lysates (1:50, 1:500; 1:5,000 and 1:10,000 dilutions) and conditioned medium from Tzb-, C75- and Tzb + C75-treated and control untreated SK-Br3 and SK-Br3/Tzb100 breast cancer cells were used to quantitate HER2 and EGFR protein expression in cell cultures. A standard curve was generated by using standard solutions as per the manufacturer's instructions. The concentrations of HER2 and EGFR in test samples (in nanograms of HER2 and EGFR per milligram of total protein) were determined by interpolation of the sample absorbances from the standard curve. Each experiment was performed in duplicate wells.

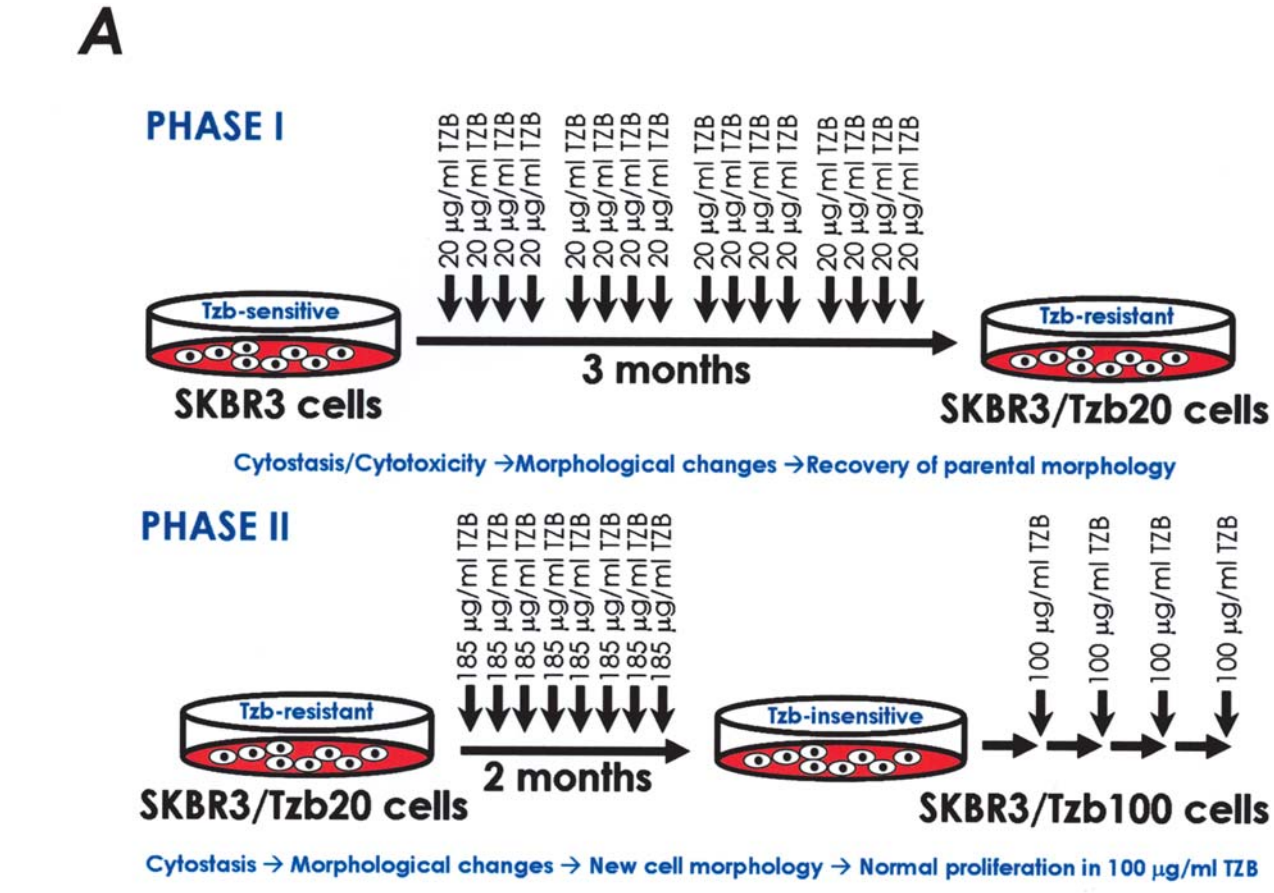
Semi-quantitative RT-PCR analysis of HER2 mRNA expression. Following treatments with Tzb, C75 or Tzb + C75, cells were harvested with Ultraspec™ RNA and total RNA was isolated according to the manufacturer's instructions (Biotecx Laboratories Inc., Madrid, Spain). Of total RNA, 1 μg was reverse transcribed (RT) and amplified (PCR) by an Access RT-PCR system (Promega) using 1 mM of specific primers for HER2 (sense: 5'-GGGCTGGCCCGATGTATTTGAT-3'; antisense: 5'-ATAGAGGTTGTCGAAGGCTGGGC-3'). As an internal control, ß-actin was used. The RT reaction was carried out for 45 min at 48˚C. HER2 and ß-actin cDNAs were amplified with 20 cycles to ensure that the PCR reactions were performed in the linear range using the following PCR profile: 96˚C for 30 sec, 60˚C for 1 min and 68˚C for 2 min. The PCR products were separated on a 2% agarose gel and detected by ethidium bromide staining.

Apoptosis. The induction of apoptosis was assessed using the Cell Death Detection ELISAPLUS kit obtained from Roche Diagnostics (Barcelona, Spain). This kit uses a photometric enzyme immunoassay that quantitatively determines the formation of cytoplasmic histone-associated DNA fragments (mono- and oligo-nucleosomes) after apoptotic cell death. For determination of apoptosis by ELISA, cells were treated with Tzb, C75 or Tzb + C75 for 72 h in 96-well plates. The induction of apoptosis was evaluated using cytosolic fractions obtained from pooled adherent and floating cells by assessing the enrichment of nucleosomes in the cytoplasm (by using anti-histone biotin and anti-DNA peroxidase antibodies). Briefly, the 96-well plates were centrifuged (200 x g) for 10 min. The supernatant was discharged, lysis buffer was added, and samples were incubated at room temperature (RT) for 30 min as described in the manufacturer's protocol. Anti-histone biotin and anti-DNA peroxidase antibodies were added to each well and incubated at RT for 2 h. After three washes, the peroxidase substrate was added to each well, and the plates were read at 405 nm at multiple time intervals. The enrichment of histone-DNA fragments in treated cells was expressed as fold increase in absorbance as compared with control (vehicle-treated) cells.

Statistics. Two-group comparisons were performed by the Student t-test for paired and unpaired values. Comparisons of means of ≥3 groups were performed by ANOVA, and the existence of individual differences, in the case of significant F values at ANOVA, was tested by Scheffé's multiple contrasts.

Results

Development of high-dose trastuzumab-conditioned (resistant) SKBR3 breast cancer cells (SKBR3/Tzb100 cells). In order to generate a preclinical model of acquired resistance to Tzb, SKBR3 cells, a widely used tumor cell *in vitro* model characterized by exhibiting natural HER2 gene amplification, HER2 receptor protein overexpression and HER2-dependency for cell proliferation and survival (36), were exposed for approximately 3 months to 20 μg/ml Tzb (Fig. 1A, top panel). At this point and as previously reported by Nahta *et al* (37), cells regained a morphology similar to the parental line. SKBR3/Tzb20 cells were then continuously grown in culture medium supplemented with 185 μg/ml Tzb over a period time of at least two months (Fig. 1A, bottom panel). These doses were chosen because clinical trials in humans have used concentrations of ~20 μg/ml Tzb and peak plasma concentrations of 185 μg/ml Tzb (38). Cells are now maintained in 100 μg/ml Tzb, a concentration of Tzb at which parental cells are strongly impaired in their metabolic status (Fig. 1B, left panel). Indeed, when sensitivity to Tzb was determined by treating SKBR3 and SKBR/Tzb100 cells with serial dilutions of the antibody for 3 days and growth inhibition was measured using a tetrazolium salt (MTT)-based quantification of metabolically viable cells, the IC_{30} (30% growth inhibitory) value for Tzb was found to significantly



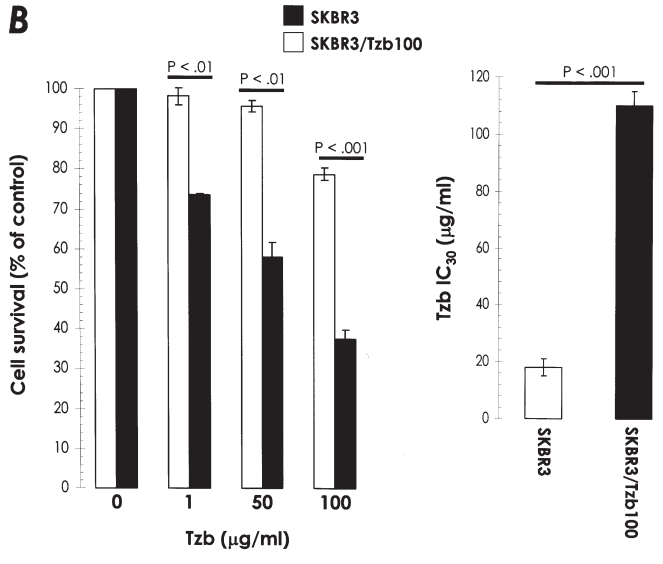


Figure 1. (A) Scheme for development of a new model of breast cancer autoresistance to Tzb. (B) Chronic exposure to Tzb significantly decreased Tzb efficacy in HER2-overexpressing SKBR3 breast cancer cells. (Left) Dose response curves of Tzb-conditioned SKBR3/Tzb100 cells and Tzb-sensitive SKBR3 parental cells to graded concentrations of Tzb (i.e., 1, 10 and 100 μg/ml Tzb). Briefly, cells seeded in 96-well plates (2,000-3,000 per well) were cultured in triplicate in the absence or presence of graded concentrations of Tzb, which was not renewed during the entire period of cell exposure. Once control (untreated) wells reached confluency, cells were exposed to MTT reagent and optical density at 570 nm was measured in a microplate reader. The cell viability effects from exposure of cells to Tzb were analysed by generating concentration-effect curves as a plot of the fraction of unaffected (surviving) cells versus Tzb concentration. Dose-response curves were plotted as percentages of the control cells' absorbance (=100%), which was obtained from wells treated with appropriate concentrations (bacteriostatic water v/v) of Tzb vehicle that were processed simultaneously. Values shown are means (columns) and 95% confidence intervals (bars) of three independent experiments carried out in triplicate for each concentration. (Right) IC_{50} values were designated for the concentrations of Tzb (in μg/ml) decreasing absorbance values at 570 nm by 50%, as determined by interpolation using the MTT-based colorimetric growth viability assay (see Materials and methods). Values shown are means (columns) and 95% confidence intervals (bars) of three independent experiments.

increase from 18±3 μg/ml in Tzb-sensitive SKBR3 parental cells to 110±5 μg/ml in Tzb-conditioned (high-dose resistant) SKBR3/Tzb100 cells (Fig. 1B, right panel).

Tzb-sensitive SKBR3 control cells (Fig. 2A) and Tzb-conditioned SKBR3/Tzb100 cells (Fig. 2B) were growth-inhibited in a dose-dependent fashion following the addition of graded concentrations of the FASN inhibitor C75. Interestingly, high-dose Tzb-conditioned SKBR3/Tzb100 cells showed a remarkably increased sensitivity (~3-fold) to the cytotoxic effects occurring upon C75-induced inhibition of FASN enzymatic activity (Fig. 2B, right panel). These results reveal that, upon acquisition of Tzb resistance, SKBR3 breast cancer does not exhibit cross-resistance to chemical FASN inhibitors but rather gains sensitivity to FASN inhibition.

FASN inhibition reverted HER2 'super-expression' in Tzb-resistant breast cancer cells. We previously demonstrated that the presence of high levels of HER2, rather than FASN over-expression, is a/the molecular determinant for hypersensitivity to FASN blocker-induced cytotoxicity in human breast cancer

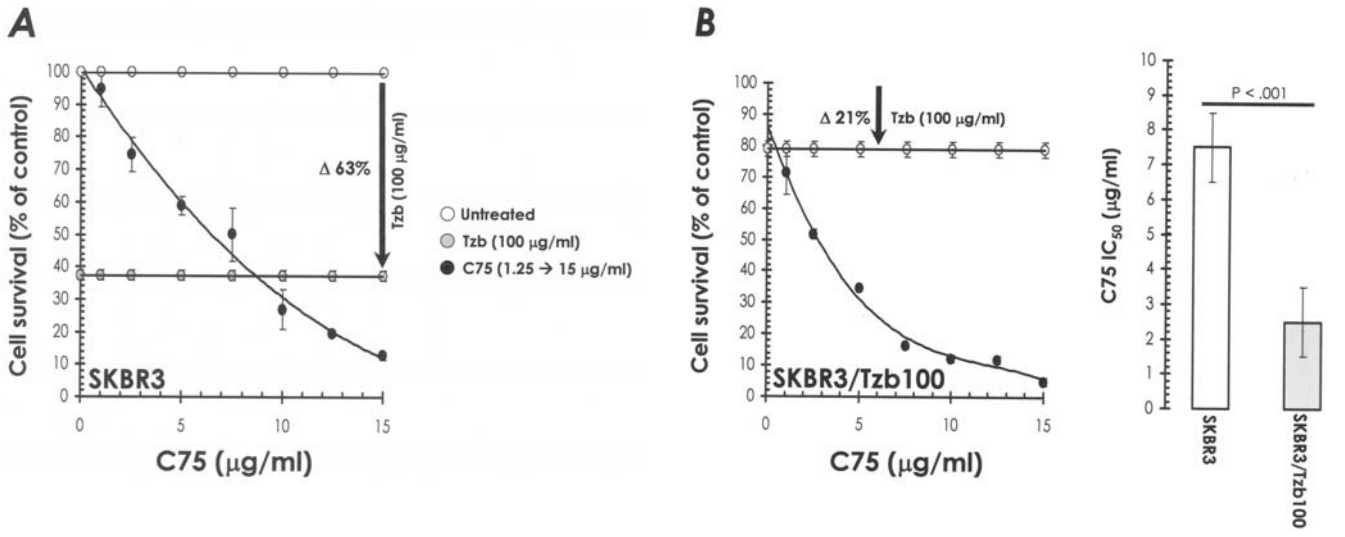


Figure 2. Tzb-resistant SKBR3/Tzb100 cells did not exhibit cross-resistance to the FASN inhibitor C75. Dose response curves of Tzb-sensitive SKBR3 parental cells (A) and Tzb-conditioned SKBR3/Tzb100 cells (B) to graded concentrations of the FASN blocker C75. Briefly, cells seeded in 96-well plates (2,000-3,000 per well) were cultured in triplicate in the absence or presence of graded concentrations of C75, which was not renewed during the entire period of cell exposure. Once control (untreated) wells reached confluency, cells were exposed to MTT reagent and optical density at 570 nm was measured in a microplate reader. The cell viability effects from exposure of cells to C75 were analysed by generating concentration-effect curves as a plot of the fraction of unaffected (surviving) cells versus C75 concentration. Dose-response curves were plotted as percentages of the control cells' absorbance (=100%), which was obtained from wells treated with appropriate concentrations (DMSO v/v) of C75 vehicle that were processed simultaneously. Values shown are means (columns) and 95% confidence intervals (bars) of three independent experiments performed in triplicate for each concentration. For comparison, the data also indicate the effects of 100 μg/ml Tzb on the cell viability of SKBR3 and SKBR3/Tzb100 cells. C75 IC_{50} values shown in panel B (right) were designated for the concentrations of C75 (in μg/ml) decreasing absorbance values at 570 nm by 50%, as determined by interpolation using the MTT-based colorimetric growth viability assay (see Materials and methods). Values shown are means (columns) and 95% confidence intervals (bars) of three independent experiments.

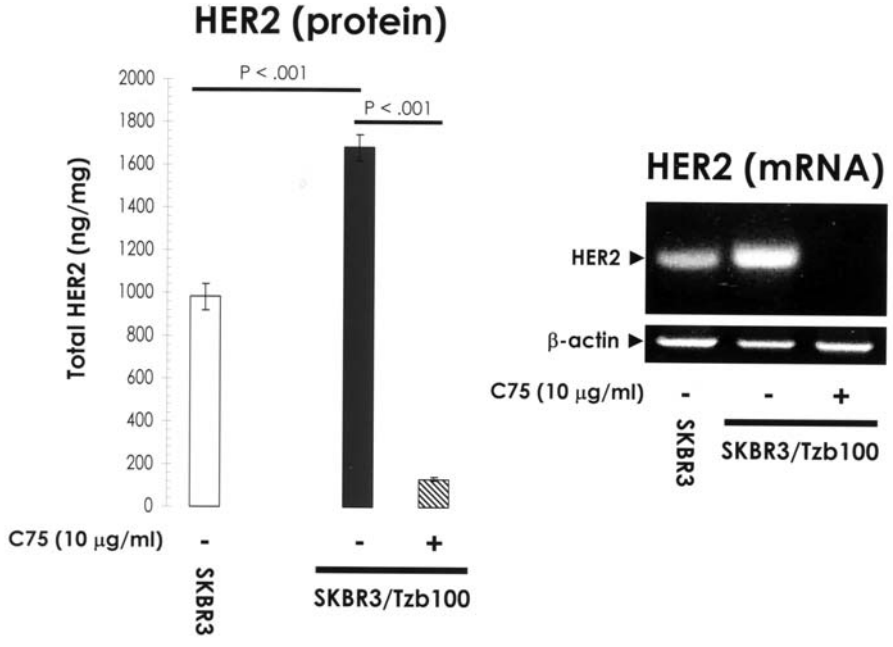


Figure 3. Chronic exposure to Tzb induces super-expression of HER2 at the transcriptional level. (Left) The Oncogene Science HER2 microtiter ELISA was used according to the manufacturer's instructions to compare HER2 concentrations in Tzb-sensitive SKBR3 parental cells and Tzb-conditioned SKBR3/Tzb100 cells in the absence or presence of 10 μg/ml C75. Results are means (columns) and 95% confidence intervals (bars) of three independent experiments performed in triplicate. One-factor ANOVA was used to analyze differences in the expression level of HER2 protein between the various treatment groups and the control cells. All statistical tests were two-sided. (Right) Of total RNA from SKBR3 and SKBR3/Tzb100 cells, 2 μg was subjected to RT-PCR analyses with specific oligos for HER2 and ß-actin as described in Materials and methods. A representative RT-PCR analysis is shown. Equivalent results were obtained in three independent experiments.

cells (23,27). Since FASN protein levels were not significantly up-regulated in Tzb-conditioned SKBR3/Tzb100 cells (data not shown), we speculated that C75 hypersensitivity occurring in SKBR3/Tzb100 cells might relate to changes in the expression levels of HER2. To test this hypothesis, HER2-specific ELISA assays were performed to quantitatively assess HER2 protein

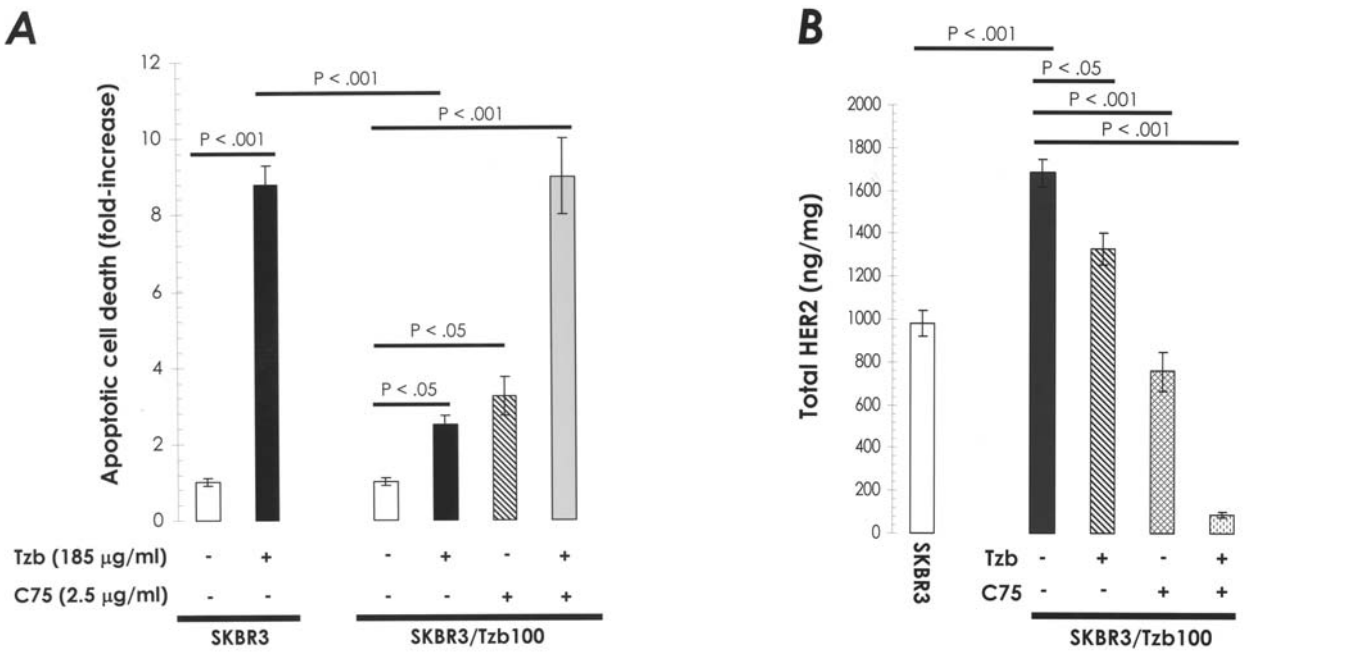


Figure 4. (A) Effects of C75-induced inhibition of FASN activity on Tzb-induced apoptotic cell death in Tzb-conditioned SKBR3/Tzb100 cells. Quantification of apoptosis-related cell death in Tzb-resistant SKBR3/Tzb100 cells treated with Tzb in the absence or presence of the FASN blocker C75 was determined by Cell Death ELISA as described in Materials and methods. The enrichment of histone-DNA fragments was expressed as fold-increase in absorbance by comparing with control (vehicle-treated) cells using the following formula: $(A_{405}\text{-}A_{490})$ treated/$(A_{405}\text{-}A_{490})$ untreated. Data are the mean (columns) and 95% confidence intervals (bars) of three independent experiments performed in duplicate. One-factor ANOVA was used to analyze differences in the percentage of apoptosis between the various treatment groups and the control group. All statistical tests were two-sided. (B) Effects of C75-induced inhibition of FASN activity on Tzb-induced down-regulation of HER2 in Tzb-conditioned SKBR3/Tzb100 cells. Overnight serum-starved SKBR3/Tzb100 cells were cultured in DMEM-0.1% FBS supplemented with 185 μg/ml Tzb in the absence or presence of 2.5 μg/ml C75 for 72 h. The Oncogene Science HER2 microtiter ELISA was used according to the manufacturer's instructions to compare HER2 concentrations in cell pellets. Results are means (columns) and 95% confidence intervals (bars) of three independent experiments performed in triplicate. One-factor ANOVA was used to analyze differences in the expression level of HER2 protein between the various treatment groups and the control cells. All statistical tests were two-sided.

levels in SKBR3/Tzb100 cells. This resistant pool, when maintained in the absence of Tzb for 72 h, likewise exhibited HER2 protein levels notably higher than those found in SKBR3 parental cells (~2-fold increase; Fig. 3, left panel). Since we previously demonstrated that Tzb treatment leads to transcriptional up-regulation of HER2 gene expression in SKBR3 cells (39), we here envisioned that chronic exposure to Tzb might also promote up-regulatory effects on the transcriptional rate of HER2 oncogene. Accordingly, RT-PCR analyses clearly revealed that Tzb-resistant SKBR3/Tzb100 cells exhibited higher levels of HER2 mRNA (~2 fold) when compared to Tzb-sensitive SKBR3 parental cells (Fig. 3, right panel). Interestingly, this transcriptional 'HER2 super-expression', which has not been reported in earlier Tzb-resistant breast cancer models (16-18), was decreased upon C75-induced inhibition of FASN activity to, and even below, the baseline mRNA HER2 levels found in Tzb-sensitive SKBR3 cells. Indeed, no mRNA HER2 signal was detected upon exposure to 10 μg/ml C75 (Fig. 3, left panel). At the protein level, treatment with 10 μg/ml C75 dramatically suppressed HER2 expression by 92% (Fig. 3, right panel).

Pharmacological blockade of FASN activity re-sensitizes SKBR3/Tzb100 cells to Tzb. We speculated that the lower sensitivity to Tzb observed in SKBR3/Tzb100 cells was not simply the result of changes in Tzb-insensitive cell proliferation, but might actually be due to a FASN-enhanced anti-apoptotic machinery. To address this question, cells were exposed to Tzb in the absence or presence of C75, apoptotic cell death was measured by a Cell Death ELISA that detects apoptosis-induced DNA-histone fragmentation, and the x-fold increase in apoptosis was calculated by comparing the ELISA optical density readings of treated samples, with the values of untreated cells as 1.0. Whereas SKBR3 parental cells were likewise exquisitely sensitive to the pro-apoptotic effects of Tzb (up to 8.8-fold increase versus 1.0-fold in untreated SKBR3 cells; Fig. 4A), Tzb-conditioned SKBR3/Tzb100 cells, which were maintained in the absence of Tzb for 72 h before the onset of treatments, exhibited a very low degree of apoptosis upon exposure to 100 μg/ml Tzb (up to 2.5-fold increase versus 1.0-fold in untreated SKBR3/Tzb100 cells). Low-dose C75 (i.e., 2.5 μg/ml), as a single agent, increased apoptotic cell death by 3.25-fold in SKBR3/Tzb100 cells. Interestingly, SKBR3/Tzb100 cells exhibited the highest extent of apoptotic cell death following concurrent exposure to Tzb and C75 (up to 9.0-fold increase versus 1.0-fold in untreated SKBR3/Tzb100 cells; Fig. 4A). When analyzing the anti-HER2 effects of combining Tzb and C75 we observed that, while Tzb treatment was still able to reduce 'HER2 super-expression' by 21% in SKBR3/Tzb100 cells, a reduction comparable to that observed in Tzb-sensitive SKBR3 parental cells, it drastically down-regulated HER2 expression by 95% when combined with a sub-optimal dose of C75 (i.e., 2.5 μg/ml), which, as a single agent, reduced 'HER2 super-expression' in SKBR3/Tzb100 cells by 55% (Fig. 4B). These results, altogether, strongly support the notion that pharmacological

blockade of FASN activity synergistically re-sensitizes Tzb-conditioned SKBR3/Tzb100 cells to the tumoricidal and anti-HER2 effects of Tzb.

Discussion

It is likely that combining the anti-HER2 monoclonal antibody Tzb with other biological agents and therapeutic agents for targeting HER2, including monoclonal antibodies, tyrosine kinase inhibitors (TKIs), and vaccines, should increase the magnitude and duration of breast cancer responses to Tzb. Alternatively, HER2 overexpression can also be repressed by attenuating the promoter activity of HER2. The rationale is that it will be more efficient to reduce HER2 levels by preventing the transcription of 2-10 gene copies than trying to neutralize up to 10^6 HER2 receptor molecules commonly found in HER2-overexpressing cancer cells. Few attempts, however, have been made to explore this promising anti-HER2 approach. One such strategy is based on the finding that the HER2 gene can be repressed by the adenovirus E1A gene (41,42). A phase I clinical trial of E1A therapy showed that intracavitary injection of the E1A gene complexed with DC-Chol cationic liposome (DCC-E1A; Targeted Genetics Corp., Seattle, WA, USA) was accompanied by HER2 down-regulation, increased apoptosis, and reduced proliferation in patients with breast cancer (43).

Another strategy based on the observation that over-expression of the Ets protein PEA3 reduced HER2 expression by transcriptionally repressing HER2 promoter activity, has successfully been examined in preclinical trials (30,32). Ovarian tumor-derived cell lines with either low expression or overexpression of HER2 growing as xenografts in nude mice were treated with daily injections of liposome-conjugated PEA3 expression plasmid (31). Significant reductions in tumor growth were observed in the HER2-overexpressing groups, with some mice surviving over a year, whereas the growth of the tumors with low expression was unaffected (31). PEA3 coupled with the liposome SN2 has recently been demonstrated to exhibit therapeutic effects in mice bearing tumors induced by breast cancer cells naturally exhibiting HER2 gene amplification and overexpression (44). Although the above results provide evidence for the antitumor activity of HER2 transcriptional repressors, systemic drugs or gene therapy vectors to specifically repress HER2 promoter activity need further evaluation before they can proceed to Tzb-based patient trials.

We recently hypothesized that cancer-associated FASN hyperactivity may play a key role determining the efficacy of Tzb against HER2-overexpressing cancer cells. For this purpose, we analyzed the cytotoxic interactions between chemical FASN blockers and Tzb in a panel of cancer cell lines naturally expressing high, moderate, and low levels of HER2. Pharmacological and siRNA-mediated inhibition of FASN activity was found to dramatically enhance the efficacy of Tzb in a dose-dependent manner. The most significant changes were seen in HER2- and FASN-overexpressing SKBR3 breast cancer cells, in which co-exposure to the FASN blocker cerulenin sensitized them to Tzb by 200-fold (22,25-27). Similarly, concurrent RNAi-induced attenuation of the FASN and HER2 genes synergistically induced apoptotic cell death in SKBR3 cells. These effects were accompanied by a dramatic suppression of HER2 overexpression, and the synergistic magnitude of this HER2 down-regulation related to the different anti-HER2 mechanisms of action of Tzb and FASN blockade. Thus, while Tzb targets the ECD of HER2 and promotes its degradation, FASN inhibition mitigates HER2 expression through the inhibitory binding of the transcriptional repressor PEA3 to the HER2 promoter (22,25-27). Considering that cancer-associated FASN acts on HER2 protein levels, at least in part, via regulation of the transcriptional activity of the HER2 gene through a positive regulatory PEA3-binding DNA motif at the HER2 promoter, we here hypothesized that this mechanism of action should not be affected by the mechanisms of resistance described for Tzb-based anti-HER2 immunotherapy. Interestingly, upon development of a new preclinical model of high-dose Tzb-conditioned SKBR3 cells, we found significant transcriptional up-regulatory changes in the expression levels of HER2-coded $p185^{HER2}$ oncoprotein in SK-Br3/Tzb100 cells relative to Tzb-sensitive parental cells. Supporting our hypothesis, Tzb-conditioned HER2-overexpressing breast cancer cells not only retained but further gained sensitivity to FASN inhibition; i.e., chronic exposure to Tzb appeared to trigger a significant increase in the ultimate mechanism of action (i.e., HER2 gene transcription) through which FASN inhibition shuts-down HER2 expression and, as a result, SKBR/Tzb100 cells become exquisitely sensitive to FASN inhibition. Indeed, when we analyzed both the anti-HER2 and the tumoricidal effects of combining FASN inhibitors and Tzb in Tzb-conditioned SKBR3/Tzb100 cells, both a synergistic down-regulation of HER2 and a synergistic enhancement of apoptotic cell death were noticeably observed.

This study not only supports the notion that FASN-driven cellular signaling actively participates in the maintenance and/or enhancement of HER2-induced malignant transformation (45,46), but further reveals for the first time that FASN blockade-induced transcriptional inhibition of HER2 expression may represent a new molecular strategy in the management of Tzb-resistant breast cancer disease.

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