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# Anti-tumor effects of retinoids combined with trastuzumab or tamoxifen in breast cancer cells: induction of apoptosis by retinoid/trastuzumab combinations

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**Abstract**

**Introduction:** HER2 and estrogen receptor (ER) are important in breast cancer and are therapeutic targets of trastuzumab (Herceptin) and tamoxifen, respectively. Retinoids inhibit breast cancer growth, and modulate signaling by HER2 and ER. We hypothesized that treatment with retinoids and simultaneous targeting of HER2 and/or ER may have enhanced anti-tumor effects.

**Methods:** The effects of retinoids combined with trastuzumab or tamoxifen were examined in two human breast cancer cell lines in culture, BT474 and SKBR3. Assays of proliferation,

apoptosis, differentiation, cell cycle distribution, and receptor signaling were performed.

**Results:** In HER2-overexpressing/ER+ BT474 cells, combining all-trans retinoic acid (atRA) with tamoxifen or trastuzumab synergistically inhibited cell growth, and altered cell differentiation and cell cycle. Only atRA/trastuzumab-containing combinations induced apoptosis. BT-474 and HER2-overexpressing/ER- SKBR3 cells were treated with a panel of retinoids (atRA, 9-cis-retinoic acid (9-cis-RA), 13-cis-retinoic acid (13-cis-RA), or *N*-(4-hydroxyphenyl) retinamide (fenretinide) (4-HPR)) combined with trastuzumab. In BT-474 cells, none of the single agents except 4-HPR induced apoptosis, but again combinations of each retinoid with trastuzumab did induce apoptosis. In contrast, in SKBR3 cells, the single retinoid agents did cause apoptosis; this was only modestly enhanced by addition of trastuzumab. The retinoid drug combinations altered signaling by HER2 and ER. Retinoids were inactive in trastuzumab-resistant BT474 cells.

**Conclusions:** Combining retinoids with trastuzumab maximally inhibits cell growth and induces apoptosis in trastuzumab-sensitive cells. Treatment with such combinations may have benefit for breast cancer patients.

## Introduction

HER2 and estrogen receptor (ER) play critical roles in the clinical care of breast cancer patients as both prognostic factors and therapeutic targets. Approximately 25% of invasive breast tumors have overexpression/amplification of HER2, which is an adverse prognostic factor [1, 2]. Trastuzumab (Herceptin), a humanized monoclonal antibody against the extracellular domain of

HER2 [3-5], has shown significant therapeutic benefit in the treatment of patients with HER2-overexpressing breast cancer [6-20]. Approximately 60% of primary breast cancers are ER+ [21, 22]. The selective estrogen receptor modulator (SERM) tamoxifen is highly effective standard therapy for all stages of endocrine-responsive breast cancer.

Retinoids inhibit growth of breast cancer cell lines in culture and inhibit breast tumor growth in animal models. Retinoid signals are mediated through the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs), with each family represented by three distinct receptor genes designated as $\alpha$, $\beta$, and $\gamma$ [23-25]. All-trans retinoic acid (atRA) preferentially binds RARs but not RXRs [23-25]. However, atRA can be converted intracellularly to 9-cis-RA, an RXR ligand [26]. 9-cis-RA and 13-cis-RA bind both RARs and RXRs. *N*-(4-hydroxyphenyl) retinamide (4-HPR, fenretinide) is a synthetic analog of atRA [23-25] that has also shown anti-tumor activity, but may have differing mechanisms of action. Following stimulation by retinoids, RAR-RXR heterodimers and RXR-RXR homodimers can form [23-25]. The receptor dimers bind to retinoic acid response elements (RAREs) or retinoid X response elements (RXREs) in the promoter sequences of target genes and modulate gene transcription [23-25].

Effects of retinoids on signaling by ER and HER2 have been reported. Inhibition of breast tumor cell growth by retinoids is greater for ER+ than ER- cells [27], which may be partly related to alterations in retinoid metabolism [28]. Some studies have found that RA increased the amount of ER in MCF7 breast cancer cells [29] although others reported that RA down regulated ER in this cell line [30]. Regardless, activated RARs have been observed to exert anti-estrogenic effects by directly or indirectly impairing binding of ER to estrogen response elements (EREs) [31]. Conversely, the N-terminal region of ER$\alpha$ modulates the transcriptional activity of RAR [32].

Both RARα and RARß have been implicated in the anti-proliferative effects of retinoids against breast cancer. RARα expression is correlated positively with ER and with RA sensitivity, and is inducible by estrogen [27]. RARß has been ascribed tumor suppressor type activity and is often down regulated in breast cancer; it is inducible by atRA and inducibility correlates with atRA sensitivity [27]. In both ER+ T47D cells and ER- SKBR3 cells, some evidence suggests that RARα is the receptor solely sufficient for the growth inhibition, cell cycle arrest, apoptosis, and modulation of RAR levels [33].

Inhibition of breast cancer cell growth by atRA and 4-HPR has been associated with down-regulation of HER2; while atRA induced morphologic changes consistent with differentiation, 4-HPR induced those of apoptosis [34]. Another study demonstrated that RA can induce differentiation of cultured breast tumor cells, and this was again associated with reduction in cell surface HER2 [35]. atRA and 9-cis-RA caused decreases in HER2 and HER3, and inhibited SKBR3 cell growth with cell cycle arrest and induction of apoptosis [36], and the retinoids down regulated HER4 in T47D cells [37]; atRA also down-regulated HER2 and HER3 in MCF cells [38]. 4-HPR was reported in another study to down regulate both HER2 and the epidermal growth factor receptor (EGFR, HER1) [39]. In contrast, stimulation of SKBR3 cells with EGF or heregulin (HRG) ß1 up-regulates RARα expression [40], yet resistance to atRA-induced growth inhibition has been reported for HER2-overexpressing breast cancer cells (either HER2 transfected MCF7 or naturally overexpressing BT474 or MDA-MB-453 cells); pretreatment for several days with trastuzumab could sensitize these latter two cell lines to inhibition by atRA [41, 42]. Trastuzumab treatment increased RARE binding activity measured by electrophoretic mobility shift assay in a HER2-overexpressing cell line [41]. Co-amplification of RARα with HER2 has been reported in human breast tumors [43]. Finally, retinoids have been found to

delay the onset of mammary tumors in HER2 transgenic mice [44, 45], and a SERM/rexinoid combination was synergistic in the prevention or treatment of such tumors, despite the ER- status of such tumors [46].

Given the known interactions of retinoids with ER and HER2, we hypothesize that treatment with retinoids and simultaneous targeting of HER2 and/or ER may be a fruitful approach to treating breast cancer. As models, we have used ER+ (BT474) and ER- (SKBR3) HER2-overexpressing human breast cancer cell lines. Here we examine the effects of various retinoids (atRA, 9-cis-RA, 13-cis-RA, and 4-HPR), trastuzumab, tamoxifen, or combinations of these drugs on proliferation, cell cycle, differentiation, and apoptosis in BT474 and SKBR3 cells. Since retinoids, trastuzumab, and tamoxifen are individually agents that possess anti-tumor activity toward breast cancer, combinations of these drugs may translate into improved therapy for breast cancer patients. Here, we report synergistic inhibition of cell proliferation for combinations of these drugs, but apoptosis-inducing activity only of the retinoid/trastuzumab combinations.

## Materials and methods

### *Drugs*

Trastuzumab (Herceptin) was a gift of Genentech (South San Francisco, CA), supplied as a stock solution in phosphate-buffered saline (PBS). Tamoxifen citrate, atRA, 9-cis-RA, 13-cis-RA, and 4-HPR were purchased from Sigma-Aldrich (St. Louis, MO); stock solutions of these drugs were prepared in 100% ethanol (EtOH) and kept light-protected. HRGβ1 was purchased

from R&D Systems (Minneapolis, MN) and reconstituted in PBS.

*Cell culture*

BT474 [47] and SKBR3 cells were obtained from American Type Culture Collection (Manassas, VA). BT474 cells, which are ER+, estrogen dependent, and HER2 overexpressing [48-50], were cultured in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO), 2 mM L-glutamine (GIBCO), 10 µg/ml bovine insulin (Sigma-Aldrich), and penicillin/streptomycin (GIBCO) at 37°C in a 5% $CO_2$/95% air humidified incubator. SKBR3 cells, which are ER- and HER2 overexpressing, were cultured in McCoy's 5A medium with L-glutamine (GIBCO), supplemented with 15% fetal bovine serum (GIBCO) and penicillin/streptomycin (GIBCO).

*WST-1 colorimetric cell proliferation assay*

BT474 or SKBR3 cells were seeded in 96-well plates at 10,000 or 5,000 cells, respectively, per well. On the following day, the cells were treated with vehicle (EtOH + PBS) or drug(s). In each independent experiment, eight replicate wells of cells were used for each treatment. On day 6 following treatment, the WST-1 proliferation assay was performed according to the protocol provided by the manufacturer (Roche Applied Science, Indianapolis IN). Results were expressed as percent of control (vehicle-treated cells).

*Analysis of drug interactions*

Drug interaction results from the WST-1 proliferation assay were examined by the method of Chou and Talalay [51] using the commercially available software CalcuSyn [52, 53] (Biosoft,

Ferguson, MO). The Chou-Talalay method is based on the median-effect equation for dose-effect relationship: $f_a/f_u = (D/D_m)_m$, which can be linearly transformed as log ($f_a/f_u$) = m log (D) - m log ($D_m$), where $f_a$ = fraction affected, $f_u$ = fraction unaffected, D = dose, $D_m$ = dose that produces median-effect ($IC_{50}$), and m = coefficient signifying the sigmoidicity of the curve (or slope in linear transformation). For examining the effect of multiple drugs, a Combination Index (CI) is calculated based on the doses that have equivalent effects. The formula CI = ($D1_c$/D1) + ($D2_c$/D2) is used for calculating the CI of two drugs; the formula CI = ($D1_c$/D1) + ($D2_c$/D2) + ($D3_c$/D3) is used for determining the CI of three drugs. D1, D2, and D3 are the doses for each drug alone that inhibit a certain percentage; $D1_c$, $D2_c$, and $D3_c$ are the doses for each drug in a combination that inhibit the same percentage. The CI is a quantitative measurement of the degree of interaction between two or more drugs. A CI less than one indicates synergism between the drugs, equal to one indicates additivity, and greater than one denotes antagonism.

*Analysis of cell cycle and detection of apoptosis by determination of sub-G1 DNA peak*

BT474 or SKBR3 cells were seeded in 25 $cm^2$ flasks at 1 x $10^6$ or 0.5 x $10^6$ cells, respectively, per flask. On the following day, the cells were treated with vehicle (EtOH + PBS) or drug(s). At designated time points, floating cells in the growth media were collected, and adherent cells were trypsinized and collected. The pooled floating and adherent cells were washed twice with cold PBS. The washed cells were then resuspended in 2 ml of cold PBS, fixed by three stepwise additions of 2 ml each of cold 95% EtOH, and stored at 4°C. For analysis of cell cycle and sub-G1 DNA peak [54], the fixed cells were pelleted by centrifugation, incubated with 1 mg/ml ribonuclease A (Sigma-Aldrich) in PBS at 37°C for 30 minutes, and stained on ice with 50 µg/ml propidium iodide (Sigma-Aldrich) in PBS for 1 hour. The cell cycle distribution

(percentages of cells in G0/G1, S, and G2/M phases) and the percentage of cells in sub-G1 DNA peak were determined by flow cytometry.

*Detection of apoptosis by annexin V assay*

BT474 cells were seeded in 25 cm2 flasks at $10^6$ cells per flask. On the following day, cells were treated with drugs or vehicle. At designated time points, floating cells in the growth media were collected, and adherent cells were trypsinized and collected. The pooled floating and adherent cells were washed with cold PBS. The cells were then stained using Vybrant Apoptosis Assay Kit #2 (Molecular Probes, Eugene, OR). Briefly, the cells were incubated with Alexa Fluor 488 annexin V and propidium iodide in 1x Annexin-Binding Buffer (provided with the kit) for 15 minutes at room temperature. The percentages of annexin V and propidium iodide positive cells were determined by flow cytometry.

*Detection of differentiation by Nile red staining of neutral lipids*

BT474 or SKBR3 cells were seeded in 25 $cm^2$ flasks at 1 x$10^6$ or 0.5 x $10^6$ cells, respectively, per flask. On the following day, the cells were treated with vehicle (EtOH + PBS), or drug(s). On day 6 following treatment, the cells were collected by trypsinization, washed with PBS, and stained with 100 ng/ml Nile red fluorescent dye (Sigma-Aldrich) in PBS for 5 minutes at room temperature. The stained cells were then washed twice with PBS, resuspended in PBS, and analyzed by flow cytometry [55].

*Immunoblot experiments*

Immunoblotting was performed on cell extracts by standard techniques using the following

antibodies. Antibodies to RARα (sc-551), RARß (sc-552), RXRα (sc-553), RXRß (sc-742) and HER2 (sc-284) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to AKT, phospho-AKT, MAPK and phospho-MAPK were from Cell Signaling Technology (Beverly, MA). Antibody to phospho-HER2 (Tyr-1248) (c-erbB-2/HER-2/*neu* phospho-specific Ab-18) was from NeoMarkers (Fremont, CA) and antibody to actin was from Sigma (St. Louis, MO).

*Measurement of estrogen receptor transcriptional activity by dual luciferase reporter assay*

BT-474 cells were seeded in 6-well plates at $10^6$ cells per well. On the following day, the cells were transiently co-transfected with Renilla luciferase reporter vector plasmid pRL-CMV (Promega, Madison, WI) (a control to normalize for transfection efficiency) and a plasmid containing three consensus EREs fused to a firefly luciferase reporter vector (3 x ERE-TATA-Luc) described previously [56, 57] using the TransFast transfection reagent (Promega) according to the manufacturer's protocol. Briefly, each well of cells was incubated at 37 degrees C for 30 min in serum-free RPMI media containing 1000 ng of 3 x ERE-TATA-Luc reporter vector, 20 ng of Renilla luciferase reporter vector pRL-CMV, and TransFast transfection reagent. Following incubation, the transfection mixture was removed, and normal growth media (including 10% FBS without charcoal stripping) was added to the cells, followed immediately by addition of experimental drugs. Drug treatment was with vehicle (EtOH and PBS), 1 uM Faslodex, 1 uM atRA, 1 ug/ml trastuzumab, 1 uM TAM, or the various drug combination at the same concentrations. Two days following transfection and treatment, the dual luciferase reporter assay was performed using the Promega Dual Luciferase Reporter Assay System according to the manufacturer's protocol. The firefly luciferase activities of the treated cells were normalized to their Renilla luciferase activities and are expressed as percentage of activity of untreated cells.

*Production of trastuzumab-resistant BT474 cells*

Trastuzumab-resistant BT474 cells were selected as described [58] by long-term culture in media containing trastuzumab at 100 µg/ml. Cells were maintained in the same trastuzumab-containing media unless otherwise indicated.

## Results

*Analysis of Interactions Between atRA, trastuzumab, and tamoxifen on cell proliferation*

ER+/HER2-overexpressing BT474 cells were treated with atRA, trastuzumab or tamoxifen at a range of doses (0.2 to 10 µM for atRA and tamoxifen, and 0.2 to 10 µg/ml for trastuzumab) and with various combinations of the three drugs at fixed dose ratios. On day 6 following treatment, the effects of the single agents and drug combinations on BT474 cell growth were examined by the WST-1 proliferation assay. The results from WST-1 assays were expressed as a percentage of control growth, and drug interactions were analyzed by the Chou-Talalay method. Each single agent demonstrated dose-dependent inhibition of cell proliferation (Tables 1-4). All combinations of atRA/trastuzumab, of atRA/tamoxifen, of trastuzumab/tamoxifen (except dose of 0.2), and of the atRA/trastuzumab/tamoxifen triple combination examined were synergistic (most were strongly synergistic with CIs less than 0.3 or very strongly synergistic with CIs less than 0.1) (Tables 1-4).

*Analysis of Cell Cycle Following Drug Treatment*

The cell cycle distribution of BT474 cells was analyzed following treatment with single agents or various combinations of atRA, tamoxifen or trastuzumab. Each drug individually is known to cause G1 cell cycle accumulation. Compared to untreated and vehicle-treated cells, single agents and various combinations of the three drugs led to an enhanced accumulation of cells in G0/G1 phase coupled with a reduction of cells in S phase of cell cycle (Figure 1). In general, the drug combinations produced lower percentage of drugs in S phase than did single agents.

*Analysis of Differentiation Following Drug Treatment*

Differentiation of BT474 cells was determined by Nile red fluorescent dye staining of neutral lipids on day 6 following treatment with HRGβ1 (positive control), single agents, or various combinations of atRA, tamoxifen or trastuzumab. Compared to untreated and vehicle-treated cells, HRGβ1, single agents, or various drug combinations led to an increase in neutral lipid production (Figure 2). Treatment with the atRA/trastuzumab, trastuzumab/tamoxifen, and atRA/trastuzumab/tamoxifen combinations resulted in greater neutral lipid production than the respective single agents alone ($p<0.05$) (Figure 2). The triple combination induced the greatest degree of differentiation ($p<0.05$) (Figure 2).

*Analysis of Apoptosis Following Drug Treatment*

To detect apoptotic cells, both floating and adherent BT474 cells were examined by annexin V staining and sub-G1 DNA peak analysis following treatment with single agents or various combinations of the three drugs. Both annexin V staining and sub-G1 DNA peak analysis demonstrate that only the atRA/trastuzumab and atRA/trastuzumab/tamoxifen combinations

induced apoptosis (Figure 3). The atRA/trastuzumab combination resulted in 3%, 9%, and 16% annexin V positive cells (Figure 3a), and 3%, 13%, and 26% cells in sub-G1 DNA peak (Figure 3b) on days 2, 4, and 6, respectively. The atRA/trastuzumab/tamoxifen combination induced greatest percentage of apoptotic cells (3%, 11%, and 25% annexin V positive cells, and 3%, 13%, and 36% cells in sub-G1 DNA peak on days 2, 4, and 6, respectively) (Figure 3). Therefore, while neither the single agents nor the atRA/tamoxifen or trastuzumab/tamoxifen combinations induced apoptosis, the atRA/trastuzumab and atRA/trastuzumab/tamoxifen combinations did result in apoptosis.

Given the unique ability of the atRA/trastuzumab combination to induce apoptosis in ER+ BT474 cells, and the known interaction between retinoids and ER signaling discussed above, it was of interest to extend these experiments to ER-/HER2-overexpressing cells. In addition, it was of interest to compare the effects of other retinoids to those of atRA.

*Effect of other retinoids with trastuzumab on cell proliferation of ER+ and ER- cells*

HER2-overexpressing ER+/BT474 or ER-/SKBR3 cells were treated with each of the following single agents: 1 μg/ml trastuzumab, 1 μM atRA, 1 μM 9-cis-RA, 1 μM 13-cis-RA, 1 μM 4-HPR, 2.5 μM 4-HPR, or 5 μM 4-HPR, or with trastuzumab/retinoid combinations. On day 6 following treatment, the effects of the single agents and drug combinations on BT474 or SKBR3 cell growth were examined by the WST-1 proliferation assay. The results from WST-1 assays were expressed as a percentage of untreated cells. The combinations of trastuzumab with the various retinoids showed greater growth inhibition than the single agents alone in both BT474 (Figure 4a) and SKBR3 (Figure 4b) cells, with the exception of 4-HPR, which showed minimal ability to enhance trastuzumab-mediated growth inhibition in both cell lines.

*Effect of other retinoids with trastuzumab on cell cycle of ER+ and ER- cells*

The cell cycle distribution of BT474 or SKBR3 cells was analyzed on day 2 following treatment with single agents or combinations of the drugs. Each drug individually is known to cause G1 cell cycle accumulation. In BT474 cells, compared to untreated and vehicle-treated cells, the single agents trastuzumab and 4-HPR, and the combinations of trastuzumab with the various retinoids, led to an enhanced accumulation of cells in G0/G1 phase coupled with a reduction of cells in S phase of cell cycle (Figure 5). In general, the drug combinations produced a lower percentage of cells in S phase than did single agents alone in BT474 cells (Figure 5). In SKBR3 cells, all single agents resulted in reduced S phase (4-HPR required higher concentrations), though there was only a modest effect of adding trastuzumab to 4-HPR and no additional effect of adding trastuzumab to the other retinoids (Figure 5). Unlike the BT474 cells, in SKBR3 cells none of the drugs or their combinations had a significant impact on G1 phase; rather, the retinoids (excluding 4-HPR) caused an increase in G2/M phase, which was just slightly enhanced by addition of trastuzumab to any of the retinoids (Figure 6).

*Effect of other retinoids with trastuzumab on differentiation of ER+ and ER- cells*

Differentiation of BT474 or SKBR3 cells was determined by Nile red fluorescent dye staining of neutral lipids on day 6 following treatment with HRGβ1 (positive control), single agents, or combinations of the drugs. In BT-474 cells, trastuzumab and single retinoid agents only slightly increased neutral lipid production, though it was significantly enhanced by adding trastuzumab to retinoids (other than 4-HPR) (Figure 7). In SKBR3 cells, single agent retinoids induced significantly greater neutral lipid production, with the exception of 4-HPR; however

addition of trastuzumab to retinoids did not enhance the effect of the retinoids, except for 4-HPR (Figure 7).

*Effect of other retinoids with trastuzumab on apoptosis of ER+ and ER- cells*

To detect apoptotic cells, both floating and adherent BT474 or SKBR3 cells were examined by sub-G1 DNA peak analysis following treatment for 6 days with single agents or combinations of the drugs. In BT474 cells, sub-G1 DNA peak analysis demonstrated that only the combinations of trastuzumab with retinoids induced apoptosis; none of the single agents (except 4-HPR at higher concentrations) induced apoptosis; 4-HPR alone induced apoptosis at concentrations higher than 1 μM (2.5 μM or 5 μM), and this was not enhanced by trastuzumab (Figure 8). In contrast, in SKBR3 cells, the single retinoid agents did cause apoptosis, with 4-HPR having a much weaker effect compared to the other retinoids; the addition of trastuzumab to the retinoids produced a small enhancement in the induction of apoptosis (Figure 8).

*Effect of drugs on receptor signaling*

The effect of treatment of BT474 cells with atRA, trastuzumab, or both, on receptor signaling, was examined (Figure 9). Single agent trastuzumab at 1 μg/ml resulted in a moderate decrease in total levels of HER2, and, as expected, a more significant decrease in HER2 activity as reflected by level of HER2 autophosphorylation. Treatment with atRA at 1μM had no effect on HER2 expression level or degree of phosphorylation, and, when added to 1μg/ml trastuzumab, did not have a significant effect on HER2 expression level or activity. Single agent atRA also did not significantly affect AKT or MAPK expression or activity. trastuzumab treatment resulted in partial inhibition of AKT and MAPK activity; while addition of atRA to

trastuzumab had no further effect on AKT activity, the combination did appear to result in a small further decrement in MAPK activity.

Single agent atRA at 1μM caused a small decrease in level of RARα (Figure 9). Trastuzumab at 1μg/ml had a similar effect, and the combination resulted in the greatest decrease in expression level of this receptor. Treatment with atRA did not appear to affect levels of RARß, RXRα or RXRß, however trastuzumab caused a small decrement in expression of RARß that was enhanced when combined with atRA (Figure 9).

ER transcriptional activity was examined using an ERE assay in BT474 cells. Treatment with single agent atRA caused a profound inhibition of ERE activity, comparable to the ER down-regulator Faslodex (Figure 10). Single agent trastuzumab caused partial inhibition of ERE activity, comparable to that of tamoxifen, implicating peptide growth factor signaling pathway driven ER activation in these cells; however, adding trastuzumab to tamoxifen did not further inhibit ERE activity. Adding trastuzumab, tamoxifen or both to atRA could not further inhibit ERE activity beyond that of atRA alone.

*Effect of atRA on growth of trastuzumab-resistant BT474 cells*

Given the ability of the atRA/ trastuzumab combination to synergistically induce apoptosis in BT474 cells under conditions where neither agent alone could do so, it was of interest to examine the activity of atRA in trastuzumab-resistant BT474 cells. Resistance was induced in these cells by long term culture in trastuzumab-containing media. As shown in Figure 11, atRA did not affect the growth of trastuzumab-resistant BT474 cells, whether used in the presence or absence of trastuzumab in the media. In fact, removal of trastuzumab from the media did not affect growth of these cells. Slight growth inhibition was observed when trastuzumab-resistant

cells were treated with 10μM of the EGFR/HER2 dual tyrosine kinase inhibitor laptinib analog GW2974 in the presence of trastuzumab-containing media, though this required doses much greater than those typically required to inhibit growth of trastuzumab-sensitive cells; the addition of atRA to GW2974 was not able to enhance growth inhibition observed with the latter agent alone.

**Discussion**

HER2 and ER play critical roles in breast cancer and are validated therapeutic targets in this disease. Retinoids have also been shown to inhibit breast cancer growth. We have demonstrated that combining atRA with trastuzumab, tamoxifen, or both results in strong synergistic growth inhibition of BT474 human breast cancer cells. To elucidate the molecular mechanisms underlying this synergistic growth inhibition, we examined the effects of single agents and various drug combinations on cell cycle, differentiation, and apoptosis. We found that treatment with the atRA/trastuzumab and atRA/trastuzumab /tamoxifen combinations caused induction of apoptosis, which was not observed for single drugs or the trastuzumab/tamoxifen or atRA/tamoxifen combinations.

Since we observed that combining atRA with trastuzumab uniquely resulted in apoptosis, we examined the effects of other retinoids with trastuzumab, and in both ER+ (BT474) and ER- (SKBR3) HER2-overexpressing human breast cancer cells. We found that in BT474 cells, while none of the single agents (except 4-HPR) induce apoptosis, the combinations of various retinoids with trastuzumab also results in apoptosis. In contrast, in SKBR3 cells, the single agent retinoids (other than 4-HPR) do induce apoptosis (weakest for 4-HPR), and adding trastuzumab to the retinoids gives only a small enhancement of that effect. Hence the pan-retinoid receptor agonists

9-cis-RA and 13-cis-RA behave similarly to atRA, while 4-HPR has a different activity profile. A recent study reported synergistic growth inhibition and induction of apoptosis for the combination of trastuzumab and 9-cis-RA in hepatocellular cells [59], suggesting application to a broader range of malignancies; that report demonstrated that trastuzumab inhibited phosphorylation of RXRα and enhanced 9-cis-RA induced RARE and RXRE activity.

The single agents employed in our study have been reported previously to promote accumulation of cells in G0/G1 phase of the cell cycle [36, 60-65]. Our results confirm these findings. Compared to untreated and vehicle-treated cells, the combinations of trastuzumab with various retinoids lead to enhanced accumulation of BT474 cells in G0/G1 phase and SKBR3 cells in G2/M phase, coupled with reduction of cells in S phase. We further demonstrate that the combinations generally lead to a greater reduction of cells in S phase of cell cycle than respective single agents in BT474 cells.

Retinoids have also been demonstrated to modulate breast cancer cell growth through differentiation as well as apoptosis [34, 35], and to cooperate with heregulin to induce morphologic differentiation (branching morphogenesis) in 3D culture [66]. We have found that the single agent retinoids, trastuzumab and tamoxifen individually induce differentiation but not apoptosis in BT474 cells. The combinations of various retinoids and trastuzumab result in greater differentiation than respective single agents in BT474 cells. Compared to untreated and vehicle-treated cells, the single retinoid agents alone induce greater differentiation and greater apoptosis in SKBR3 cells than in BT474 cells. The combinations of various retinoids and trastuzumab result in greater apoptosis than, but similar differentiation as, respective single agents alone in SKBR3 cells.

The combinations of trastuzumab and various retinoids do induce apoptosis in both BT474

and SKBR3 cells. Our findings are consistent with previous studies that show sensitivity to atRA is decreased in HER2-overexpressing breast cancer cells [41, 42]. Consequently, we find targeting of HER2 by trastuzumab in the presence of retinoids induces apoptosis and greater differentiation in BT474 cells; the potentiation of retinoid-induced apoptosis by trastuzumab was modest in SKBR3 cells. The capacities of retinoids to induce differentiation and apoptosis are thus enhanced when trastuzumab inhibits signaling by HER2. Through the induction of apoptosis, greater differentiation, and effects on cell cycle, the combinations of trastuzumab and various retinoids resulted in greater growth inhibition than single agents alone in both BT474 and SKBR3 cells.

Numerous previous studies have suggested promise for the combination of retinoids with anti-estrogens. Tamoxifen was found to potentiate the effect of atRA to inhibit estrogen-induced growth of MCF7 cells [31]. In a rat carcinogen-induced mammary tumor model, the rexinoid bexarotene (Targretin) was able to induce complete remission of the majority of established tumors, and its combination with tamoxifen was more effective than either alone [67]. In this model, there was also some evidence that adding the retinoid to tamoxifen after the development of tamoxifen resistance may restore some sensitivity to tamoxifen, since response rates were higher than when tamoxifen was discontinued and Targretin used instead [68].

A number of clinical trials have explored the therapeutic potential of retinoids in breast cancer patients or as prevention agents. Fenretinide, Targretin, 9-cis-RA, 13-cis-RA and atRA have been examined in clinical trials. A small phase II trial of 13-cis-RA in 18 heavily pretreated (chemotherapy and endocrine therapy refractory) advanced breast cancer patients yielded no objective responses [69]. Fenretinide had relatively mild and reversible toxicity in a small phase II trial in patients with advanced disease but also showed no clinical activity [70]. A small phase

II trial of atRA in patients with hormone refractory metastatic breast cancer showed it to be relatively well tolerated, but noted only 1 partial response among 14 evaluable patients, though there was marked interpatient variability in pharmacokinetics [71]. A large phase III secondary prevention trial using fenretinide for five years after surgical treatment for ductal carcinoma *in situ* or stage I breast cancer revealed no statistically significant effect on the prevention of second contralateral or ipsilateral breast malignancies in the group as a whole, or in distant metastases or survival, although intriguingly there was a reduction of contralateral and ipsilateral breast cancer among pre-menopausal patients in the study [72], which may suggest a specific interaction with estrogen signaling; at a 15-year follow up that study continued to show the same trend [73].

Given the disappointing results for retinoids as single agents in advanced disease, combinations with other agents become of interest. The combinations of tamoxifen with atRA, retinyl acetate, 9-cis-RA, fenretinide, targretin and retinyl palmitate have been studied in clinical trials. A pilot breast cancer chemoprevention trial using fenretinide in combination with tamoxifen is being conducted [74]. This agent is also being tested as secondary chemoprevention (of contralateral breast cancer) as a single agent by the Milan group. In patients with advanced disease in a small phase I trial of the 9-cis-RA/tamoxifen combination, the dose limiting toxicities were headache, hypercalcemia and noncardiogenic pulmonary edema, and of 9 assessable patients there was 1 partial and 1 complete response, both in patients who had ER+ tumors and previous tamoxifen therapy [75]. A phase I/II trial of tamoxifen with or without fenretinide in ER+ or PR+, previously untreated metastatic breast cancer revealed no significant toxicity and improvement or stabilization of disease in 12 of 15 patients [76]. In a phase I/II trial of the atRA/tamoxifen combination in patients with potentially hormone responsive advanced disease, the dose-limiting toxicity was headache and dermatologic toxicity,

and 2 of 7 patients with measurable disease responded while 7 of 18 patients with nonmeasurable but evaluable disease had stable disease [77]. Targretin has been tested in patients with metastatic breast cancer, as monotherapy and in combination with tamoxifen for tamoxifen-resistant patients, however response rates were low, on the order of 3-6%, though up to 20% of patients had some clinical benefit [78]. In a phase II study of tamoxifen plus high-dose retinyl acetate in postmenopausal patients with advanced breast cancer, toxicity was generally mild and an overall response rate of 38.5% was reported [79]. In a phase II trial of the fenretinide/tamoxifen combination specifically in advanced disease patients with ER- tumors or patients with ER+ tumors previously treated with tamoxifen, no objective responses were observed although 3 patients had prolonged stable disease [80]. A recent report with a 2 x 2 trial design found that either low-dose tamoxifen or fenretinide could lower risk of breast neoplasms compared to placebo, but curiously their combination could not, suggesting potential antagonism, although the study was underpowered to detect true differences [81]. A pilot phase II study of interferon-β/retinyl palmitate/tamoxifen in patients with advanced disease showed a clinical response rate of 55% [82]. No reported studies have evaluated the therapeutic effects of a retinoid/trastuzumab combination in a clinical trial, and our results suggest such a strategy could be of benefit.

**Conclusions**

In summary, the combinations of various retinoids with trastuzumab, tamoxifen or both shows strong synergistic inhibition of proliferation accompanied by cell cycle delay, differentiation, and, for retinoid/trastuzumab combinations, apoptosis in both ER+ and ER- human breast cancer cells. The retinoid/trastuzumab combination resulted in enhanced inhibition

of MAPK signaling and down-regulation of RARα and RARß. Treatment with a retinoid and simultaneous inhibition of HER2 and/or ER signaling may thus hold promise as therapy for breast cancer patients.

## Abbreviations

ER, estrogen receptor; SERM, selective estrogen receptor modulator; RAR, retinoic acid receptor; RXR, retinoid X receptor; atRA, all-trans retinoic acid; 9-cis-RA, 9-cis-retinoic acid; 13-cis-RA, 13-cis-retinoic acid; 4-HPR, *N*-(4-hydroxyphenyl) retinamide (fenretinide); RARE, retinoic acid response element; RXRE, retinoid X response element; ERE, estrogen receptor response element; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; HRG, heregulin (neuregulin).

## Competing interests

Drs. Koay, Narayan, Harris and Mrs. Zerillo declare that they have no competing interests. Dr. DiGiovanna has received royalties from DAKO and NeoMarkers, and served in consulting/advisory board roles for Genentech and NovoNordisk.

## Authors' contributions

MPD conceived of the study, participated in its design, and helped to draft the manuscript. DCK and CZ conducted the experiments; DCK primarily drafted the manuscript, and CZ drafted sections of the manuscript. MN and LNH developed the trastuzumab-resistant BT474 cell line. All authors read and approved the final manuscript.

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**Figure legends**

*Figure 1.* Cell cycle analysis. BT474 cells were either untreated or treated with vehicle (EtOH+PBS), 1 μM atRA, 1 μg/ml trastuzumab, 1 μM tamoxifen, 1 μM atRA + 1 μg/ml trastuzumab (Tzmab), 1 μM atRA + 1 μM tamoxifen (Tam), 1 μg/ml trastuzumab (Tzmab) + 1 μM tamoxifen (Tam), or 1 μM atRA + 1 μg/ml trastuzumab (Tzmab) + 1 μM tamoxifen (Tam). On day 2 following treatment, both floating and adherent cells were collected and fixed. The percentages of cells in G0/G1, S, and G2/M phases of cell cycle were determined by flow cytometric analyses. The results shown are the mean of three independent experiments ± standard error.

*Figure 2.* Differentiation of BT474 cells as determined by Nile red staining of neutral lipids. BT474 cells were either untreated or treated with vehicle (EtOH+PBS), 50 ng/ml heregulin β1, 1 μM atRA, 1 μg/ml trastuzumab, 1 μM tamoxifen, 1 μM atRA + 1 μg/ml trastuzumab (Tzmab), 1 μM atRA + 1 μM tamoxifen (Tam), 1 μg/ml trastuzumab (Tzmab) + 1 μM tamoxifen (Tam), or 1 μM atRA + 1 μg/ml trastuzumab (Tzmab) + 1 μM tamoxifen (Tam). On day 6 following treatment, the cells were collected and stained with the fluorescent dye Nile red. The fluorescence intensities of the cells were analyzed by flow cytometry and expressed relative to the intensity of the untreated cells. The results shown are the mean of eight independent experiments ± standard error.

*Figure 3.* Detection of apoptotic BT474 cells by annexin V staining (a) and sub-G1 DNA peak analysis (b). BT-474 cells were either untreated or treated with vehicle (EtOH+PBS), 1 μM atRA, 1 μg/ml trastuzumab, 1 μM tamoxifen, 1 μM atRA + 1 μg/ml trastuzumab (Tzmab), 1 μM

atRA + 1 µM tamoxifen (Tam), 1 µg/ml trastuzumab (Tzmab) +1 µM tamoxifen (Tam), or 1 µM atRA + 1 µg/ml trastuzumab (Tzmab) + 1 µM tamoxifen (Tam). On days 2, 4, and 6 following treatment, both floating and adherent cells were collected and examined by annexin V staining (a) or sub-G1 DNA peak analysis (b). The percentages of annexin V positive cells (a) or cells in sub-G1 DNA peak (b) were determined by flow cytometry. The results shown are the mean of three independent experiments ± standard error.

*Figure 4.* WST-1 proliferation assay of BT474 (a) or SKBR3 (b) cells either untreated or treated for 6 days with vehicle (EtOH+PBS), trastuzumab (Tzmab), various retinoids, or their combinations. The results from WST-1 assays are expressed as a percentage of untreated cells. Each value is the mean of three independent experiments (with 8 replicate wells for each treatment) ± standard error.

*Figure 5.* Cell cycle analysis of BT474 cells either untreated or treated for 2 days with vehicle (EtOH+PBS), trastuzumab (Tzmab), various retinoids, or their combinations. On day 2 following treatment, both floating and adherent cells were collected and fixed. The percentages of cells in G0/G1 (a), S (b), and G2/M (c) phases of cell cycle were determined by flow cytometric analyses. The results shown are the mean of three independent experiments ± standard error.

*Figure 6.* Cell cycle analysis of SKBR3 cells either untreated or treated for 2 days with vehicle (EtOH+PBS), trastuzumab (Tzmab), various retinoids, or their combinations. On day 2 following treatment, both floating and adherent cells were collected and fixed. The percentages of cells in G0/G1 (a), S (b), and G2/M (c) phases of cell cycle were determined by flow cytometric

analyses. The results shown are the mean of three independent experiments ± standard error.

*Figure 7.* Differentiation of BT474 (a) or SKBR3 (b) cells as determined by Nile red staining of neutral lipids. BT474 (a) or SKBR3 (b) cells were either untreated or treated with vehicle (EtOH+PBS), heregulin β1, trastuzumab (Tzmab), various retinoids, or their combinations. On day 6 following treatment, the cells were collected and stained with the fluorescent dye Nile red. The fluorescence intensities of the cells were analyzed by flow cytometry and expressed relative to the intensity of the untreated cells. The results shown are the mean of three independent experiments ± standard error.

*Figure 8.* Detection of apoptotic BT474 (a) or SKBR3 (b) cells by sub-G1 DNA peak analysis. BT474 (a) or SKBR3 (b) cells were either untreated or treated with vehicle (EtOH+PBS), trastuzumab (Tzmab), various retinoids, or their combinations. On day 6 following treatment, both floating and adherent cells were collected and examined by sub-G1 DNA peak analysis. The percentages of cells in sub-G1 DNA peak were determined by flow cytometry. The results shown are the mean of three independent experiments ± standard error.

*Figure 9.* Effect of atRA, trastuzumab or the combination on HER2 expression level and activity, AKT expression level and activity, MAPK expression level and activity, and expression levels of RARα, RARß, RXRα and RXRß in BT474 cells. Drugs used at indicated concentrations. pHER2, pMAPK and pAKT represent phospho-HER2, MAPK and AKT, respectively.

*Figure 10*. ER transcriptional activity in BT474 cells transiently co-transfected with 3xERE-TATA luciferase and CMV-Renilla luciferase vectors following treatment with 1 uM Faslodex, 1 uM atRA, 1 ug/ml trastuzumab (Tzmab), 1 uM tamoxifen (Tam), or their combinations. The firefly luciferase activities of the treated cells have been normalized to their Renilla luciferase activities and are expressed as percentage of activity of untreated cells. The results shown are the mean of 3 independent experiments ± standard error.

*Figure 11*. WST-1 proliferation assay of trastuzumab-resistant BT474 cells treated for 6 days with 10μM GW2974, 1 uM atRA, or their combination, in the presence or absence of trastuzumab (Tzmab) in the culture media. Each bar is normalized to the growth of Control cells in trastuzumab-containing media. Each value is the mean of 3 independent experiments (with 3 replicate wells for each treatment) ± standard error. C = Control = Vehicle treated.

**Table 1. WST-1 proliferation assay for BT-474 cells treated with atRA and trastuzumab[a]**

| | fraction affected (Fa) | | | |
|---|---|---|---|---|
| **dose** | **atRA** | **trastuzumab** | **atRA+Tzmab** | **combination index (CI)** |
| 0.2 | 0.17 ±0.052 | 0.26 ±0.009 | 0.56 ±0.021 | 0.19 ±0.057 |
| 0.4 | 0.28 ±0.056 | 0.51 ±0.006 | 0.79 ±0.037 | 0.01 ±0.003 |
| 0.6 | 0.33 ±0.058 | 0.58 ±0.012 | 0.83 ±0.049 | 0.01 ±0.007 |
| 1 | 0.46 ±0.046 | 0.62 ±0.026 | 0.86 ±0.042 | 0.01 ±0.005 |
| 5 | 0.52 ±0.061 | 0.64 ±0.023 | 0.85 ±0.052 | 0.07 ±0.046 |
| 10 | 0.55 ±0.058 | 0.66 ±0.030 | 0.87 ±0.048 | 0.10 ±0.067 |

[a]The doses of atRA and trastuzumab (Tzmab) are in μM and μg/ml, respectively. Each value is the mean of three independent experiments (with 8 replicate wells for each treatment) ± standard error.

**Table 2. WST-1 proliferation assay for BT-474 cells treated with atRA and tamoxifen[a]**

| | fraction affected (Fa) | | | |
|---|---|---|---|---|
| **dose** | **atRA** | **tamoxifen** | **atRA+Tam** | **combination index (CI)** |
| 0.2 | 0.21 ±0.012 | 0.16 ±0.059 | 0.33 ±0.015 | 0.53 ±0.099 |
| 0.4 | 0.26 ±0.075 | 0.23 ±0.049 | 0.46 ±0.020 | 0.27 ±0.066 |
| 0.6 | 0.37 ±0.026 | 0.29 ±0.075 | 0.51 ±0.035 | 0.24 ±0.062 |
| 1 | 0.47 ±0.043 | 0.33 ±0.032 | 0.60 ±0.027 | 0.16 ±0.048 |
| 5 | 0.51 ±0.067 | 0.42 ±0.029 | 0.62 ±0.050 | 0.64 ±0.072 |
| 10 | 0.53 ±0.071 | 0.59 ±0.045 | 0.69 ±0.050 | 0.61 ±0.106 |

[a]The doses of atRA and tamoxifen (Tam) are in μM. Each value is the mean of three independent experiments (with 8 replicate wells for each treatment) ± standard error.

**Table 3. WST-1 proliferation assay for BT-474 cells treated with trastuzumab and tamoxifen[a]**

| | fraction affected (Fa) | | | |
|---|---|---|---|---|
| **dose** | **trastuzumab** | **tamoxifen** | **Tzmab+Tam** | **combination index (CI)** |
| 0.2 | 0.27 ±0.006 | 0.20 ±0.028 | 0.39 ±0.020 | 1.27 ±0.212 |
| 0.4 | 0.49 ±0.009 | 0.21 ±0.022 | 0.60 ±0.038 | 0.26 ±0.072 |
| 0.6 | 0.58 ±0.012 | 0.34 ±0.040 | 0.69 ±0.026 | 0.13 ±0.027 |
| 1 | 0.64 ±0.017 | 0.36 ±0.021 | 0.75 ±0.021 | 0.10 ±0.017 |
| 5 | 0.66 ±0.012 | 0.48 ±0.015 | 0.82 ±0.022 | 0.19 ±0.047 |
| 10 | 0.68 ±0.012 | 0.64 ±0.012 | 0.90 ±0.012 | 0.07 ±0.016 |

[a]The doses of trastuzumab (Tzmab) and tamoxifen (Tam) are in μg/ml and μM, respectively. Each value is the mean of three independent experiments (with 8 replicate wells for each treatment) ± standard error.

**Table 4. WST-1 proliferation assay for BT-474 cells treated with atRA, trastuzumab, and tamoxifen[a]**

| | fraction affected (Fa) | | | | |
|---|---|---|---|---|---|
| **dose** | **atRA** | **trastuzumab** | **tamoxifen** | **atRA +Tzmab+Tam** | **combination index (CI)** |
| 0.2 | 0.20 ±0.028 | 0.24 ±0.017 | 0.10 ±0.063 | 0.58 ±0.017 | 0.15 ±0.014 |
| 0.4 | 0.29 ±0.041 | 0.48 ±0.032 | 0.15 ±0.036 | 0.84 ±0.007 | 0.01 ±0.001 |
| 0.6 | 0.37 ±0.023 | 0.58 ±0.012 | 0.25 ±0.087 | 0.89 ±0.010 | 0.01 ±0.001 |
| 1 | 0.46 ±0.046 | 0.63 ±0.023 | 0.29 ±0.055 | 0.90 ±0.006 | 0.01 ±0.003 |
| 5 | 0.55 ±0.038 | 0.65 ±0.015 | 0.37 ±0.055 | 0.89 ±0.009 | 0.06 ±0.011 |
| 10 | 0.57 ±0.041 | 0.66 ±0.030 | 0.59 ±0.048 | 0.90 ±0.003 | 0.09 ±0.020 |

[a]The doses of atRA, trastuzumab (Tzmab), and tamoxifen (Tam) are in μM, μg/ml, and μM, respectively. Each value is the mean of three independent experiments (with 8 replicate wells for each treatment) ± standard error.

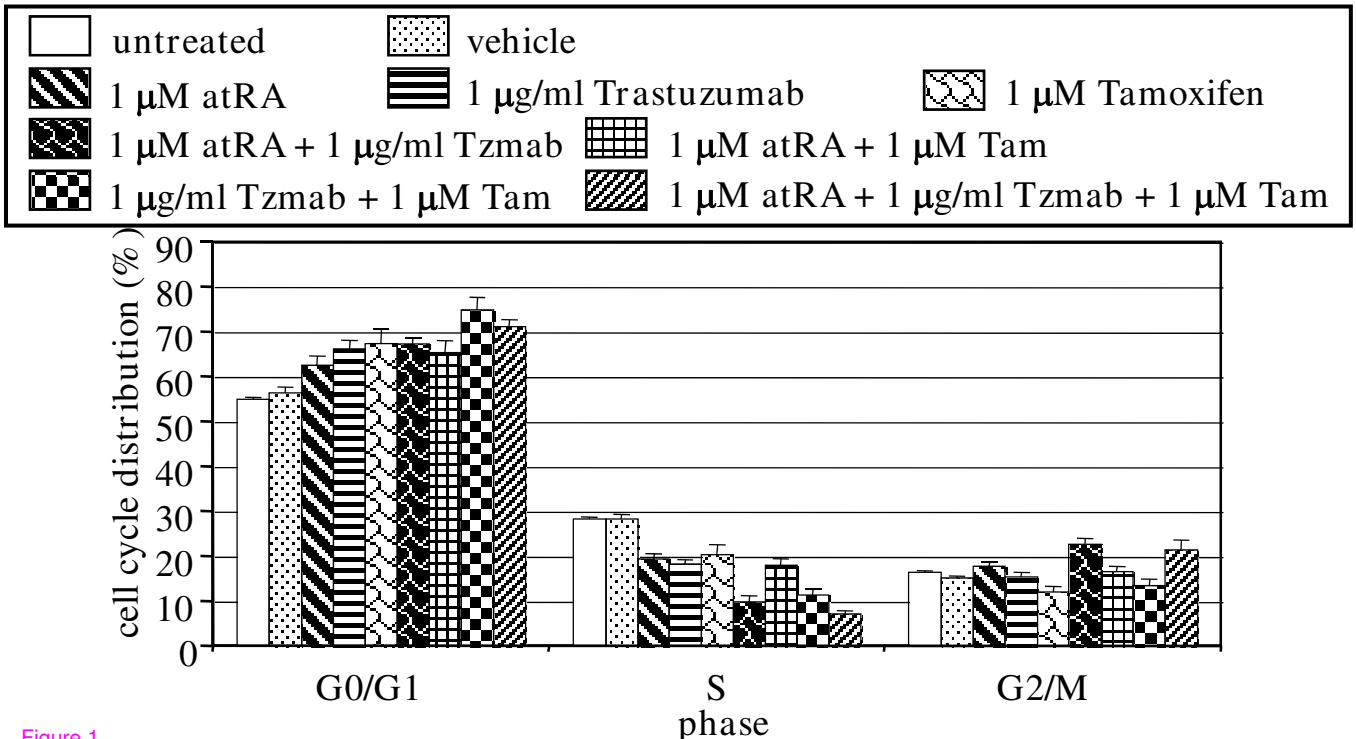

BT-474 Cells
untreated
vehicle
1 μM atRA
1 μg/ml Trastuzumab
1 μM Tamoxifen
1 μM atRA + 1 μg/ml Tzmab
1 μM atRA + 1 μM Tam
1 μg/ml Tzmab + 1 μM Tam
1 μM atRA + 1 μg/ml Tzmab + 1 μM Tam
cell cycle distribution (%)
90
80
70
60
50
40
30
20
10
0
G0/G1
S
G2/M
phase

Figure 1

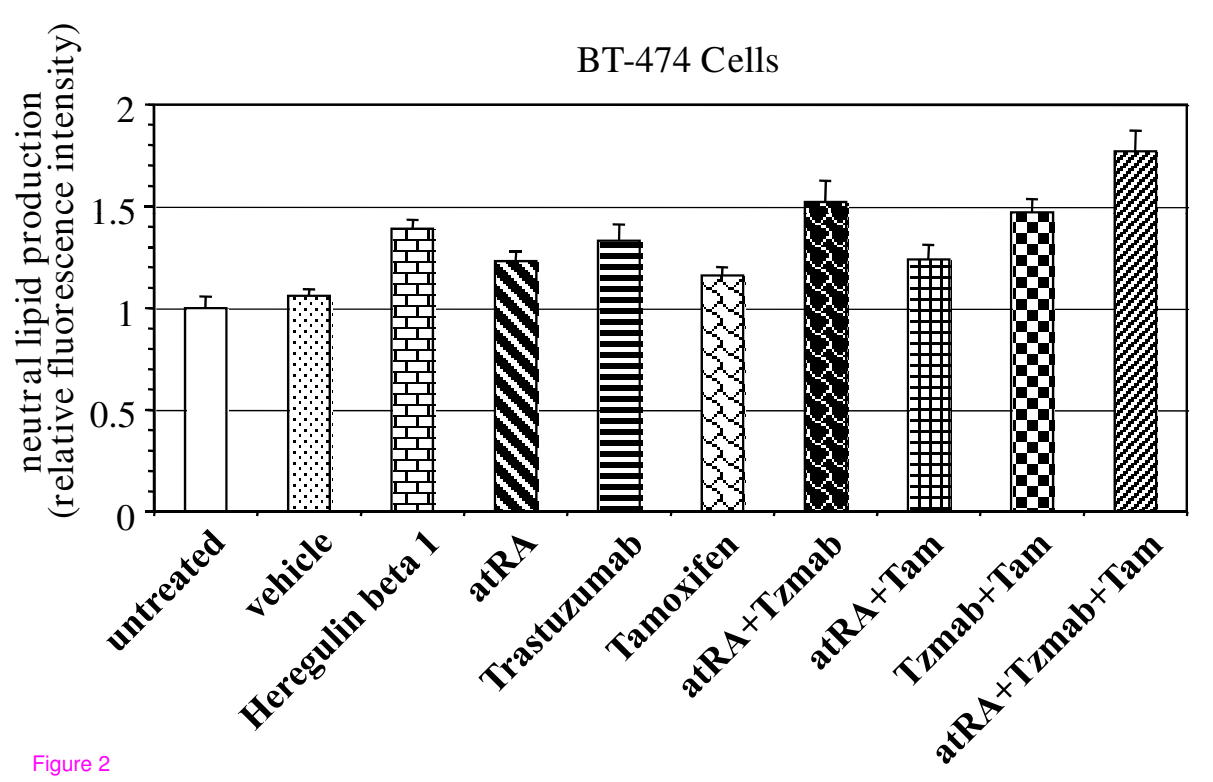

BT-474 Cells
neutral lipid production
(relative fluorescence intensity)
2
1.5
1
0.5
0
untreated
vehicle
Heregulin beta 1
atRA
Trastuzumab
Tamoxifen
atRA+Tzmab
atRA+Tam
Tzmab+Tam
atRA+Tzmab+Tam

Figure 2

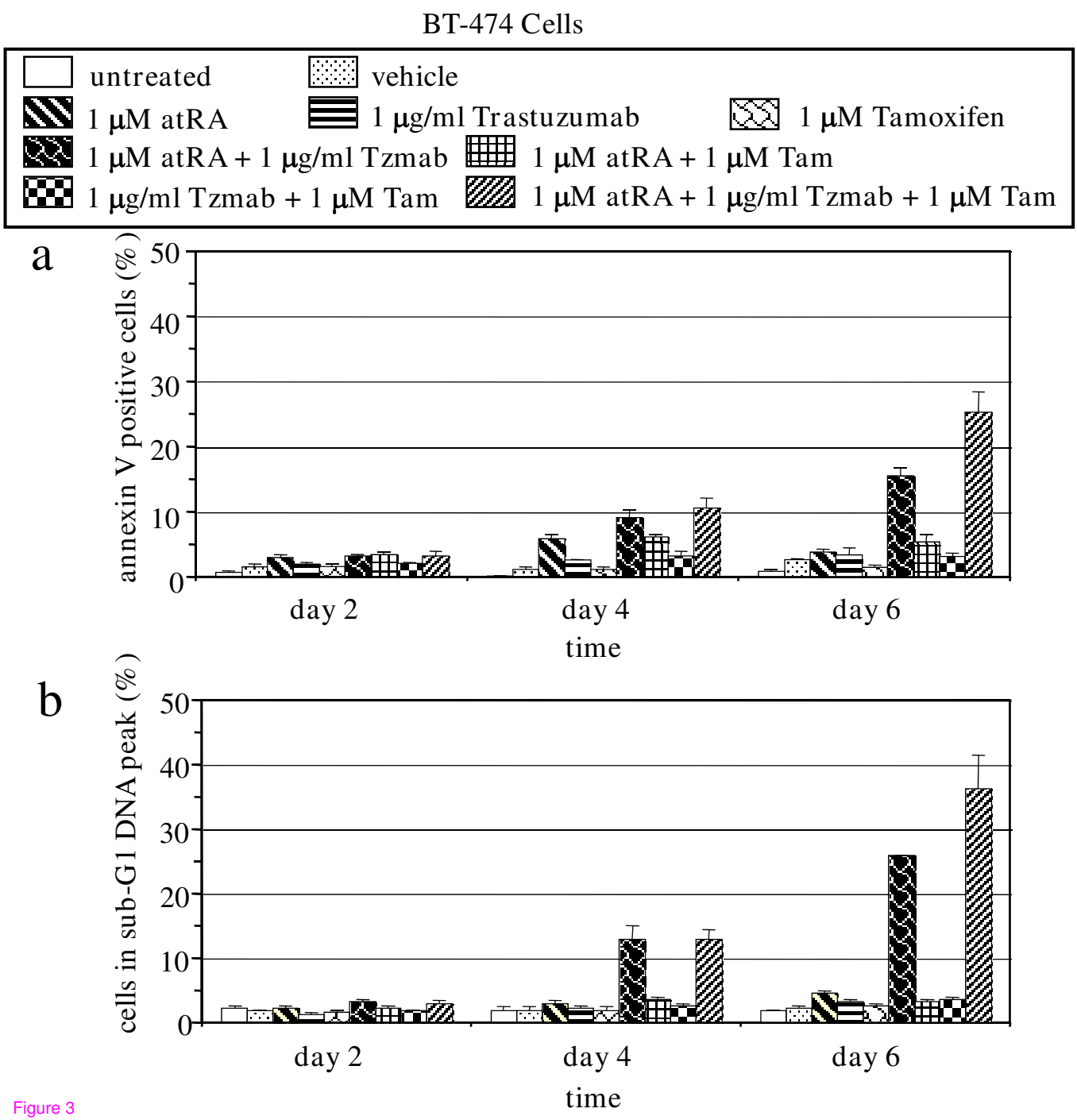

BT-474 Cells
untreated
vehicle
1 μM atRA
1 μg/ml Trastuzumab
1 μM Tamoxifen
1 μM atRA + 1 μg/ml Tzmab
1 μM atRA + 1 μM Tam
1 μg/ml Tzmab + 1 μM Tam
1 μM atRA + 1 μg/ml Tzmab + 1 μM Tam
a
annexin V positive cells (%)
0
10
20
30
40
50
day 2
day 4
day 6
time
b
cells in sub-G1 DNA peak (%)
0
10
20
30
40
50
day 2
day 4
day 6
time

Figure 3

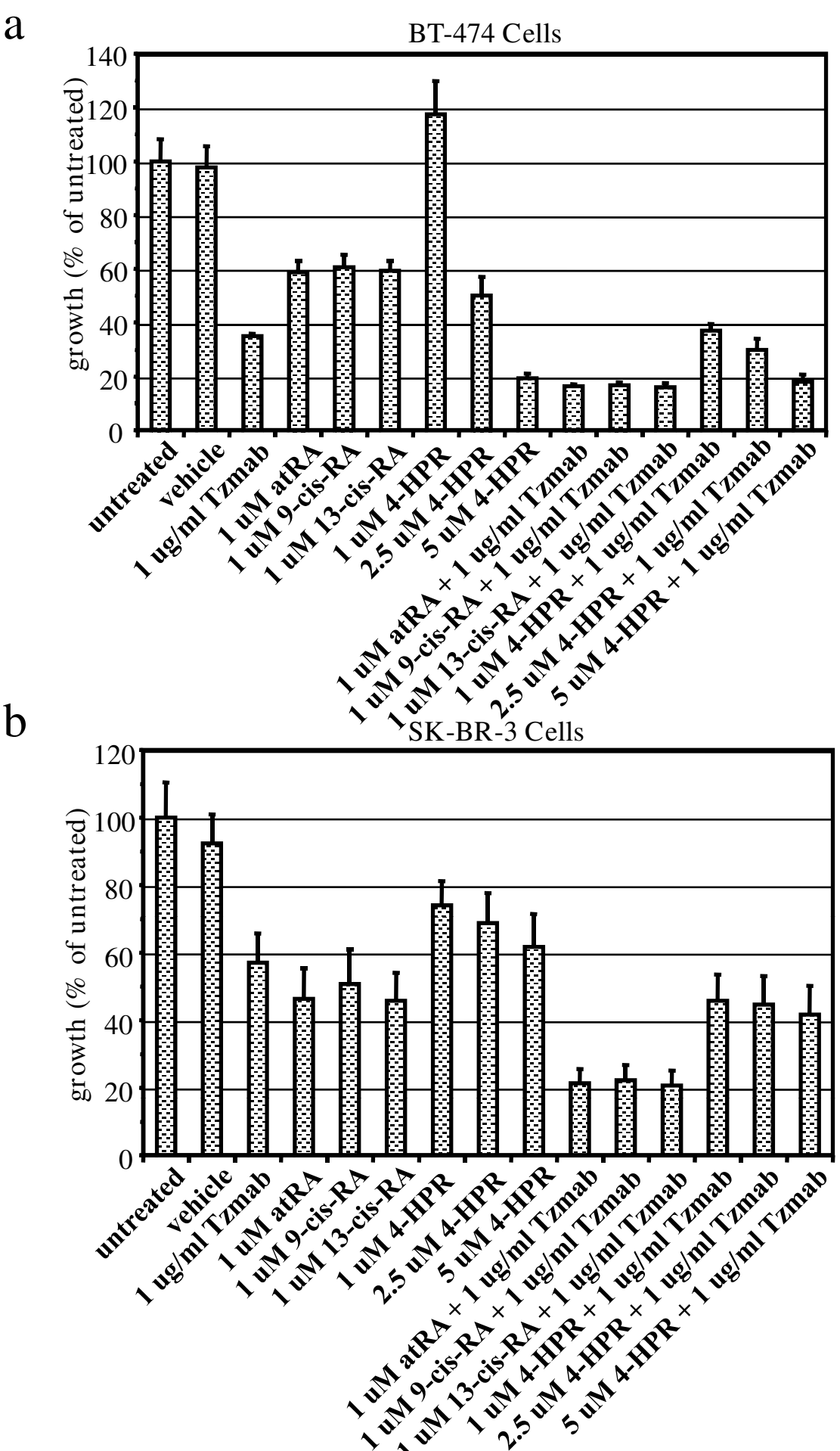

a
BT-474 Cells
growth (% of untreated)
0
20
40
60
80
100
120
140
untreated
vehicle
1 ug/ml Tzmab
1 uM atRA
1 uM 9-cis-RA
1 uM 13-cis-RA
1 uM 4-HPR
2.5 uM 4-HPR
5 uM 4-HPR
1 uM atRA + 1 ug/ml Tzmab
1 uM 9-cis-RA + 1 ug/ml Tzmab
1 uM 13-cis-RA + 1 ug/ml Tzmab
1 uM 4-HPR + 1 ug/ml Tzmab
2.5 uM 4-HPR + 1 ug/ml Tzmab
5 uM 4-HPR + 1 ug/ml Tzmab
b
SK-BR-3 Cells
growth (% of untreated)
0
20
40
60
80
100
120
untreated
vehicle
1 ug/ml Tzmab
1 uM atRA
1 uM 9-cis-RA
1 uM 13-cis-RA
1 uM 4-HPR
2.5 uM 4-HPR
5 uM 4-HPR
1 uM atRA + 1 ug/ml Tzmab
1 uM 9-cis-RA + 1 ug/ml Tzmab
1 uM 13-cis-RA + 1 ug/ml Tzmab
1 uM 4-HPR + 1 ug/ml Tzmab
2.5 uM 4-HPR + 1 ug/ml Tzmab
5 uM 4-HPR + 1 ug/ml Tzmab

Figure 4

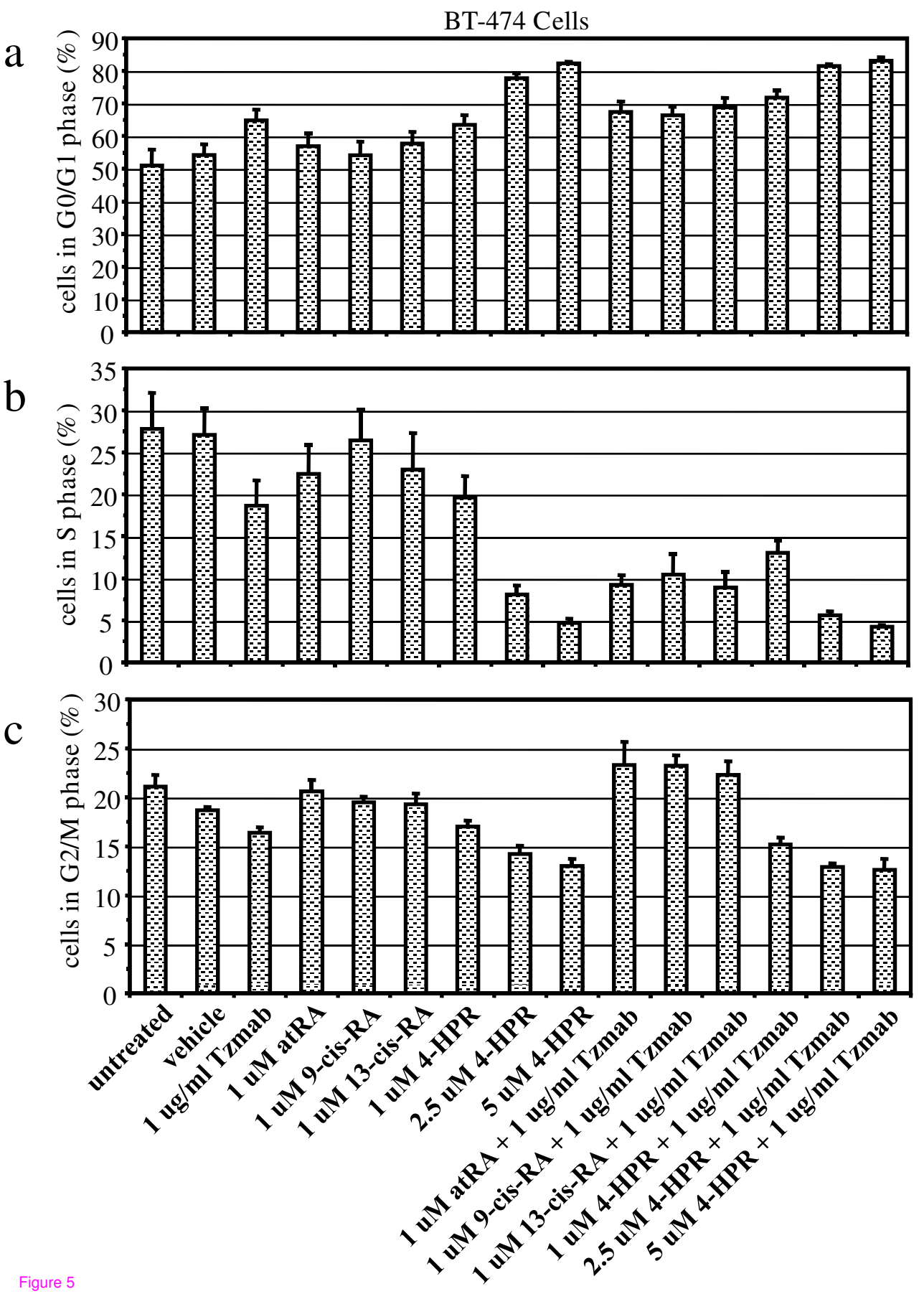

BT-474 Cells
a
cells in G0/G1 phase (%)
0
10
20
30
40
50
60
70
80
90
b
cells in S phase (%)
0
5
10
15
20
25
30
35
c
cells in G2/M phase (%)
0
5
10
15
20
25
30
untreated
vehicle
1 ug/ml Tzmab
1 uM atRA
1 uM 9-cis-RA
1 uM 13-cis-RA
1 uM 4-HPR
2.5 uM 4-HPR
5 uM 4-HPR
1 uM atRA + 1 ug/ml Tzmab
1 uM 9-cis-RA + 1 ug/ml Tzmab
1 uM 13-cis-RA + 1 ug/ml Tzmab
1 uM 4-HPR + 1 ug/ml Tzmab
2.5 uM 4-HPR + 1 ug/ml Tzmab
5 uM 4-HPR + 1 ug/ml Tzmab

Figure 5

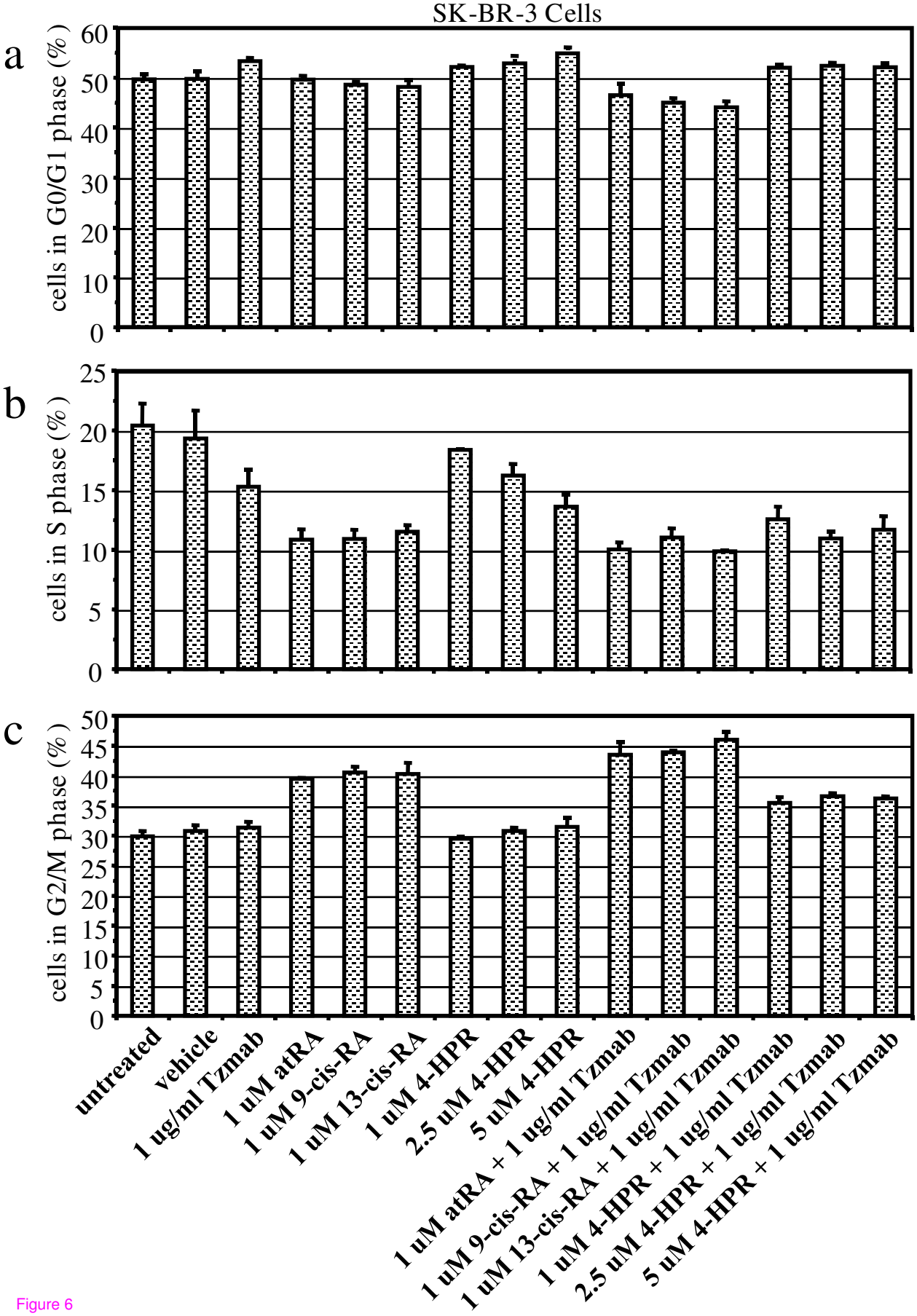

SK-BR-3 Cells
a
cells in G0/G1 phase (%)
60
50
40
30
20
10
0
b
cells in S phase (%)
25
20
15
10
5
0
c
cells in G2/M phase (%)
50
45
40
35
30
25
20
15
10
5
0
untreated
vehicle
1 ug/ml Tzmab
1 uM atRA
1 uM 9-cis-RA
1 uM 13-cis-RA
1 uM 4-HPR
2.5 uM 4-HPR
5 uM 4-HPR
1 uM atRA + 1 ug/ml Tzmab
1 uM 9-cis-RA + 1 ug/ml Tzmab
1 uM 13-cis-RA + 1 ug/ml Tzmab
1 uM 4-HPR + 1 ug/ml Tzmab
2.5 uM 4-HPR + 1 ug/ml Tzmab
5 uM 4-HPR + 1 ug/ml Tzmab

Figure 6

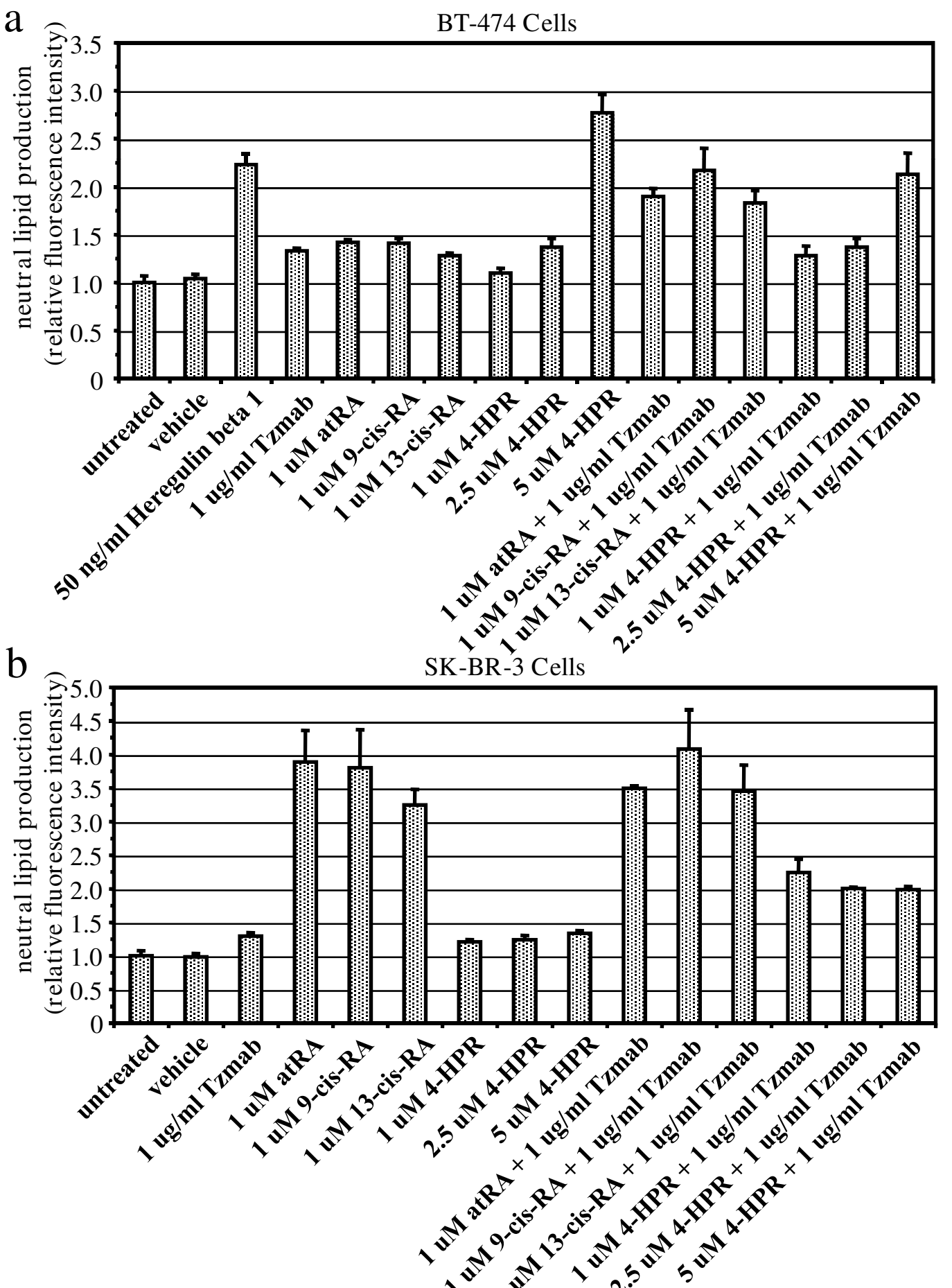

a
BT-474 Cells
neutral lipid production
(relative fluorescence intensity)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
untreated
vehicle
50 ng/ml Heregulin beta 1
1 ug/ml Tzmab
1 uM atRA
1 uM 9-cis-RA
1 uM 13-cis-RA
1 uM 4-HPR
2.5 uM 4-HPR
5 uM 4-HPR
1 uM atRA + 1 ug/ml Tzmab
1 uM 9-cis-RA + 1 ug/ml Tzmab
1 uM 13-cis-RA + 1 ug/ml Tzmab
1 uM 4-HPR + 1 ug/ml Tzmab
2.5 uM 4-HPR + 1 ug/ml Tzmab
5 uM 4-HPR + 1 ug/ml Tzmab
b
SK-BR-3 Cells
neutral lipid production
(relative fluorescence intensity)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
untreated
vehicle
1 ug/ml Tzmab
1 uM atRA
1 uM 9-cis-RA
1 uM 13-cis-RA
1 uM 4-HPR
2.5 uM 4-HPR
5 uM 4-HPR
1 uM atRA + 1 ug/ml Tzmab
1 uM 9-cis-RA + 1 ug/ml Tzmab
1 uM 13-cis-RA + 1 ug/ml Tzmab
1 uM 4-HPR + 1 ug/ml Tzmab
2.5 uM 4-HPR + 1 ug/ml Tzmab
5 uM 4-HPR + 1 ug/ml Tzmab

Figure 7

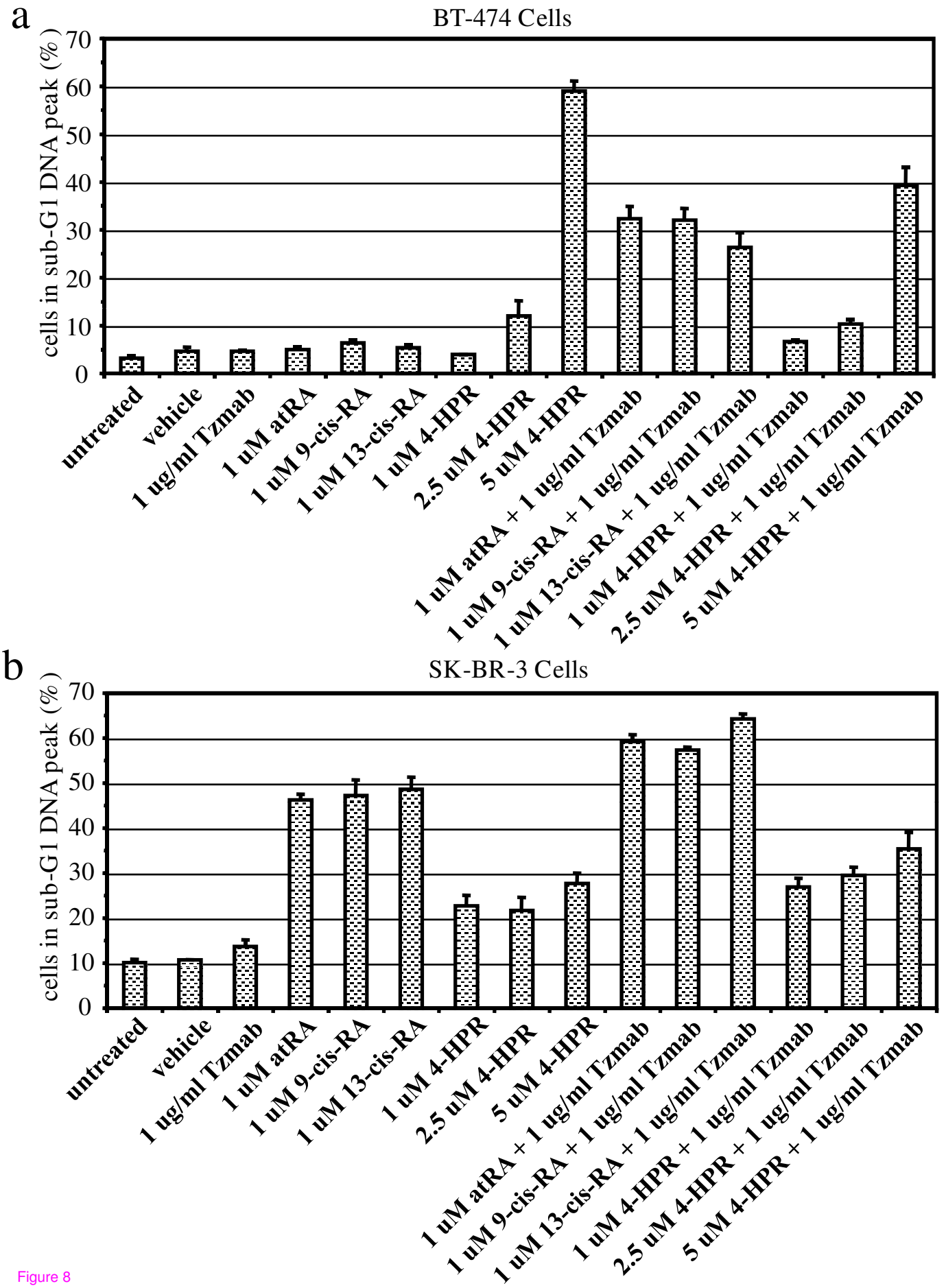

a
BT-474 Cells
cells in sub-G1 DNA peak (%)
0
10
20
30
40
50
60
70
untreated
vehicle
1 ug/ml Tzmab
1 uM atRA
1 uM 9-cis-RA
1 uM 13-cis-RA
1 uM 4-HPR
2.5 uM 4-HPR
5 uM 4-HPR
1 uM atRA + 1 ug/ml Tzmab
1 uM 9-cis-RA + 1 ug/ml Tzmab
1 uM 13-cis-RA + 1 ug/ml Tzmab
1 uM 4-HPR + 1 ug/ml Tzmab
2.5 uM 4-HPR + 1 ug/ml Tzmab
5 uM 4-HPR + 1 ug/ml Tzmab
b
SK-BR-3 Cells
cells in sub-G1 DNA peak (%)
0
10
20
30
40
50
60
70
untreated
vehicle
1 ug/ml Tzmab
1 uM atRA
1 uM 9-cis-RA
1 uM 13-cis-RA
1 uM 4-HPR
2.5 uM 4-HPR
5 uM 4-HPR
1 uM atRA + 1 ug/ml Tzmab
1 uM 9-cis-RA + 1 ug/ml Tzmab
1 uM 13-cis-RA + 1 ug/ml Tzmab
1 uM 4-HPR + 1 ug/ml Tzmab
2.5 uM 4-HPR + 1 ug/ml Tzmab
5 uM 4-HPR + 1 ug/ml Tzmab

Figure 8

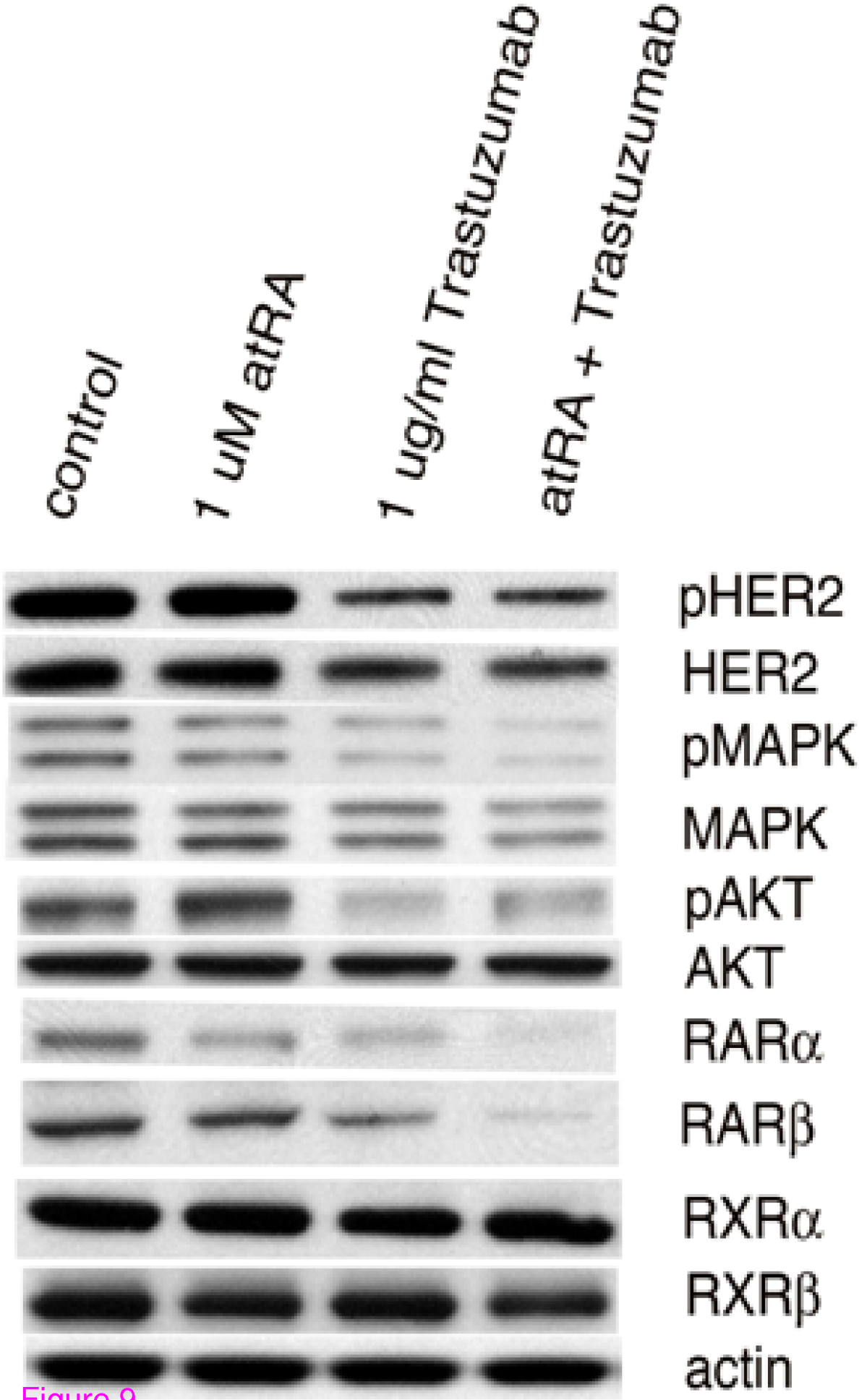

control
1 uM atRA
1 ug/ml Trastuzumab
atRA + Trastuzumab
pHER2
HER2
pMAPK
MAPK
pAKT
AKT
RARα
RARβ
RXRα
RXRβ
actin

Figure 9

Figure 10

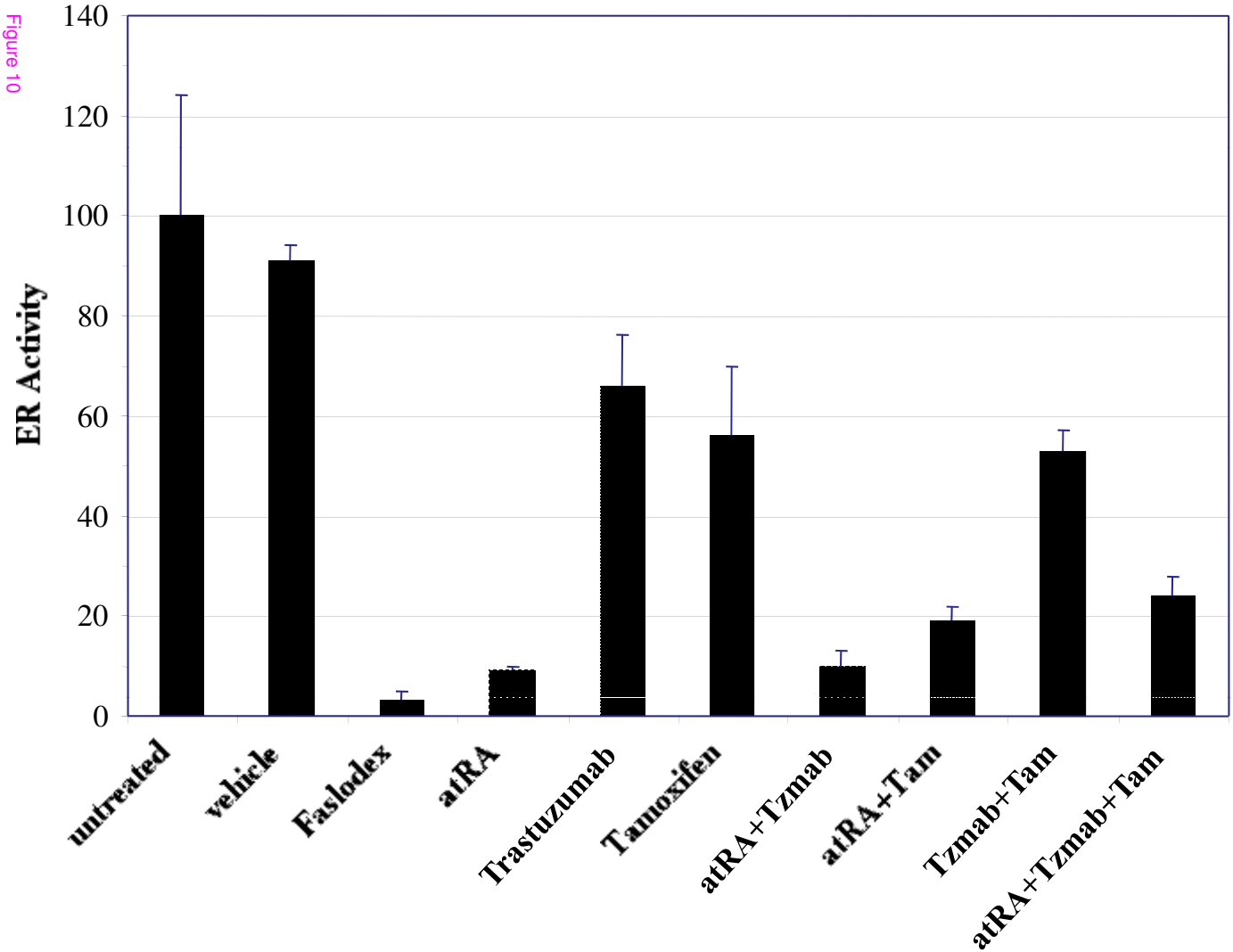
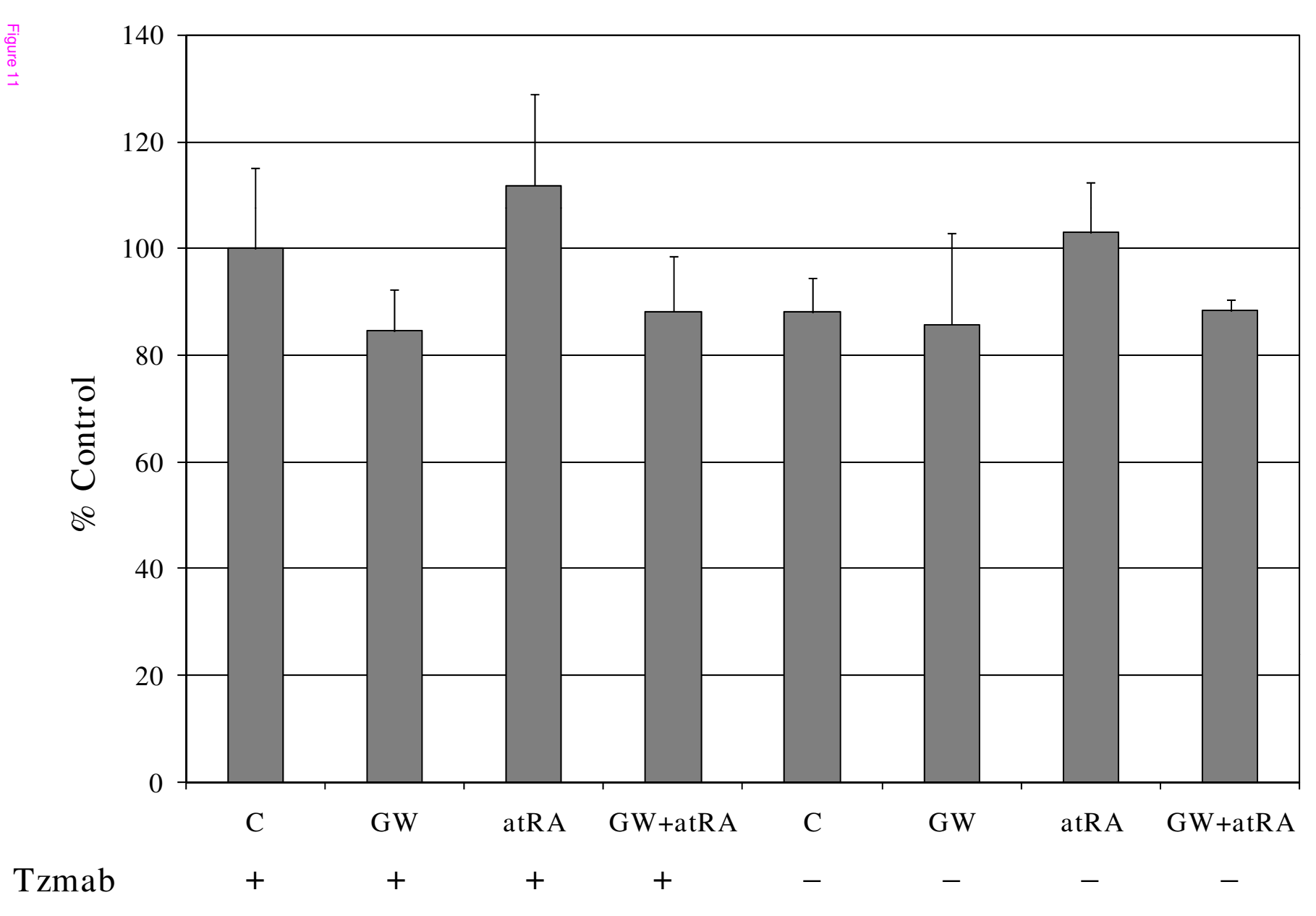

ER Activity
140
120
100
80
60
40
20
0
untreated
vehicle
Faslodex
atRA
Trastuzumab
Tamoxifen
atRA+Tzmab
atRA+Tam
Tzmab+Tam
atRA+Tzmab+Tam

Figure 11


140
120
100
80
60
40
20
0
% Control
C
GW
atRA
GW+atRA
C
GW
atRA
GW+atRA
Tzmab
+
+
+
+
–
–
–
–