Trastuzumab Down-Regulates Bcl-2 Expression and Potentiates Apoptosis Induction by Bcl-2/Bcl-X_L Bispecific Antisense Oligonucleotides in *HER-2* Gene–Amplified Breast Cancer Cells

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ABSTRACT

Purpose: **To investigate the possible existence of an antiapoptotic cross-talk between HER-2 and antiapoptotic Bcl-2 family members.**

Experimental Design: **Bcl-2 and Bcl-X_L expression and apoptosis induction were analyzed in *HER-2* gene–amplified (BT474) and nonamplified (ZR 75-1) breast cancer cell lines exposed to trastuzumab, alone or in combination with either Bcl-2/Bcl-X_L bispecific antisense oligonucleotides (AS-4625) or the small-molecule Bcl-2 antagonist HA14-1.**

Results: **In addition to HER-2 and epidermal growth factor receptor, trastuzumab down-regulated Bcl-2, but not Bcl-X_L, protein, and mRNA expression in BT474 cells. Interestingly, trastuzumab-induced down-regulation of HER-2 and Bcl-2 was also observed in three of five and two of three breast cancer patients undergoing trastuzumab treatment, respectively. Despite Bcl-2 down-regulation, however, trastuzumab only marginally increased the rate of apoptosis (7.3 ± 3.5%). We therefore investigated whether a combination of AS-4625 and trastuzumab might increase proapoptotic efficiency. AS-4625 treatment of BT474 cells decreased both Bcl-2 and Bcl-X_L expression, resulting in a 21 ± 7% net apoptosis induction; the combination of AS-4625 followed by trastuzumab resulted in a significantly stronger induction of apoptosis (37 ± 6%, $P < 0.01$) that was not observed with the reverse treatment sequence (trastuzumab followed by AS-4625). Similar results were obtained with the Bcl-2 antagonist HA14-1; indeed, exposure of BT474 cells to HA14-1 followed by trastuzumab resulted in a striking proapoptotic synergism (combination index = 0.58 ± 0.18), as assessed by isobologram analysis.**

Conclusions: **Altogether our findings suggest that combined targeting of HER-2 and Bcl-2 may represent a novel, rational approach to more effective breast cancer therapy.**

INTRODUCTION

Recognition of the oncogenic potential of the human epidermal growth factor receptor-2 (HER-2, also known as ErbB2) represents one of the major advances in the understanding of breast cancer biology and profoundly impacts clinical management of breast cancer patients (1). HER-2 gene amplification/protein overexpression occurs in approximately 30% of breast cancer cases and is frequently, although not invariably, associated with an aggressive, therapy-resistant phenotype and poor clinical outcome (1–3). Trastuzumab is a humanized monoclonal antibody that binds with high affinity to the ectodomain of the HER-2 protein and has consistently shown clinical activity in advanced breast cancer, either as a single agent or combined with standard chemotherapy regimens (4–6). Although trastuzumab approval for advanced breast cancer treatment represents an important addition to our therapeutic armamentarium, several issues remain unresolved: first, it is presently unknown which, if any, of the hypothesized mechanisms of action (reviewed in ref. 7) plays a major role in the clinical antitumor efficacy of trastuzumab; second, the majority of patients with advanced disease are resistant to trastuzumab (4) and essentially none of the initially sensitive patients are cured. Therefore, further insights into the mode of action of trastuzumab could ultimately lead to a more effective clinical application of this promising anticancer agent.

The relationships between HER-2 and Bcl-2 family members in the regulation of breast cancer cell survival and chemoresistance are presently unclear. The Bcl-2 family includes both pro- and antiapoptotic members that act as master regulators of mitochondrial homeostasis and cell survival/apoptosis (8–11). Despite ongoing controversy on their prognostic role in breast cancer (3, 12–14), Bcl-2 and Bcl-X_L have been consistently reported to be expressed in a high (~70%) proportion of breast cancer cases (12); in experimental breast cancer model systems, the expression of antiapoptotic Bcl-2 family members causes increased resistance to drug-induced apoptosis and en-

Received 5/8/04; revised 7/27/04; accepted 8/4/04.
Grant support: In part by grants from the Italian Ministry of Health (M. Milella, D. Del Bufalo, and M. Mottolese), the Italian Association for Cancer Research (M. Milella, D. Del Bufalo), and the Lega Italiana per la Lotta ai Tumori (M. Mottolese). D. Trisciuoglio is the recipient of a fellowship of the Italian Foundation for Cancer Research.
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hanced angiogenesis and metastatic potential (15–19) whereas their inhibition potently inhibits tumor growth, induces apoptosis, and sensitizes to the action of conventional cytotoxics both *in vitro* and *in vivo* (20–24). Thus, in breast cancer, as well as in many other malignancies, Bcl-2 and Bcl-X_L seem to be very promising targets for therapeutic intervention (25, 26). Indeed, simultaneous targeting of both Bcl-2 and Bcl-X_L by bispecific antisense oligonucleotides (AS; ref. 27) results in a profound inhibition of tumor growth and synergistically enhances the cytotoxicity of drugs commonly used in clinical breast cancer treatment (28, 29).

Whereas studies in clinical breast cancer series have documented an inverse relationship between the levels of HER-2 and Bcl-2 expression (30, 31), mechanistic studies have shown that *HER-2* gene transfer into MCF-7 breast cancer cells strongly up-regulates Bcl-2 and Bcl-X_L expression (32, 33) and that AS-mediated HER-2 down-regulation also suppresses Bcl-2 expression (23). The purpose of the present study was to assess whether HER-2 blockade by trastuzumab attenuates Bcl-2/Bcl-X_L expression and increases the susceptibility of breast cancer cells to apoptosis induction.

MATERIALS AND METHODS

Cell Cultures and *In vitro* Treatment. The human breast carcinoma cell lines BT474 and ZR 75-1 were cultured in DMEM supplemented with 10% fetal calf serum, glutamine, penicillin, and streptomycin sulfate at 37°C under 5% CO_2-95% air. Cell culture reagents were purchased from Invitrogen (Milan, Italy). Trastuzumab (Herceptin, kindly provided by Roche, Monza, Italy) for clinical and *in vitro* use was stored at 4°C and adjusted to the final concentration with culture medium. PD98059 and LY294002 were purchased from Calbiochem (La Jolla, CA). The oligonucleotides 4625 (AS-4625, fully complementary to the Bcl-2 mRNA and with three mismatching nucleotides to the Bcl-X_L mRNA) and 4626 (SC-4626, scrambled sequence control) were used as described previously (27, 34). The small-molecule Bcl-2 antagonist HA14-1 (35) was purchased from Sigma Chemical Co. (St. Louis, MO). For all experiments, exponentially growing cells were exposed to different treatments for various periods of time (3 to 72 hours), washed, assayed for cell viability (by trypan blue exclusion test) and counted with a Coulter Counter (Kontron Instruments, Milan, Italy). Aliquots of drug-treated and control cells were differentially processed according to the analyses to be done. Cell cycle and apoptosis analysis were done with a FACScan flow cytometer as described previously (36, 37).

Western Blot and Semiquantitative Reverse Transcription-PCR Analysis. Cells were rinsed three times with ice-cold PBS, harvested, and centrifuged at 4°C; cell pellets were then lysed by incubation on ice for 30 minutes in lysis buffer containing 50 mmol/L Tris-HCl (pH 8.0), 250 mmol/L NaCl, 1 mmol/L EDTA, 100 mmol/L NaF, 1 mmol/L $NaVO_4$, 10 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 1% NP40. Western blots were prepared by standard procedures with the following antibodies: anti-HER-2, anti-epidermal growth factor receptor (EGFR), anti-Bcl-X_L (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Bcl-2 (DAKO Corp., Carpinteria, CA), and anti-β-tubulin (Oncogene Science, Cambridge, MA). Immunoreactivity was detected by ECL chemiluminescence reaction (Amersham Pharmacia Biotech, Milan, Italy).

For semiquantitative reverse transcription (RT)-PCR, BT474 cells were treated with trastuzumab for different times (3 to 24 hours). RNA was isolated with the TRIZOL reagent (Life Technologies, Inc., Paisley, United Kingdom), and equal amounts of RNA were analyzed for the expression of Bcl-2 and glyceraldehyde-3-phosphate dehydrogenase by RT-PCR (25 cycles) with a Platinum Quantitative RT-PCR kit (Life Technologies, Inc.). PCR products were separated on a 2% agarose gel. PCR conditions were incubation at 95°C for 5 minutes, followed by 25 cycles at 95°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes. The following primers were used for RT-PCR amplifications: 5′-GTGAACTGGGGGAGGATTGT-3′ (Bcl-2 forward primer) and 5′-GGAGAAATCAAACAGAGGCC-3′ (Bcl-2 reverse primer); 5′-CCAAGGTCATCCATGACAAC-3′ (glyceraldehyde-3-phosphate dehydrogenase forward primer) and 5′-TTACTCCTTGGAGGCCATGT-3′ (glyceraldehyde-3-phosphate dehydrogenase reverse primer).

Clinical Trastuzumab Treatment, Immunohistochemistry, and Fluorescent *In situ* Hybridization Analysis. Clinical specimens were obtained by either biopsy or fine-needle aspiration from women with advanced breast cancer undergoing single-agent trastuzumab (4 mg/kg intravenous loading dose, followed by 2 mg/kg intravenous dose weekly for 6 weeks) in the context of an Institutional Review Board-approved "phase II window" study. After providing written informed consent to participate in the study, patients underwent biopsy or fine-needle aspiration of the same metastatic lesion 1 week before the start of treatment and the day after the 7th trastuzumab administration.

HER-2 and Bcl-2 protein expression were evaluated with the polyclonal antibody A0485 and the monoclonal antibody 124 (Dako Cytomation, Milan, Italy), respectively, on 5-μm sections of routinely fixed paraffin embedded blocks or on cytological specimens sampled from fine-needle aspiration. Tumors were considered HER-2 positive when at least 10% of the neoplastic cells displayed a distinct plasma membrane staining (2+/3+ score) and Bcl-2 positive when a cytoplasmic immunoreactivity was observed in >20% of the neoplastic cells.

For fluorescent *in situ* hybridization (FISH) analysis, the PathVysion HER-2 DNA probe kit (Vysis, Downers Grove, IL) was used. The LSI HER-2 (Spectrum Orange) probe contains DNA sequences specific for the *HER-2* human gene locus and hybridizes to 17q11.2-q12 region of human chromosome 17. The chromosome 17 enumeration probe (CEP 17, Spectrum Green) contains α-satellite DNA that hybridizes to the D17Z1 locus. After pretreatment, the procedure "Hybrite System" (Vysis) was used. The slides were processed with Olympus BX60 fluorescence microscope equipped with a 100-watt mercury lamp. Separate band pass filters were used for the detection of the HER-2 probe signal (Spectrum Orange), CEP 17 probe signal (Spectrum Green), and 4′,6-diamidino-2-phenylindole counterstain. Fluorochrome signals were captured individually, and images were generated via computer with Quips Genetic Workstations and Imaging Software (Vysis). At least 100 well-defined nuclei were scored for each hybridization. The amplification was defined as a HER-2 to CEP 17 ratio >2.

Statistical Analysis. For *in vitro* experiments, differences between treatment groups were analyzed with a two-tailed Student's *t* test for paired samples. Synergism, additivity, and antagonism were assessed by isobologram analysis with a fixed-ratio experimental design and the Chou-Talalay method (38). Results were analyzed with the Calcusyn software (Biosoft, Cambridge, United Kingdom) and combination indexes (CI) were appropriately derived. With this method, CI values <0.9, $>0.9 < 1.2$, and >1.2 indicate synergism, additive effect, and antagonism, respectively.

RESULTS

Trastuzumab Inhibits Cell Growth and Down-Regulates HER-2 and EGFR Protein Expression in Breast Cancer Cell Lines with *HER-2* Gene Amplification. Two human breast cancer cell lines, BT474 and ZR 75-1, were used in this study as a model for *HER-2* gene-amplified and -nonamplified breast cancer, respectively.

Exposure of BT474 cells to clinically relevant concentrations of trastuzumab resulted in a dose- and time-dependent inhibition of cell growth (47 ± 12% and 54 ± 5% growth inhibition after 72 hours of exposure to 10 and 25 μg/mL trastuzumab, respectively; Fig. 1*A*) and cell cycle progression (percentage of cells in the S phase of the cell cycle at 48 hours: 23 ± 6%, 11.5 ± 1%, and 8.5 ± 2% in control-, 10 μg/mL trastuzumab-, and 25 μg/mL trastuzumab-treated cells, respectively; Fig. 1*B* and *C*). However, trastuzumab treatment at the highest dose of 25 μg/mL induced little or no increase in the percentage of apoptotic cells (7.3 ± 3.5% net apoptosis induction at 48 hours), as detected by annexin V binding (Fig. 1*D* and *E*). Conversely, trastuzumab treatment did not affect cell growth, proliferation, and survival of the HER-2–negative cell line ZR 75-1 (data not shown). Trastuzumab-mediated inhibition of BT474 cell growth was accompanied by down-regulation of HER-2 and EGFR protein expression, as detected by Western blot analysis (Fig. 2*A*). Conversely, HER-2 expression was barely detectable in ZR 75-1 cells and was not affected by trastuzumab treatment (Fig. 2*A*).

Overall these results confirm previous findings of selective HER-2/EGFR protein down-regulation and cell growth inhibi-

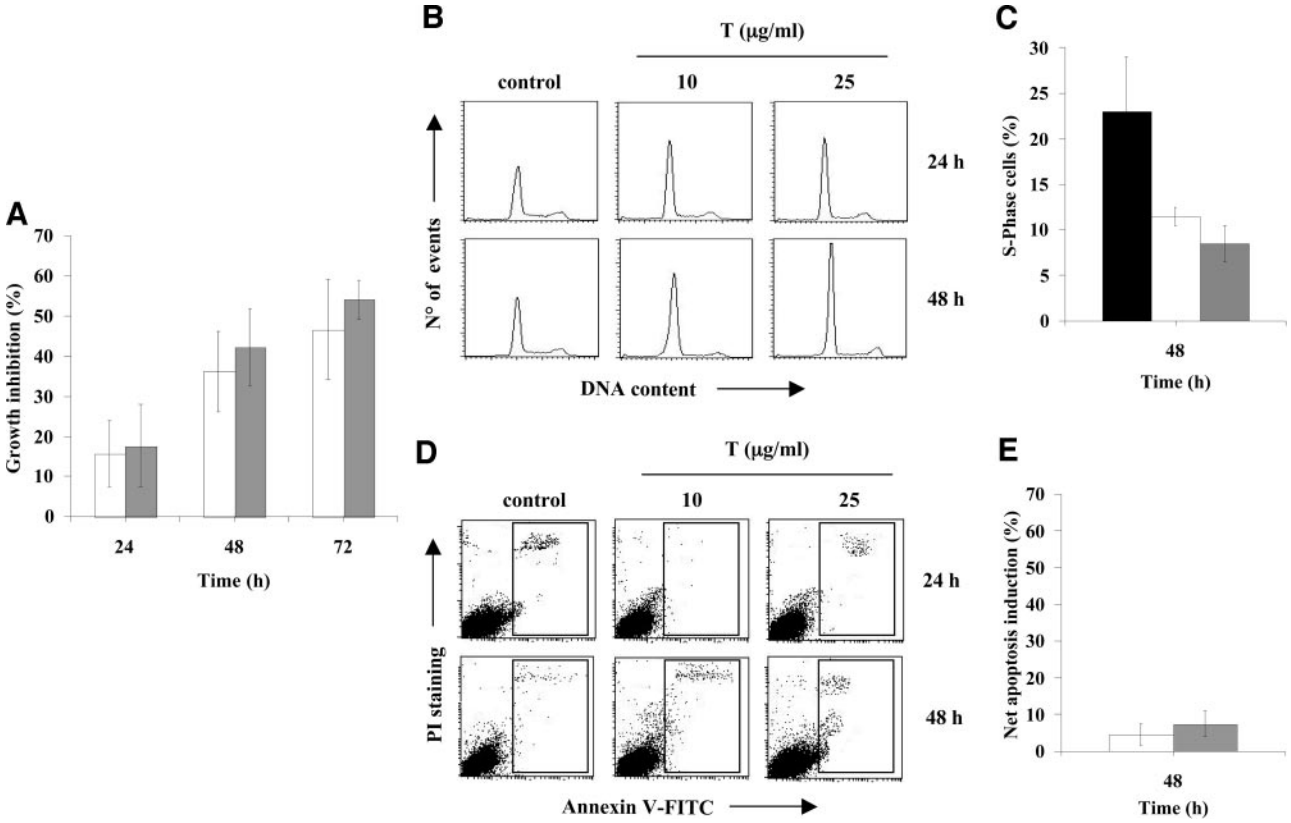

Fig. 1 Effects of trastuzumab on cell growth, cell cycle distribution, and apoptosis of BT474 cells. Cells were exposed to 10 μg/mL (*white columns*) and 25 μg/mL (*gray columns*) trastuzumab for up to 72 hours and then assessed for cell viability (by trypan blue exclusion counting), cell cycle distribution (by PI staining), and apoptosis (by annexin V binding). *A*, growth-inhibitory effects are expressed as percentage of cell growth inhibition induced by trastuzumab relative to untreated control cells. Results represent the average ± SD of four independent experiments. *B*, cell distribution in the different phases of cell cycle. Results from one experiment representative of three are shown. *C*, percentage of cells in the S phase of the cell cycle in untreated control (*black columns*) and trastuzumab-treated cells. Results represent the average ± SD of three independent experiments. *D*, cytofluorometric analysis of apoptotic cells by annexin V/PI staining; Annexin V-positive cells are highlighted in the box. Results from one experiment representative of three are shown. *E*, net apoptosis induction by trastuzumab (% apoptosis in trastuzumab-treated cells minus % apoptosis in control cells). Results represent the average ± SD of three independent experiments.

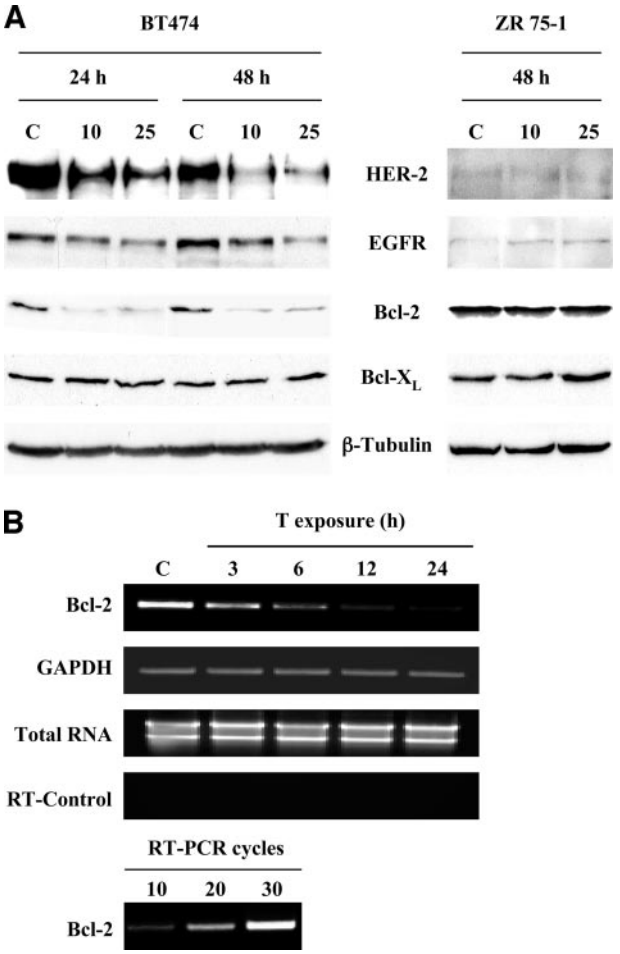

Fig. 2 Effects of trastuzumab on HER-2, EGFR, Bcl-2, and Bcl-XL expression levels in BT474 and ZR 75-1 cells. *A*, BT474 and ZR 75-1 cells were exposed to trastuzumab (10 and 25 μg/mL) for the indicated times, lysed, and assayed for HER-2, EGFR, Bcl-2, and Bcl-X_L protein expression by Western blot analysis. β-Tubulin expression is shown as protein loading and blotting control. Results from one experiment representative of three are shown. *B*, BT474 cells were exposed to trastuzumab (25 μg/mL) for the indicated times and then equal amounts of RNA (RNA input) were analyzed by RT-PCR (25 cycles) for the expression of Bcl-2 and glyceraldehyde-3-phosphate dehydrogenase. RT control lanes represent PCR in the absence of reverse transcription. Results of one experiment representative of two are shown.

tion by trastuzumab in *HER-2* gene-amplified breast cancer cells, with little or no effect on apoptosis induction.

Trastuzumab Down-Regulates Bcl-2, but not Bcl-X_L, in HER-2 Overexpressing Breast Cancer Cells. We next analyzed the effects of trastuzumab on the expression of two major antiapoptotic Bcl-2 family members, Bcl-2 and Bcl-X_L. BT474 cells were exposed to trastuzumab (10–25 μg/mL) for up to 48 hours, and Bcl-2 and Bcl-X_L protein levels were then analyzed by Western blotting. As shown in Fig. 2*A*, both trastuzumab concentrations selectively decreased Bcl-2 protein expression, without affecting Bcl-X_L, at 24 and 48 hours. Conversely, neither Bcl-2 nor Bcl-X_L protein levels were modulated by trastuzumab treatment in ZR 75-1 cells (Fig. 2*A*). In BT474 cells, semiquantitative PCR analysis revealed a trastuzumab (25 μg/mL)-induced reduction in Bcl-2 mRNA levels, which was already detectable at 3 hours and was maximal at 24 hours (Fig. 2*B*), suggesting that trastuzumab affects Bcl-2 expression either at the transcriptional or at the mRNA stability level.

Because the mitogen activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) and the phosphoinositide 3-kinase/AKT pathways are two major downstream mediators of HER-2 overexpression-supported proliferation and survival that are known to be inhibited after trastuzumab treatment (39), we investigated their involvement in the observed trastuzumab-mediated inhibition of Bcl-2 expression. To this purpose, BT474 cells were exposed to selective pharmacologic inhibitors of MEK activation (PD98059) and phosphoinositide 3-kinase–enzymatic activity (LY294002) for up to 48 hours at the concentrations of 20 and 10 μmol/L, respectively. Under these conditions, both PD98059 and LY294002 inhibited BT474 cell growth by approximately 50% (Fig. 3*A*), but neither one affected Bcl-2 expression (Fig. 3*B*), thus ruling out MEK/ERK or phosphoinositide 3-kinase inhibition as the main mechanism of trastuzumab-induced Bcl-2 down-regulation.

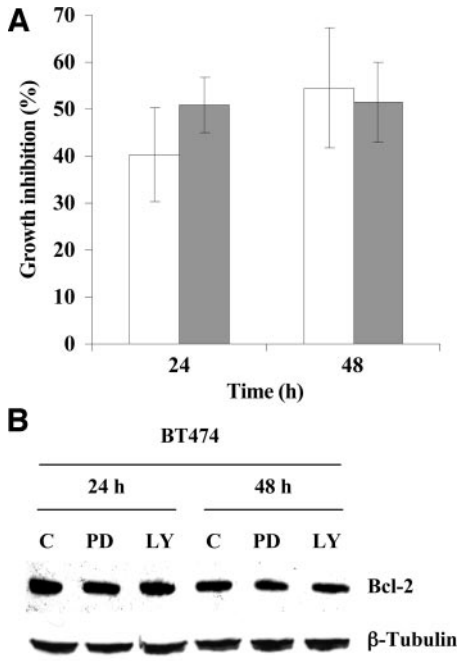

Fig. 3 Effects of MEK (PD98059) and phosphoinositide 3-kinase (LY294002) inhibitors on Bcl-2 protein expression in BT474 cells. Cells were exposed to vehicle control (DMSO, C) PD98059 (20 μmol/L, PD), or LY294002 (10 μmol/L, LY), for the indicated times and then assayed for cell viability (by trypan blue exclusion counting) and Bcl-2 protein expression (by Western blot analysis). *A*, growth-inhibitory effects are expressed as percentage of cell growth inhibition induced by PD (*white columns*) or LY (*gray columns*) relative to vehicle-treated control cells. Results represent the average ± SD of three independent experiments. *B*, Western blot analysis of Bcl-2 protein expression. β-Tubulin expression is shown as protein loading and blotting control. Results from one experiment representative of three are shown.

Table 1 Modulation of HER-2 and Bcl-2 expression in metastatic breast cancer patients undergoing trastuzumab treatment

Patient no.	Metastatic site(s)	Time of assessment	HER-2 expression *	HER-2 gene amplification †	Bcl-2 expression ‡
1	Liver	Baseline	3+	No	Positive
		After trastuzumab	0	No	Negative
2	Skin	Baseline	3+	Yes	Positive
		After trastuzumab	1+	Yes	Negative
3	Lymph nodes	Baseline	3+	Yes	Positive
		After trastuzumab	3+	Yes	Positive
4	Skin	Baseline	3+	Yes	Negative
		After trastuzumab	3+	Yes	Negative
5	Skin and lymph nodes	Baseline	3+	Yes	Negative
		After trastuzumab	1+	Yes	Negative

* IHC score system (Herceptest).
† FISH analysis.
‡ IHC.

HER-2 and Bcl-2 Are Down-Regulated in Metastatic Breast Cancer Patients Treated with Trastuzumab. We next sought confirmation of our cell line findings about modulation of Bcl-2 expression in breast cancer patients undergoing trastuzumab treatment. All patients had estrogen/progesterone receptor-negative breast cancer, with the exception of patient 1 in Table 1, who had detectable estrogen receptor expression in 20% of the cells. HER-2 and Bcl-2 expression levels were strikingly down-regulated after trastuzumab treatment in three of five and two of three evaluable patients, respectively (Table 1; Fig. 4). Interestingly, the combination of immunohistochemistry (IHC) and FISH analysis clearly showed that trastuzumab-induced decrease in HER-2 membrane expression, despite persistent gene amplification, represents true protein down-regulation rather than selective elimination of HER-2–positive cells (Table 1; Fig. 4). After 7 weeks of treatment with trastuzumab, the size of the target lesion decreased by >50% in patients 2 and 4, increased by >25% in patients 1 and 3, and was stable in patient 5 (data not shown).

Overall, these results suggest that HER-2 and Bcl-2 down-regulation may also occur *in vivo* in at least some breast cancer patients treated with trastuzumab, further adding to the potential clinical relevance of our *in vitro* findings.

Down-Regulation of Bcl-2 and Bcl-X_L by Bispecific AS Induces Apoptosis in Breast Cancer Cells: Sequence-Dependent Potentiation by Trastuzumab. Previous reports have shown that simultaneous down-regulation of both Bcl-2 and

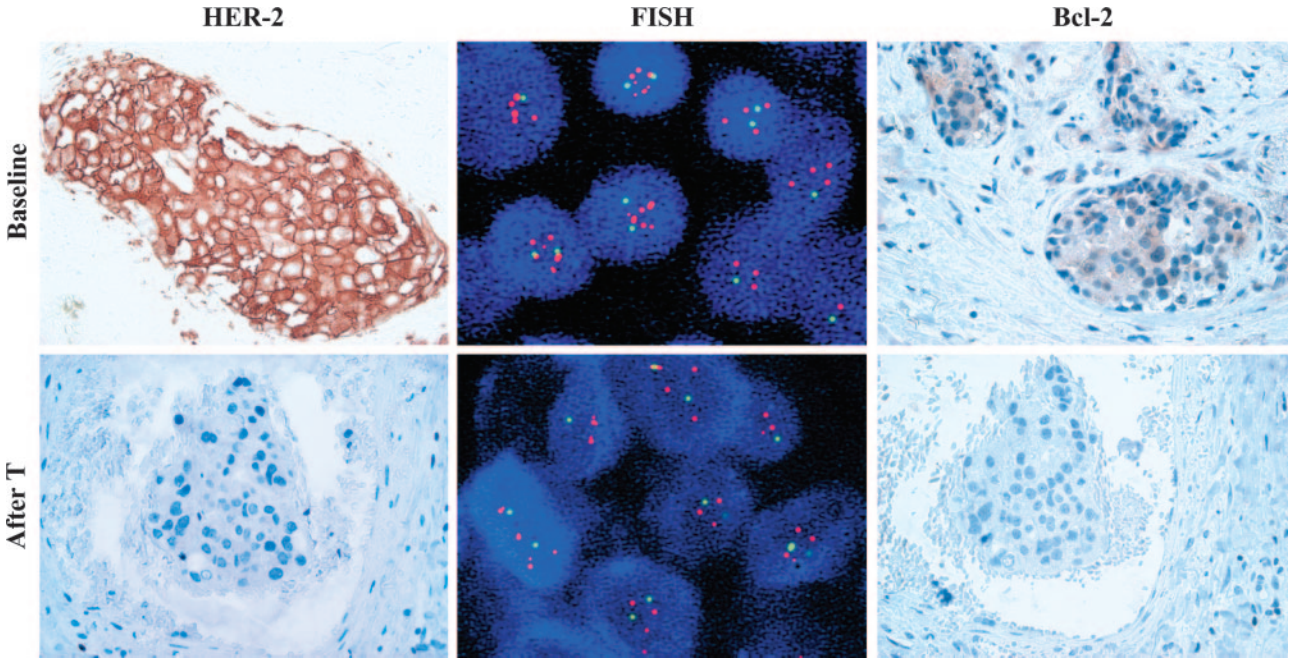

Fig. 4 *In vivo* effects of trastuzumab on HER-2 and Bcl-2 expression. Patient 2 in Table 1 was treated with trastuzumab (4 mg/kg intravenous loading dose, followed by 2 mg/kg intravenous dose weekly) for 6 consecutive weeks. HER-2 and Bcl-2 protein expression, as well as *HER-2* gene amplification, were assayed by IHC and FISH in punch biopsies of the same metastatic skin lesion obtained before treatment start (Baseline) and 24 hours after the last trastuzumab administration (After trastuzumab). In the FISH analysis, *green* and *red dots* represent chromosome 17 control hybridization and HER-2-specific hybridization, respectively.

Bcl-X_L expression by bispecific AS inhibits cell growth (both *in vitro* and *in vivo*), induces apoptosis, and sensitizes to the cytotoxic action of different chemotherapeutic drugs in several human cancer cells, including breast cancer cell lines (27–29). Trastuzumab treatment, on the other hand, selectively down-regulates Bcl-2, but not Bcl-X_L, protein expression in *HER-2* gene-amplified breast cancer cells but has little or no effect on apoptosis induction (Fig. 1). We therefore hypothesized that a combination of Bcl-2/Bcl-X_L bispecific AS and trastuzumab, by affording a more complete and prolonged suppression of the expression of antiapoptotic Bcl-2 family members and/or the simultaneous inhibition of complementary survival pathways, might increase the proapoptotic efficiency of each agent alone.

To test this hypothesis, BT474 cells were transfected with 300 nmol/L bispecific Bcl-2/Bcl-X_L AS-4625 (or a matched concentration of its scrambled sequence control, SC-4626) for 20 hours (27, 34) and then exposed to either trastuzumab (25 μg/mL) or vehicle control. Cell viability, cell cycle, and apoptosis were then assessed 24 and 48 hours after the addition of trastuzumab. As expected, AS-4625 decreased both Bcl-2 and Bcl-X_L expression, whereas trastuzumab, alone (Fig. 2) or combined with SC-4626, selectively reduced Bcl-2, but not Bcl-X_L, expression (Fig. 5). Treatment with AS-4625 alone resulted in a 21 ± 7% net apoptosis induction over SC-4626 at 48 hours, whereas trastuzumab combined with SC-4626 inhibited cell growth and cell cycle progression in a manner similar to that reported in Fig. 1 for trastuzumab alone (data not shown) but had little or no effect on the proportion of apoptotic cells as compared with SC-4626 control (Fig. 6*A* and *B*). However, the combination of AS-4625 followed by trastuzumab resulted in a significantly higher percentage of apoptotic cells (37 ± 6% net apoptosis induction at 48 hours), as compared with each treatment alone (*P* for the comparison between AS-4625 alone and AS-4625 followed by trastuzumab = 0.01; Fig. 6*B*). Conversely, pretreatment of BT474 cells with 25 μg/mL trastuzumab for 48 hours followed by transfection with AS-4625 did not increase the proportion of apoptotic cells over treatment with AS-4625 alone, as assessed 48 hours after transfection (Fig. 6*C*). In ZR 75-1 cells, AS-4625 gave results similar to those obtained in BT474 cells in terms of reduction of Bcl-2/Bcl-X_L expression and induction of apoptotic cell death, but its proapoptotic effects were not potentiated by subsequent exposure to trastuzumab (data not shown).

Trastuzumab Synergistically Potentiates Apoptosis Induced by Small-Molecule Bcl-2 Antagonists. To further confirm our results, we used the small-molecule Bcl-2 antagonist HA14-1, which interferes with Bcl-2, and possibly Bcl-X_L, function by interacting with the surface pocket responsible for the BH3-mediated binding of proapoptotic Bcl-2 family members (35). Exposure of BT474 cells to HA14-1 alone (40 μmol/L) for 72 hours resulted in a 26 ± 3% net apoptosis induction, whereas trastuzumab alone (25 μg/mL) caused little or no increase in the percentage of apoptotic cells (4 ± 3% net apoptosis induction at 48 hours; Fig. 7*A* and *B*). However, pretreatment with HA14-1 for 24 hours followed by exposure to trastuzumab for 48 hours significantly enhanced the apoptotic response of BT474 cells, resulting in a 39 ± 3% net apoptosis induction over untreated control (*P* for the comparison between HA14-1 alone and HA14-1 followed by trastuzumab = 0.02; Fig. 7*A* and *B*). We further analyzed pharmacologic interactions between HA14-1 and trastuzumab with a fixed-ratio experimental design (HA14-1:trastuzumab ratio = 2.5). Isobologram analysis formally showed that sequential exposure of BT474 breast cancer cells to HA14-1 and trastuzumab resulted in the highly synergistic (CI = 0.58 ± 0.18) induction of apoptosis over many HA14-1 and trastuzumab doses (Fig. 7*C*). Conversely, in ZR 75-1 cells the combination of HA14-1 and trastuzumab yielded results similar to those obtained with HA14-1 alone (data not shown).

Overall, our results indicate that, although relatively ineffective *per* se, trastuzumab synergistically enhances apoptosis induced by interference with Bcl-2/Bcl-X_L expression and function by either AS or small-molecule antagonists in *HER-2* gene–amplified breast cancer cells.

DISCUSSION

In this report we provide evidence that HER-2 signaling blockade by trastuzumab down-modulates the expression of the antiapoptotic Bcl-2 protein in *HER-2* gene-amplified breast cancer cell lines. trastuzumab-induced Bcl-2 down-regulation is also observed in a few metastatic breast cancer patients, further supporting *in vitro* findings. Despite its lack of proapoptotic activity when used alone, trastuzumab significantly potentiates apoptosis induction by agents (either AS or small molecule antagonists) that interfere with Bcl-2 and/or Bcl-X_L expression and function, resulting in a proapoptotic synergism that could be exploited for therapeutic purposes.

To our knowledge, this is the first evidence that trastuzumab treatment causes Bcl-2 down-regulation in HER-2–overexpressing/gene-amplified breast cancer cells. Although the relationships between HER-2 amplification/overexpression and expression of Bcl-2 family members have not been completely elucidated, independent groups have shown that forced HER-2 overexpression induces up-regulation of the Bcl-2 protein in MCF-7 breast cancer cells (32, 33); our findings of trastuzumab-

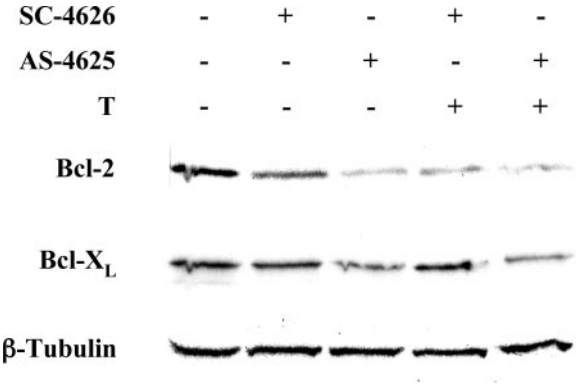

Fig. 5 Bcl-2 and Bcl-X_L protein expression in response to combined trastuzumab and AS-4625 in BT474 cells. Cells were transfected with 300 nmol/L of either SC-4626 or AS-4625 for 20 hours, followed by exposure to trastuzumab (25 μg/mL) or control medium for 48 hours. Western blot analysis of Bcl-2 and Bcl-X_L protein expression was done at the end of treatments. β-Tubulin expression is shown as protein loading and blotting control. Results from one experiment representative of three are shown.

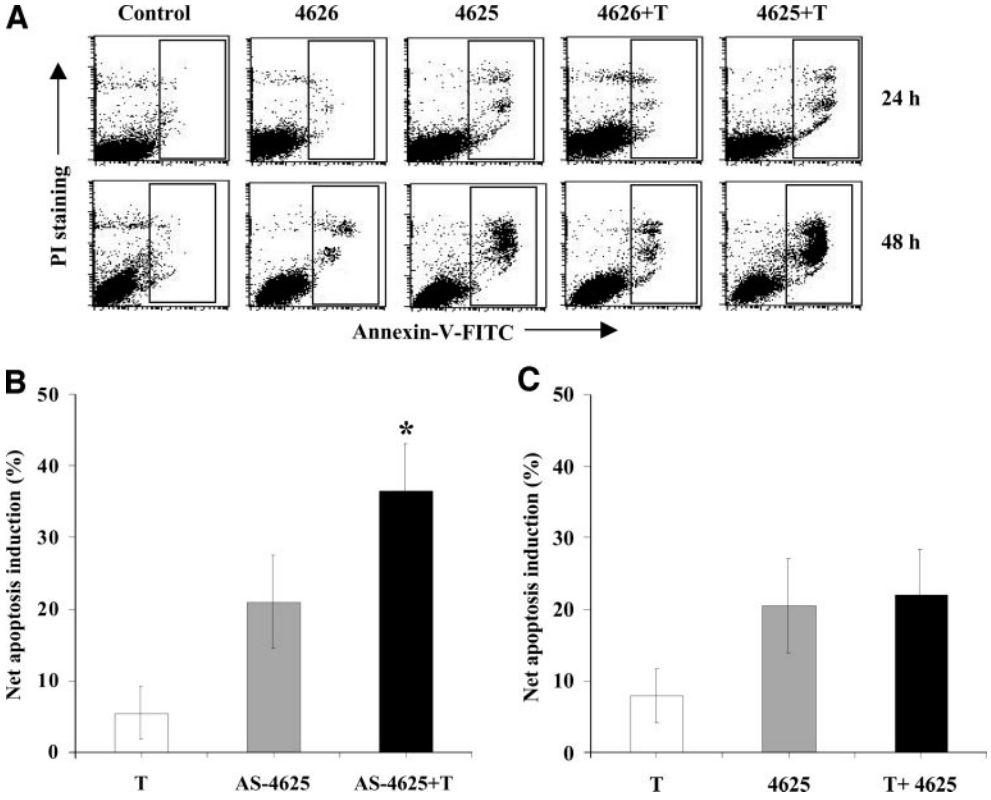

Fig. 6 Proapoptotic effects of combined trastuzumab and AS-4625 in BT474 cells. Cells were transfected with 300 nmol/L of either SC-4626 or AS-4625 for 20 hours, followed by exposure to trastuzumab (25 μg/mL) or control medium for 48 hours (*A* and *B*); in panel *C*, cells were exposed to either trastuzumab (25 μg/mL) or control medium for 48 hours, then transfected with 300 nmol/L of either SC-4626 or AS-4625, and assayed for apoptosis induction 48 hours after transfection. *A*, cytofluorometric analysis of apoptotic cells by annexin V/PI staining; annexin V-positive cells are highlighted in the *box*. Results from one experiment representative of three are shown. *B*, net apoptosis induction (% apoptosis in SC-4626+trastuzumab– (trastuzumab), AS-4625–, or AS-4625+trastuzumab–treated cells minus % apoptosis in SC-4626–treated cells). Results represent the average ± SD of three independent experiments. ∗*P* for the comparison between AS-4625 and AS-4625+trastuzumab = 0.01 by two-tailed Student's *t* test. *C*, net apoptosis induction (% apoptosis in trastuzumab+SC-4626– (trastuzumab), AS-4625–, or trastuzumab+AS-4625–treated cells minus % apoptosis in SC-4626–treated cells). Results represent the average ± SD of three independent experiments.

mediated Bcl-2 down-regulation lend support to the hypothesis that HER-2 signaling may play a causal role in Bcl-2 expression and suggest that HER-2–directed strategies may exert their apoptosis-sensitizing effects, at least in part, through a reduction in Bcl-2 levels. This view is further supported by recent evidence indicating that HER-2–directed AS also suppress Bcl-2 expression levels in a dose-dependent fashion in BT474 breast cancer cells, thereby sensitizing them to the growth-inhibitory and proapoptotic action of different classes of chemotherapeutic agents (23). Conversely, Bcl-X_L, which was also found to be increased in HER-2–transfected as compared with control vector-transfected MCF-7 cells (32, 33), did not seem to be regulated in response to trastuzumab treatment in our model system.

Despite its pronounced growth inhibitory activity and its effects on the expression of a major antiapoptotic player, such as Bcl-2, trastuzumab was largely ineffective at inducing apoptosis in *HER-2* gene-amplified breast cancer cells, as reported previously by several groups (40–45). However, when properly combined with bispecific AS that target both Bcl-2 and Bcl-X_L (AS-4625, ref. 27), trastuzumab significantly potentiated their proapoptotic action. One possible explanation for these findings is that above-threshold levels of Bcl-X_L may compensate for trastuzumab-induced reduction in Bcl-2 expression, which would be insufficient to cause irreversible commitment to apoptosis in the absence of additional cellular stresses. Indeed, increased expression of Bcl-X_L is associated with higher tumor grade and an increased number of nodal metastases (46), and its inhibition by AS efficiently induces apoptosis in cell line models (20), thus establishing a critical role for Bcl-X_L as an inhibitor of apoptosis in breast cancer. According to this hypothesis, both trastuzumab and Bcl-2/Bcl-X_L bispecific AS would converge on the regulation of mitochondrial homeostasis, with the combination possibly affording a more complete and prolonged suppression of the antiapoptotic properties of Bcl-2 and Bcl-X_L. Alternatively, in addition to Bcl-2 down-regulation, trastuzumab-mediated inhibition of complementary survival pathways, such as the mitogen-activated protein kinase cascade, may also play a role in the observed proapoptotic cooperation with Bcl-2/Bcl-X_L bispecific AS. In this regard, Davis *et al.* (47) have shown recently that Raf-1

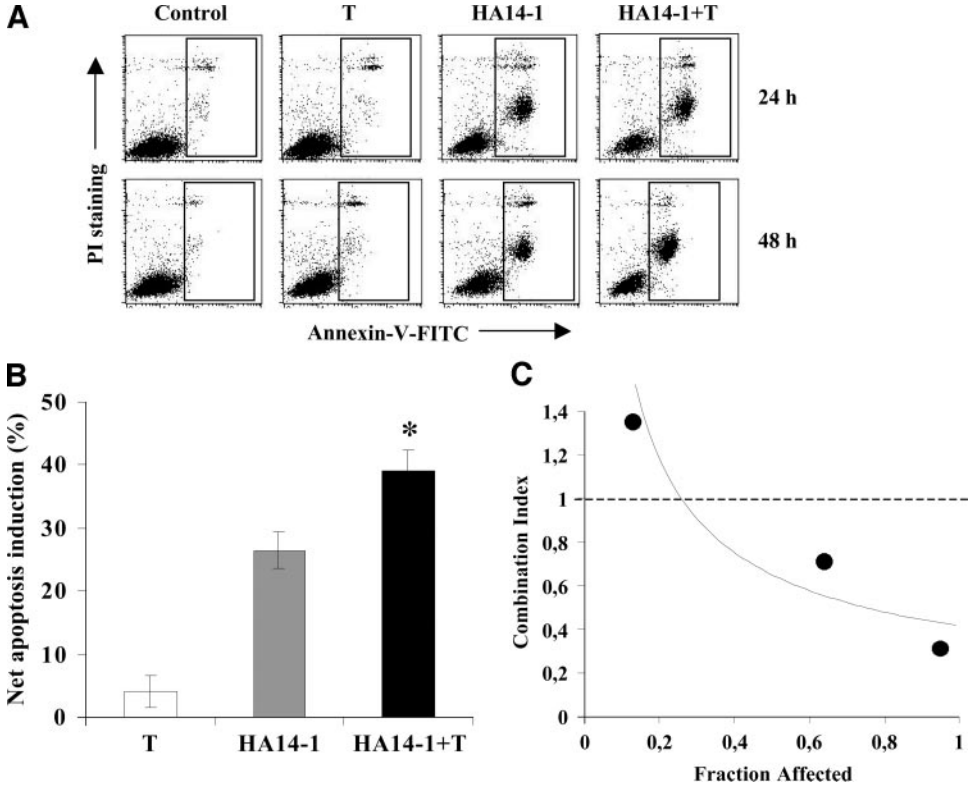

Fig. 7 Proapoptotic effects and synergism analysis of combined trastuzumab and HA14-1 in BT474 cells. Cells were exposed to either vehicle control (Control and Trastuzumab) or 40 μmol/L HA14-1 (HA14-1 and HA14-1+trastuzumab) for 24 hours, followed by trastuzumab (25 μg/mL, trastuzumab and HA14-1+trastuzumab) or control medium (Control and HA14-1). Apoptosis induction was assayed by annexin V binding 24 to 48 hours after the addition of trastuzumab. *A*, cytofluorimetric analysis of apoptotic cells by annexin V/PI staining; annexin V-positive cells are highlighted in the box. Results from one experiment representative of three are shown. *B*, net apoptosis induction (% apoptosis in trastuzumab–, HA14-1–, or HA14-1+trastuzumab–treated cells minus % apoptosis in vehicle control-treated cells). Results represent the average ± SD of three independent experiments. ∗*P* for the comparison between HA14-1 and HA14-1+trastuzumab = 0.02 by two-tailed Student's *t* test. *C*, synergism analysis was carried out with the Chou-Talalay method (38) and a fixed-ratio experimental design over a range of HA14-1 (10–80 μmol/L) and trastuzumab (5–100 μg/mL) doses. Results are shown as CI plots; the *hatched line* represents additive effects (CI = 1) and synergism and antagonism fall below and above the line, respectively. *Black circles* represent actual data points for the combination.

and Bcl-2 cooperate in inducing breast cancer cell chemoresistance, and we have shown previously that, in hematologic malignancies, simultaneous disruption of the Bcl-2 and MEK/ERK pathways synergistically induces apoptosis (48).

Interference with Bcl-2 function using an entirely different approach, namely the small molecule Bcl-2 antagonist HA14-1, also results in a striking proapoptotic synergism when combined with trastuzumab. HA14-1 was first identified through virtual molecular database screening of a collection of compounds, taking the BakBH3 peptide-binding pocket on the Bcl-2 protein as the target. In the original study (35) the three-dimensional structure of the Bcl-2 protein was modeled taking the nuclear magnetic resonance structure of the Bcl-X_L protein in the complex of Bcl-X_L and BakBH3 peptide as a template; moreover, the binding affinity of the compound to the Bcl-2 protein *in vitro* was determined by a competitive binding assay based on a fluorescent peptide, GQVGRQLAIIGDDINR, derived from the BH3 domain of Bak (Flu-BakBH3), which has been shown to bind to the surface pocket of the Bcl-2 and Bcl-X_L proteins with similarly high-affinity (K_D = 0.20 and 0.34 μmol/L, respectively). Therefore the possibility that, by design, HA14-1 could inhibit both Bcl-2 and Bcl-X_L heterodimerization with BH3-containing proapoptotic family members seems very likely and is currently the subject of ongoing investigation by several groups, including ours. Overall, these findings further support the conclusion that simultaneous disruption of apoptosis resistance mediated by HER-2 and Bcl-2 family members can lead to enhanced antitumor effects that could be exploited for therapeutic purposes. This is not surprising given that, with rare exceptions, neoplastic cell growth is the result of multiple genetic alterations (49), and thus clinically successful new therapeutic strategies will most likely rely on the mechanism-based manipulation of multiple, cross-talking pathways involved in growth and survival control.

Despite substantial advances in the understanding of the underlying biology, therapeutic approach to breast cancer patients remains a clinical challenge, especially in an advanced disease setting. Our findings demonstrating that sequential targeting of antiapoptotic Bcl-2 family members and HER-2 results in synergistic antitumor activity provides preclinical rationale for a novel, mechanism-based, therapeutic strategy to be tested in breast cancer patients.

ACKNOWLEDGMENTS

We are indebted to Drs. Alessandra Fabi, Gianluigi Ferretti, Paolo Carlini, and Paola Papaldo for help in carrying out the clinical trastuzumab protocol. We are also grateful to Prof. James A. McCubrey (Brody School of Medicine at East Carolina University, Greenville, NC) for critical reading of this report and scientific advice.

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