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Reduced Dose and Intermittent Treatment with Lapatinib and Trastuzumab for Potent Blockade of the HER Pathway in HER2/*neu*-Overexpressing Breast Tumor Xenografts

Mothaffar F. Rimawi[1,2,3], Lisa S. Wiechmann[4], Yen-Chao Wang[1], Catherine Huang[1,5], Ilenia Migliaccio[1], Meng-Fen Wu[2], Carolina Gutierrez[1], Susan G. Hilsenbeck[1,2], Grazia Arpino[1], Suleiman Massarweh[6], Robin Ward[1], Robert Soliz[1], C. Kent Osborne[1,2,3,5], and Rachel Schiff[1,2,3,5]

Abstract

Purpose: We have shown that incomplete blockade of the human epidermal growth factor (HER) pathway is a mechanism of resistance to treatment with trastuzumab (T) in HER2-overexpressing tumor xenografts. We now investigate whether the addition of lapatinib (L), a dual HER1/2 kinase inhibitor, to T results in more potent inhibition of the pathway and therefore inhibition of tumor growth, and whether reduced dose and intermittent treatment with the combination is equally effective.

Experimental Design: Nude mice bearing HER2-overexpressing MCF7/HER2–18 or BT-474 xenograft tumors were treated with L and T, alone or in various combinations with other HER inhibitors. L + T for short duration (14 and 42 days), intermittent administration (14 days on/off), and reduced dosing (half dose) was also investigated. Inhibition of tumor growth, downstream signaling, proliferation, and induction of apoptosis were assessed. All statistical tests were two-sided.

Results: L + T was the most effective regimen in both MCF7/HER2–18 and BT-474 xenografts with complete regression (CR) of tumor observed in all mice. Intermittent and reduced dose treatment ($^1/_2$ dose) resulted in high rates of CR and low rates of tumor recurrence that were comparable to full dose continuous treatment. L + T resulted in significantly reduced downstream signaling and proliferation, and increased apoptosis.

Conclusions: L + T is a potent and effective combination even when given in reduced dose or intermittent schedule potentially resulting in lower toxicity and reduced cost if translated to patients. These findings warrant timely clinical testing. *Clin Cancer Res; 17(6); 1351–61. ©2011 AACR.*

Background

Human epidermal growth factor receptor 2 (c-ErbB2, HER2/*neu*, or HER2) is a clinically important therapeutic target in patients with HER2-overexpressing breast cancers. It is a component of a robust and complex network comprised of 4 tyrosine kinase receptors, HER1–4, which can be activated by multiple ligands which induce homo- and heterodimerization. HER2 does not have a ligand and, therefore, is activated by partnering with itself or another family member (1–7). The pathway can also be activated by alterations downstream of the HER receptor layer including loss of the tumor suppressor gene *PTEN* or activating mutations in PI3K that may cause resistance to trastuzumab (T; refs. 8–10).

Trastuzumab, a humanized monoclonal antibody directed at the HER2 extracellular domain, inhibits this pathway. Its use resulted in significant reductions in recurrence and mortality in patients with HER2-positive breast cancer (11, 12). However, *de novo* and acquired drug resistance remain a clinical problem (13, 14).

Lapatinib (L), a dual HER1 and HER2 tyrosine kinase inhibitor, is approved for treatment of metastatic HER2-positive breast cancer and is being investigated in various clinical settings. It would be expected to effectively block the receptor layer by inhibiting signals generated by multiple dimer pairs (15–17). On the basis of our early report and data from other groups, L combined with T is now being studied in the clinical setting (16–20).

We investigated the effect of L alone or in combination with other anti-HER agents in 2 xenograft models and identified L + T as the most potent combination. Given concerns about the toxicity and cost of long-term treatment with these expensive agents, we further

Authors' Affiliation: [1]Lester and Sue Smith Breast Center; [2]Dan L. Duncan Cancer Center; [3]Margaret M. and Albert B. Alkek Department of Medicine, Baylor College of Medicine, Houston, Texas; [4]Montefiore–Einstein Center for Cancer Care, Montefiore Medical Center, Bronx, New York; [5]Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas; and [6]Markey Cancer Center, University of Kentucky, Lexington, Kentucky

Note: M.F. Rimawi and L.S. Wiechmann contributed equally.

Corresponding Authors: Mothaffar F. Rimawi or Rachel Schiff, Lester & Sue Smith Breast Center, Baylor College of Medicine, One Baylor Plaza, BCM660 Houston, TX 77030. Phone: 713-798-1311; Fax: 713-798-8884. E-mail: rimawi@bcm.edu or rschiff@bcm.edu

Translational Relevance

We have previously shown that combination anti-HER therapies are more effective than single agents in HER2-overexpressing breast cancer models by providing more potent blockade of downstream signaling. The present preclinical study investigates drugs targeting the pathway by different mechanisms and examines different doses and schedules in HER2-overexpressing xenografts. Lapatinib + trastuzumab was the most potent combination in blocking HER signaling, and it led to tumor eradication in most mice. Interestingly, intermittent or reduced dosing schedules were just as effective as continuous therapy.

These data provide a rationale for clinical trials of intermittent or reduced doses of lapatinib + trastuzumab therapy, strategies that, if as effective as continuous full dose treatment, would dramatically reduce the toxicity and cost of treatment.

investigated reduced dosing and intermittent scheduling of this potent combination.

Methods

Reagents, hormones, and antibodies

Sixty-day release, 17β-estradiol pellets (E2; 0.36 mg) were purchased from Innovative Research and tamoxifen citrate (Tam; 500 μg subcutaneously in peanut oil, 5 d/wk) was purchased from Sigma. L (100 mg/kg free base active ingredient via gavage in 1% Tween once a day, 5 d/wk) was provided by GlaxoSmithKline. Gefitinib (G; 100 mg/kg via gavage in 1% Tween 80 5 d/wk) was provided by AstraZeneca. T (10 mg/kg intraperitoneally in sterile H_2O twice a week) and pertuzumab (P; 12 mg/kg intraperitoneally the first week and then 6 mg/kg intraperitoneally in 1% sterile PBS weekly) were provided by Genentech. Antibodies used for immunoblotting were as follows: phosphorylated (p)-Tyr1248-HER2 (Millipore); total HER2, total and phosphorylated forms of AKT (Thr308), extracellular signal regulated kinase 1 and 2, mitogen activated protein kinase (MAPK; Thr202/Tyr204), and β-actin (Cell Signaling Technology).

Immunohistochemistry

Tumor tissue was fixed in 4% neutral-buffered formalin overnight before processing and paraffin embedding. Immunohistochemistry (IHC) was done on 4-μ sections from randomly arrayed in 4-mm core tissue arrays. Bromodeoxyuridine (BrDU) labeling of tumor cell nuclei was visualized by staining with BrDU antibody (Biogenic). Additional sections were used to stain apoptotic cells using the cleaved caspase 3/7 antibody (Cell Signaling Technology) and for activated MAPK using the p-MAPK antibody (Cell Signaling Technology) as previously described (21, 22).

Tumors were scored by percent of positive cells for BrDU and cleaved caspase 3/7 staining, and by Allred score for the activated MAPK staining (21, 23).

Tumor extracts and immunoblots

Frozen tumors from the different treatment groups were homogenized in lysis buffer containing 1% Triton X-100, 50 mmol/L HEPES, pH 7.4, 150 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EGTA, 100 mmol/L NaF, 10 mmol/L NaPPi, 10% glycerol, 1 mmol/L phenylmethylsulfonyl-fluoride, 1 mmol/L Na_3VO_4, 10 μg/mL aprotinin, and 1× protease inhibitor cocktail (Roche Molecular Biochemicals). Tumor lysates were microcentrifuged at $14,000 \times g$ for 10 minutes at 4°C. Cell supernatants were aliquoted and stored at −70°C. Protein concentration was measured by the Bio-Rad Protein Assay kit (Bio-Rad Laboratories). Equivalent amounts of protein (25 μg) from each sample were separated by electrophoresis on 8% to 16% polyacrylamide gels containing SDS (SDS-PAGE), and transferred by electroblotting onto nitrocellulose membranes (Schleicher & Schuell) followed by immunoblotting with the specific antibodies as previously described (21). For all antibodies the reaction was in Odyssey Blocking Buffer (LI-COR Biosciences) + 0.05% Tween-20 overnight at 4°C. The blots were washed 3 times in PBS with Tween-20 (PBST) and then incubated for 1 hour at room temperature with fluorescently labeled secondary antibody in Odyssey Blocking Buffer + 0.05% Tween-20. The blots were then washed in PBST, after which the labeled protein was quantified by Odyssey Infrared Imaging System. Gels were reproduced at least twice. In each tumor sample, protein levels were corrected with β-actin (protein levels/actin levels $\times 10^2$). Means of corrected expression levels were calculated and fold changes compared with the endocrine treatment alone group (without anti-HER therapy) are presented for each treatment group. Representative blots are presented.

Xenograft studies

MCF/HER2–18 (HER2 transfected) and BT-474 (gene-amplified for HER2) cells were maintained as described (21, 24, 25). Animal care was in accordance with institutional guidelines. MCF7/HER2–18 and BT-474 xenografts were established in ovariectomized 5- to 6-week-old athymic mice (Harlan Sprague Dawley) supplemented with estrogen pellets by inoculating subcutaneously (5×10^6 cells) as described previously (21, 26). When tumors reached the size of 200–250 μL (2–4 weeks), mice bearing MCF/HER2–18 xenografts were randomly allocated to continued estrogen (E2), estrogen deprivation alone (ED) by removal of the estrogen pellets, or ED + Tam (26). Mice treated with ED + Tam were then randomized to receive L or T alone, L + T, double dose L (2L), L + G, or L + P. Mice treated with E2 and mice treated by ED were randomly allocated to vehicle, L, T, or L + T. Another group was treated with continued E2, Tam, and L + T. Each treatment group contained a minimum of 12 mice. Groups of animals destined for molecular studies contained a minimum of 8 additional mice.

Animals bearing BT-474 xenografts were randomly allocated to E2, ED alone, ED + L, ED + T, ED + L + T, and E2 + L + T. Additional mice were treated with L + T for short duration (14 and 42 days), intermittent administration

(14 days on/14 days off), and dose reduction ($^1/_2$L + $^1/_2$T). Each treatment group contained a minimum of 10 mice. Groups of animals destined for molecular studies contained a minimum of 6 additional mice.

Tumor volumes were measured weekly as described previously (21, 26). Mice were sacrificed and tumors were harvested after short-term treatment (3 days) for analysis of associated biomarkers, when they reached the size of 1000 μL, or at the end of the experiments. Each tumor analyzed was from a different mouse; tumor tissues were removed from each individual mouse and kept at −190°C or formalin-fixed and paraffin embedded for later analyses.

Statistical analysis

Tumor growth curves were constructed using the mean tumor volume at each time point with error bars representing the standard error of the mean. Animals that died of other causes prior to the first animal developing a resistant tumor were not included in the calculation of tumor growth curves but were included in all other analyses. Time to treatment resistance (TTR), defined as time in days to developing tumors that are 2.5 times the volume at baseline, was calculated for each mouse. In the experiments with intermittent scheduling and reduced dosing, to detect small but significant differences, the rate of tumor progression was measured at day 315 (TP315). A tumor was considered to have progressed if 2 consecutive 10% or greater increases in tumor size were detected starting from a tumor size greater than 0.

The median TTR along with 95% CI were estimated using the Kaplan–Meier method and compared by generalized Wilcoxon test. P values for the xenograft studies were adjusted for multiple comparisons using the Hommel method to control for type I error when appropriate. Complete regression (CR) was defined as complete tumor disappearance for at least 3 consecutive measurements. CR rates were calculated based on the total number of animals treated in each group. Pairwise comparisons of tumor proliferation and survival pathways based on IHC assessments were made using nonparametric Wilcoxon rank-sum test. All statistical tests were two-sided.

Results

L+ T combination in MCF7/HER2–18 tumors

As we have previously shown, in MCF7/HER2–18 tumor xenografts concurrent targeting of the HER pathway and the estrogen receptor (ER) greatly enhances therapeutic efficacy (21). Although treatment with L alone, T alone, or L + T significantly delayed estrogen stimulated growth and prolonged TTR in this model (Fig. 1A and Table 1), the benefit was short-lived and tumor growth resumed.

Figure 1B shows the growth curves in tumors treated with tamoxifen alone or along with HER inhibitors, and Table 1 shows the CR rate and the median TTR in these tumors. As previously shown, this xenograft model is tamoxifen stimulated due to, at least in part, crosstalk between non-nuclear ER and the HER pathway (24, 27–29). When

tamoxifen-treated mice were treated with single anti-HER agents (Tam + L and Tam + T), CR occurred in some mice and TTR was significantly prolonged (Table 1). However, in mice treated with the combination (Tam + L + T), CR was observed in 86% of mice (12/14; Fig. 1B). The median TTR was significantly prolonged with this combination to 229 days (95% CI: 170–342) although resistance to this combination eventually emerged. This combination was effective even in the presence of estrogen (E2 + Tam + L + T;Fig. 1C and Table 1).

In addition, ED was used to target ER to mimic aromatase inhibitor therapy in postmenopausal patients. Figure 1D shows that the addition of L or T to ED improved the CR rate and the TTR. However, the most effective regimen was ED combined with L + T. CR was observed in 85% (11/13) of tumors and after 231 days, only 2 mice had resistant tumors ($P < 0.0001$, 0.039, and 0.035 compared with ED, ED + L, and ED + T, respectively; generalized Wilcoxon test with Hommel adjustment).

After 231 days, L + T treatment was stopped in 9 mice with no evidence of tumor and they were randomized to retreatment with estrogen (4 mice) or continued ED (5 mice), and followed for 70 additional days. Two of the 4 mice retreated with estrogen had tumor regrowth, whereas none of the mice continued on ED showed regrowth of tumors.

Other inhibitors of the HER pathway in MCF7/HER2–18 tumors

To better understand the mechanism of action and potency of L + T, we evaluated other HER targeting regimens (Fig. 1E). To exclude the possibility that L + T was more effective than L alone because of suboptimal dosing of L and based on published data suggesting that higher dose of L may be more effective (30, 31), 1 group of mice was given a double dose of L (200 mg/kg, 5 d/wk; Tam + 2L). Because L is a less potent inhibitor of HER1 than HER2, another group was treated with L and G for more potent epidermal growth factor receptor inhibition (Tam + L + G). Finally, L was combined with P (2C4; Genentech), a monoclonal antibody that inhibits HER2 dimerization, to determine if this potent inhibitor could replace T in the combination (Tam + L + P; Table 1).

As shown in Table 1 and Figure 1E, doubling the L dose (Tam + 2L) did not improve efficacy and although several animals had only small residual tumors, none achieved CR. The median TTR was 150 days [95% CI: 122–not achieved (NA)]. Adding G to L (Tam + L + G) also did not improve the CR rate (8%) or the median TTR (147 days, 95% CI: 109–NA). In comparison with Tam + L + T, the substitution of P for T (Tam + L + P) led to inferior results with lower tumor regression rate (14%) and a shorter median TTR (178 days, 95% CI: 138–NA). Although the P value adjusted for multiple comparisons did not achieve statistical significance when comparing TTR in the Tam + L + P group to the Tam + L + T group, the notable difference in CR (Tam + L + P, 14%; Tam + L + T, 86%) suggests that Tam + L + T is the superior regimen.
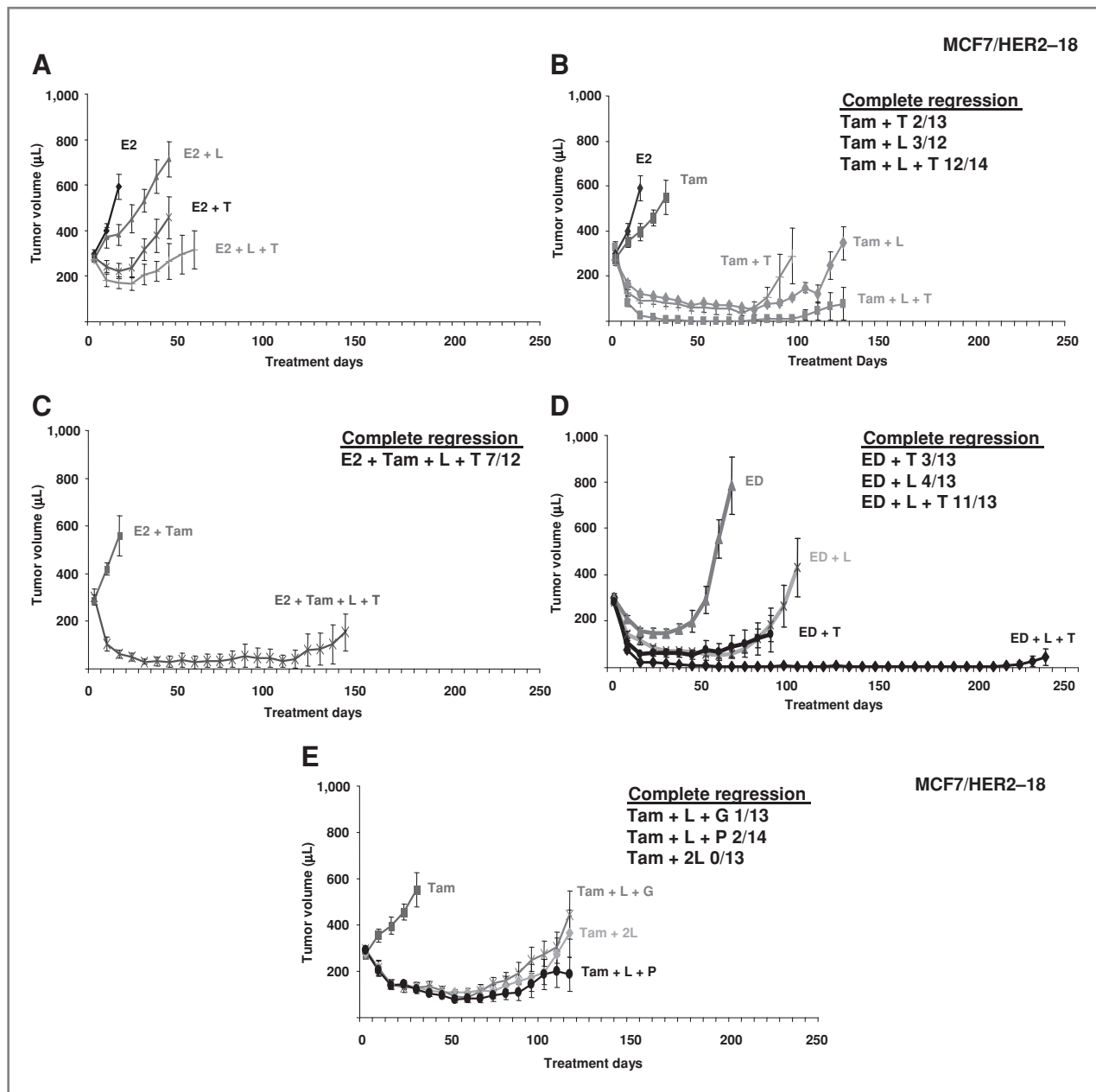
Figure 1. Growth of MCF7/HER2–18 xenograft tumors in athymic female mice treated with variable anti-HER single agents and combinations, with or without ER targeted therapy. A, E2 treatment alone or with lapatinib (E2 + L), trastuzumab (E2 + T), or their combination (E2 + L + T). B, tamoxifen treatment alone or with L (Tam + L), T (Tam + T), or their combination (Tam + L + T). C, tamoxifen treatment in the presence of estrogen with the combination (E2 + Tam + L + T). D, estrogen deprivation (ED) alone or along with L (ED + L), T (ED + T), or their combination (ED + L + T). E, tamoxifen treatment with alternative combinations of HER family inhibitors—L and gefitinib (Tam + L + G), double dose L 200 mg/kg/d (Tam + 2L), and tamoxifen with L and pertuzumab (Tam + L + P). Complete regression was defined as complete tumor regression documented on 3 consecutive weekly measurements. Results are presented as the mean tumor volume; error bars represent the standard error. B–E, for each group, the number of mice with complete tumor regression and the total number of mice are shown.

L + T combination in BT-474 tumors

The BT-474 cell line is an ER-positive human breast cancer cell line that is naturally amplified for HER2 (25). As we have shown before, established BT-474 xenograft tumors are estrogen independent despite expressing ER and grow similarly with and without estrogen (Fig. 2A; ref. 21). L and T alone led to complete tumor regression in 45% (5/11) and 91% (10/11) of ED-treated mice, respectively. However, BT-474 tumors were exquisitely sensitive to L + T. All tumors completely regressed in the presence of

Table 1. Complete tumor response and time to treatment resistance with variable treatment combinations in MCF7/HER2–18 tumor xenografts

Treatment	Complete tumor response (%)	Time to treatment resistance in days (95% CI)
E2	0	23 (14–43)
Tam	0	49 (24–60)
E2 + L	0	44 (30–56)
E2 + T	15	53 (39–68)
E2 + L + T	0	103 (42–136)
Tam + T	15	115 (90–146)
Tam + L	25	143 (122–174)
Tam + 2L	0[b]	150 (122–NA[a])
Tam + L + G	8	147 (109–NA[a])
Tam + L + P	14	178 (138–NA[a])
Tam + L + T	86	229 (170–342)
E2 + Tam + L + T	58	182 (143–185)

Abbreviations: Tam, tamoxifen; E2, estrogen; T, trastuzumab; L, lapatinib; G, gefitinib; P, pertuzumab.
[a]95% CI upper limit not achieved.
[b]Several animals had tiny nodules that did not satisfy the definition of complete response.

Figure 2. Growth of BT-474 xenograft tumors in athymic mice treated with estrogen supplementation (E2) or estrogen deprivation (ED) alone or with HER blocking agents. A, ED alone or along with lapatinib (ED + L), trastuzumab (ED + T), or their combination (ED + L + T). B, continued E2 alone or with the combination of L and T (E2 + L + T). Results are presented as the mean tumor volume; error bars represent the standard error. For each group, the number of mice with complete tumor regression and the total number of mice are shown. Complete regression was defined as complete disappearance of the tumor for 3 consecutive weeks.

estrogen (13/13) and 92% completely regressed in its absence (11/12; Fig. 2A and B, respectively). By day 231 of treatment, 4 out of the original 11 mice on the L alone arm and 2 out of 11 mice on T alone arm had developed resistant tumors. In contrast, none of the animals treated with L + T developed any tumors at any point during the experiment. This again indicates the superiority of this combination in BT-474 as well as in MCF7/HER2–18 tumors.

L + T intermittent and reduced dose regimen in BT-474 tumors

We next asked whether alternative treatment regimens of (L + T) with reduced dosing or changes in scheduling were as effective as full continuous dosing.

As shown in Figure 3A and Table 2, several control treatment groups from prior experiments were repeated to ensure reproducibility of our results (E2, ED, L, T arms, and L + T), and all yielded similar results.

Short treatment duration for just 14 days led to CR in 80% of mice and median time to CR was similar to continuous L + T (35 days in both groups, $P = 0.72$, generalized Wilcoxon test). This indicates that the treatment effect carried on beyond its brief duration, as tumors continued to shrink after treatment was stopped. Although the rate of eventual TP was clearly higher in this group (60% vs. 0%, $P = 0.001$, generalized
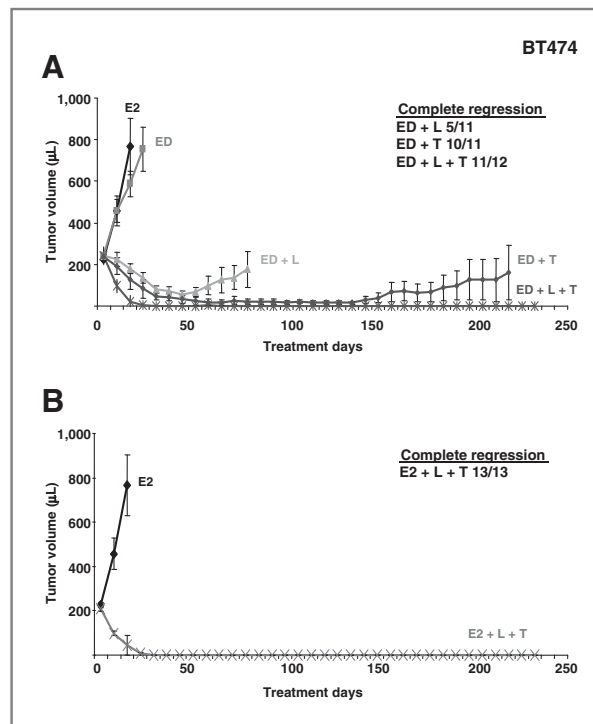
Wilcoxon test), still this brief duration of treatment resulted in prolonged tumor control with no TP in 6 out of 15 mice. Similarly, when treatment lasted just 42 days, CR rate and median time to CR were similar to full L + T but the rate of TP at 315 days was higher than with continuous L + T (27% vs. 0% TP, $P = 0.05$, generalized Wilcoxon test).

Figure 3B shows 2 alternate dosing methods, reduced or intermittent dosing. Treatment with reduced dosing, $(^{1}/_{2}L + {}^{1}/_{2}T)$ led to CR in 93% of mice. Although, median time to CR was longer compared with continuous full dose L + T (49 days vs. 35 days, respectively, $P = 0.03$, generalized Wilcoxon test), the rate of TP315 was not significantly different (13% vs. 0%, respectively, $P = 0.18$, generalized Wilcoxon test). On the contrary, intermittent treatment with L + T (14 days on treatment and 14 days off) led to CR and median time to CR similar to continuous full dose L + T (100% and 35 days in both groups). The difference in rate of TP between intermittent versus full L + T groups was not statistically significant (14% vs. 0%, respectively, $P = 0.17$, generalized Wilcoxon test).
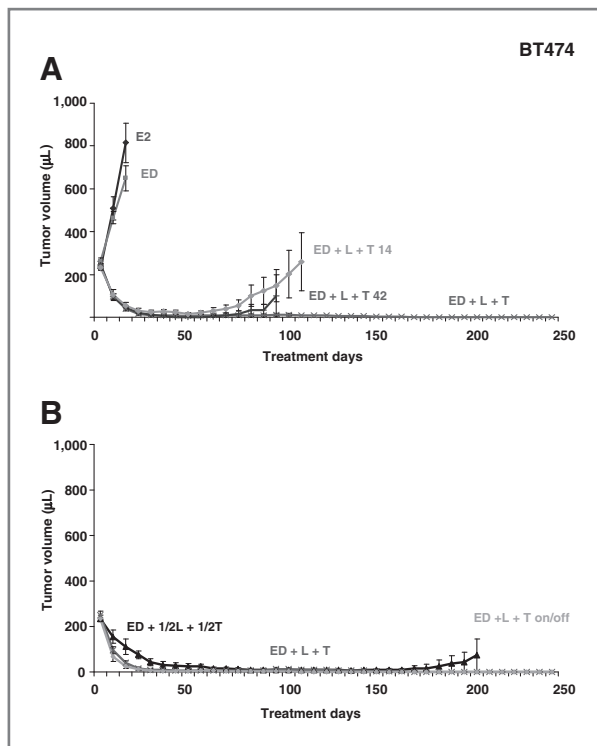
Figure 3. Growth of BT-474 xenograft tumors in athymic mice treated with estrogen supplementation (E2) or estrogen deprivation (ED) alone and with lapatinib (L), trastuzumab (T), or their combination (L + T) in various doses and schedules. A, growth of tumors in animals treated with ED plus short durations of L + T for 14 days (ED + L + T 14) and 42 days (ED + L + T 42) compared with control groups treated with E2, ED, and ED + L + T. B, growth of tumors treated with ED with full continuous L + T, reduced dose therapy ($^1/_2$L + $^1/_2$T), or intermittent therapy (ED + L + T 14 on/off). For each group, the number of mice with complete tumor regression and the total number of mice are shown. Complete regression was defined as complete disappearance of the tumor for 3 consecutive weeks.

Effect of L + T on tumor cell proliferation and apoptosis

We next examined the effects of these treatments on tumor cell proliferation, apoptosis, and key downstream signaling intermediaries in the HER pathway.

Levels of p-HER2 were suppressed in MCF7/HER2–18 tumors from mice treated with L or T (Fig. 4A and B). However, the combination L + T was more potent in inhibiting p-HER2 levels regardless of whether mice received continued estrogen, ED, or Tam (Fig. 4A). The same trends were noted in BT-474 tumors in which L and T each inhibited p-HER2 individually but were more potent in combination (Fig. 4C). Although total HER2 seems reduced in both tumor models in mice treated with the L + T combination, this is likely related in part to tumor shrinkage and tumor cell death. When corrected to pancytokeratin, total HER2 was not significantly reduced in animals treated with L + T (data not shown).

Levels of p-MAPK were assessed in MCF7/HER2–18 (Fig. 4A, B, and D) and BT-474 tumors (Fig. 4C). Levels of p-MAPK were significantly reduced in tumors from mice receiving L, or T in addition to endocrine therapy in both models (Fig. 4A, $P = 0.008$ and 0.05; Fig. 4C, $P = 0.02$ and 0.002, respectively, when compared with endocrine therapy alone; Wilcoxon rank-sum test). L + T also significantly reduced p-MAPK levels in both tumor models regardless of endocrine therapy (Fig. 4A, $P = 0.02$ compared with Tam; Fig. 4B, $P = 0.05$ compared with E2; Fig. 4C, $P = 0.002$ compared with ED; Wilcoxon rank-sum test). Interestingly, Figure 4A shows that L reduced p-MAPK more than L + T. However, p-MAPK assessment by IHC (Fig. 4D) shows that in tumors from mice treated with Tam + L + T, p-MAPK levels were significantly reduced than those observed in Tam, Tam + L, and Tam + T ($P = 0.005$, 0.019, and 0.039, respectively, Wilcoxon rank-sum test). Similar significant reductions in p-MAPK levels were observed in tumors from mice treated with Tam + L + T in the presence of estrogen (data not shown). In BT-474 tumors p-MAPK levels were suppressed in tumors treated with both T and L as single agents, and even more so with the combination (Fig. 4C). These results indicate that L + T is the most effective regimen in reducing levels of p-MAPK.

Levels of p-Akt were assessed by Western blot and were notably reduced in tumors receiving L, T, or their combination in both tumor models; MCF7/HER2–18 and BT-474 (Fig. 4A–C).

Table 2. Complete response (CR), time to complete response (TCR), and tumor progression rate at 315 days (TP315) in treatment arms with alternate dosing/scheduling of lapatinib/trastuzumab in BT-474 tumor xenografts

	Full L + T	T	L	$^1/_2$L + $^1/_2$T	L + T 42 days	L + T 14 days	L + T 14 days on/off
N (mice)	13	19	13	15	15	15	14
Median TCR	35	63	70	49	42	35	35
P^a	Ref	0.008	0.0003	0.03	0.58	0.72	0.46
CR (%)	100	89	77	93	100	80	100
TP315 (%)	0	16	46	13	28	60	14
P^a	Ref	0.13	0.009	0.18	0.05	0.001	0.17

Abbreviations: T, trastuzumab; L, lapatinib.
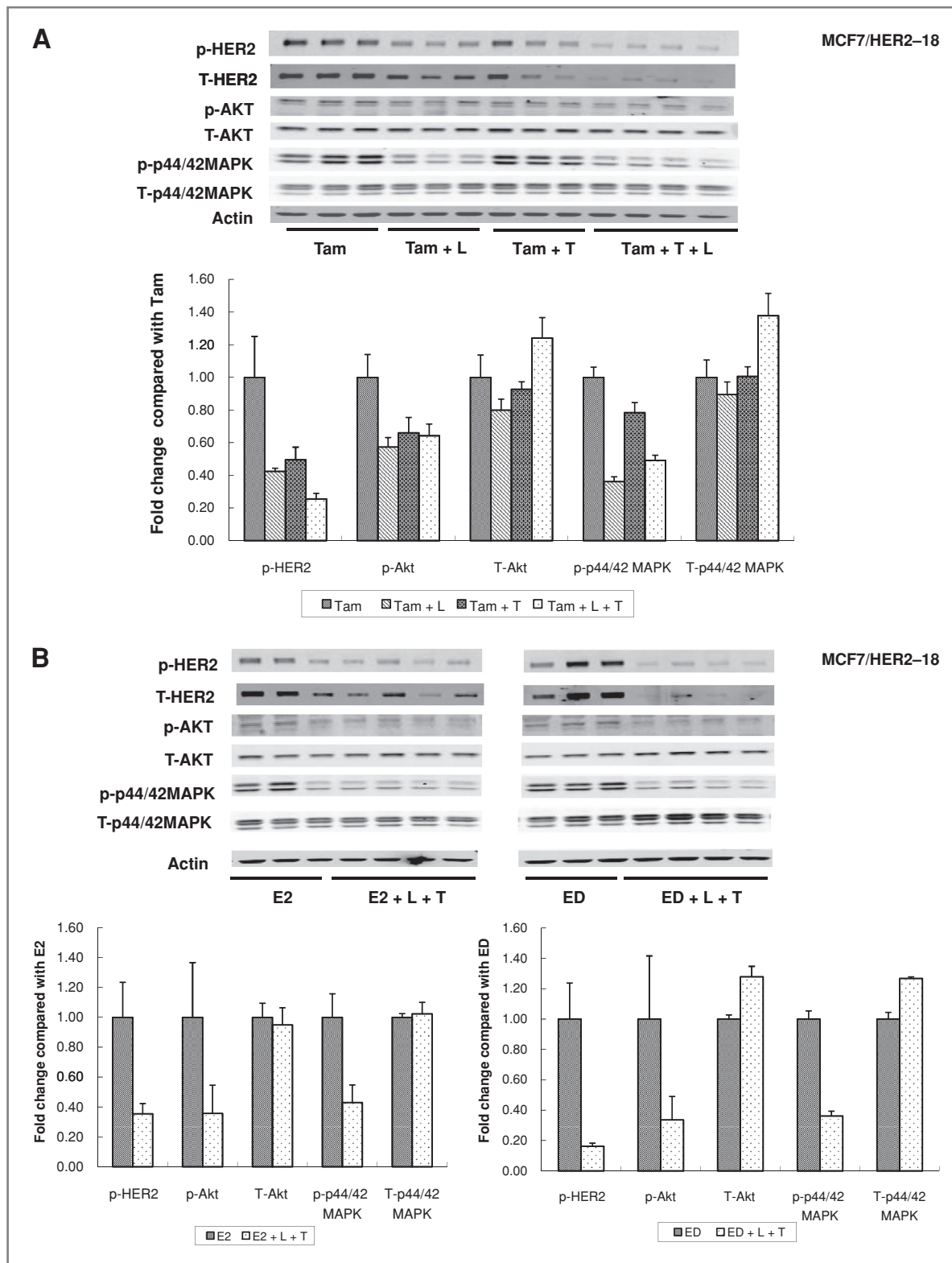[a]P value of generalized Wilcoxon test comparing other treatment groups to reference group (Ref).

Figure 4. HER2 and downstream signaling pathways were assessed in tumors from 2 cell lines grown as xenografts in athymic mice and treated for 3 days. A, MCF7/HER2–18 xenograft tumors treated with tamoxifen (Tam) alone or with lapatinib (Tam + L), trastuzumab (Tam + T), or their combination (Tam + L + T). B, MCF7/HER2–18 xenograft tumors grown with estrogen (E2) alone or with the combination of L and T (E2 + L + T) and estrogen deprivation (ED) alone or with the combination of L and T (ED + L + T).
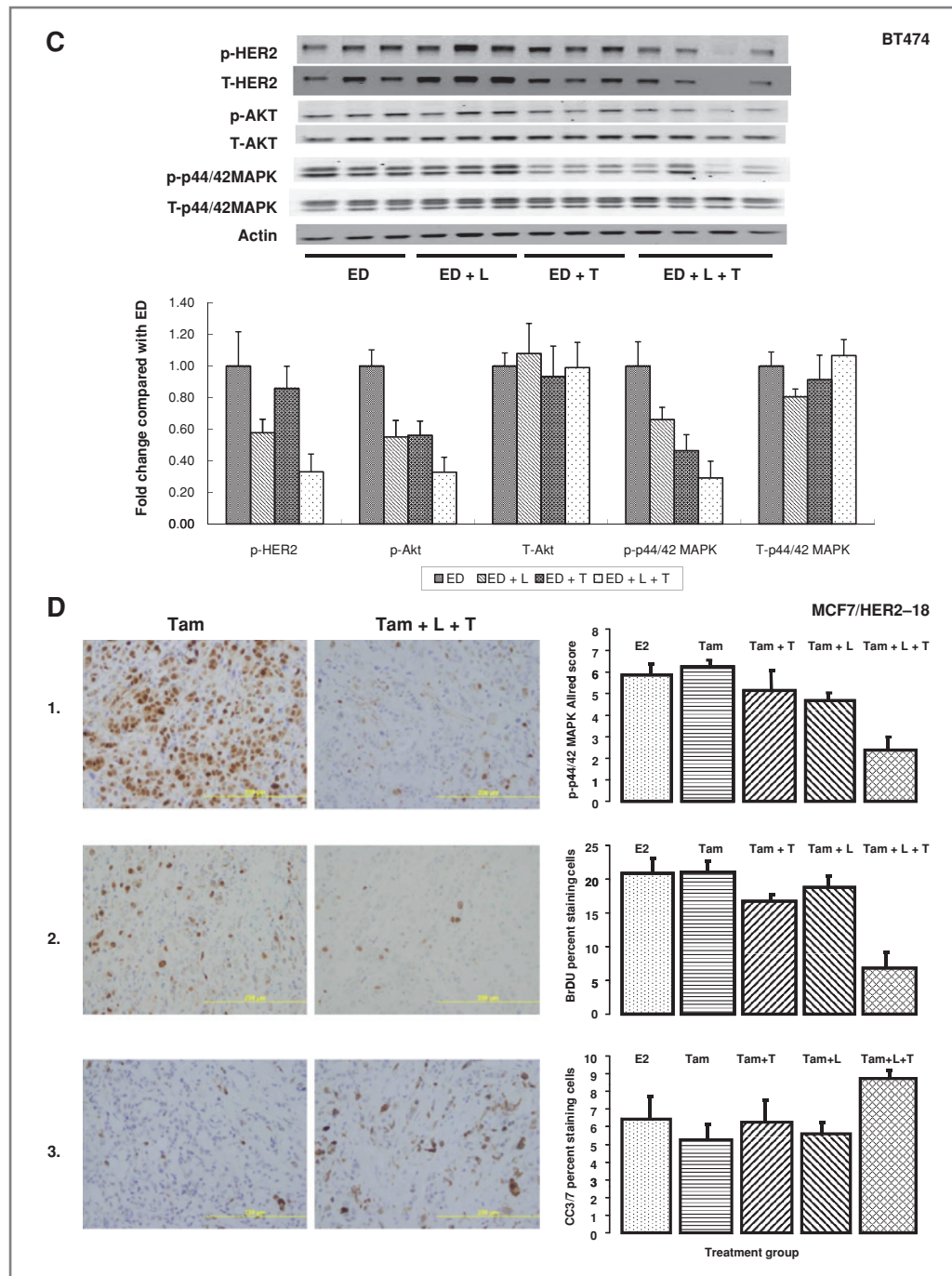
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Figure 4. C, BT-474 xenograft tumors treated with ED alone or with L (ED + L), T (ED + T), or their combination (ED + L + T). Proteins from tumor lysates were separated by SDS PAGE and subjected to immunoblot analysis with antibodies specific to total HER2, total Akt, total MAPK, p-HER2 (at Tyr1248), p-Akt (at Thr308) and p-p44/42 MAPK (at Thr202 and Tyr204), or β-actin. Protein expression and phosphorylation levels were quantified by Odyssey Infrared Imaging System. In each tumor, protein levels were normalized to the level of β-actin (the loading-control; by the formula, protein level/actin level × 100). For each treatment arm, protein expression levels were compared with control group (Tam, E2, and ED) as 1.00. D, immunohistochemistry studies on MCF7/HER2–18 xenografts: the pictures on the left are representative images of each biomarker staining. The panels on the left are quantitative representations of biomarker expression. 1, expression of p-p44/42 MAPK (at Thr202 and Tyr204) in MCF7/HER2–18 xenograft tumors treated with E2, Tam alone, or with L (Tam + L), T (Tam + T), and the combination (Tam + L + T). The length of treatment was 3 days. Levels of p-MAPK were assessed by immunohistochemical staining and reported using the Allred Score. There were at least 8 tumors from each group. 2, cell proliferation: BrDU staining was used to measure cell proliferation. Proliferation was measured in tumors, 8 from each treatment group, after 3 days of treatment. Results are the percentage of cells positive for BrDU. 3, apoptosis levels of cleaved caspase 3/7 were assessed immunohistochemically by use of an antibody against cleaved caspase 3/7 after day 3 of treatment. Apoptosis was measured in at least 8 tumors from each treatment group. Results are the percentage of cells positive for cleaved caspase 3/7. In all panels, error bars represent the standard error.

The relative effect of L + T on proliferation and apoptosis was investigated. Proliferation was assessed using BrDU uptake after 3 days of treatment in MCF/HER2–18 tumor xenografts (Fig. 4D). Neither L nor T significantly reduced the proliferative fraction when added to Tam ($P = 0.54$ and 0.10, respectively, Wilcoxon rank-sum test). However, the L + T combination caused a significant reduction in BrDU uptake in comparison to Tam alone, Tam + L, or Tam + T ($P = 0.007$, 0.007, and 0.016, respectively, Wilcoxon rank-sum test). Similar results were noted in BT-474 xenografts as well (data not shown).

Apoptosis was assessed by IHC at day 3 by an antibody that detects cleaved caspase 3 and 7. Figure. 4D shows that in MCF7/HER2–18 xenografts, neither L nor T resulted in any significant change in apoptosis ($P = 0.57$ and 0.61, respectively, Wilcoxon rank-sum test). However, when L + T were used in combination, a significant increase in apoptosis was observed over Tam alone or Tam + L ($P = 0.032$ and 0.0097, respectively, Wilcoxon rank-sum test). In BT-474 xenografts, a trend of increased apoptosis in the T and L + T treatment groups over Tam or Tam + L was noted, but was not statistically significant.

Discussion

Trastuzumab is effective in several clinical settings (11, 12), and recent data suggest that the population of patients that benefit from T therapy may be expanding (32). However, de novo and acquired resistance to T remain a challenge in clinical management. One strategy to overcome resistance is a more complete blockade of HER2 signaling using a combination of HER2 inhibitors. At the same time, identifying optimal duration of this blockade could lead to reduced treatment cost and enhanced quality of life if prolonged therapy is not necessary.

Lapatinib, a dual tyrosine kinase inhibitor of HER1 and 2, should block the HER receptors more completely than T, which is most effective in inhibiting HER2 homodimers. Furthermore, L inhibits p95, the constitutively active short form of HER2 against which T is ineffective. The data presented here using 2 different in vivo model systems show that neither L nor T alone are as effective as their combination in antagonizing HER2 related signaling pathways or inducing tumor regression. The combination of the 2 drugs provides more potent inhibition of downstream signals, more effective inhibition of cell proliferation, and possibly greater induction of apoptosis than each as a single agent. Our findings support our prior report of the remarkable efficacy of a 3-drug cocktail of HER inhibitors in tumor xenografts in mice (21) and they provide additional rationale for a combination regimen of the 2 approved drugs now in clinical testing.

Our data also provide mechanistic insights into the optimal method to block the HER pathway at the receptor level. L was expected to be a more complete inhibitor of the pathway but the antitumor effects of the drug were insufficient as a single agent in both MCF7/HER2–18 and BT-474

tumors. Adding G to L for added HER1 inhibition, doubling the L dose, and substituting P for T to block HER2 heterodimerization were much less effective in causing complete tumor regressions and delaying TTR than was the combination of L with T.

Data from our group evaluating human HER2-overexpressing breast cancer samples of patients obtained from 2 neoadjuvant clinical trials treated with single agent T or L showed that tumors with low PTEN or mutated PI3KCA are resistant to T but sensitive to L (33). These findings along with evidence from other groups and the present data indicate that L and T may exert their effect via distinct but complementary mechanisms of action (34, 35).

After demonstrating the efficacy of the combination of L and T, we investigated the effect of dose reduction, intermittent delivery, or shortened duration of the standard combination. Given the promising results from a clinical study in patients treated with short term T (36) and our prior observation that T, like chemotherapy, induces apoptosis in tumors from patients undergoing neoadjuvant therapy (37), in the current study we reasoned that a brief course of combined L + T might still be very effective.

Our results showed that even when treatment was given for a short course (14 days), tumor regression continued after stopping the therapy, and the majority of mice had a CR with 40% of mice without TP315. When treatment duration was extended to 42 days, the proportion of mice without TP315 increased to 72%. Thus, whereas longer therapy may be optimal, these very short exposures were still very effective, suggesting that shorter treatment durations should be explored in patients.

We also show that a reduction of the dose of both L and T ($\frac{1}{2}$ L + $\frac{1}{2}$ T) effectively blocks the HER pathway and is as effective as full doses in eradicating tumors. Furthermore, the schedule of intermittent therapy which lowered the dose intensity by half was just as effective.

Although it is difficult to extrapolate these treatment doses and durations directly to patients, the cumulative data suggests that these or similar strategies should be tested in patients. There is increasing emphasis on reducing toxicity from unnecessary treatment. A recent clinical trial of L + T with paclitaxel had to be stopped early due to significant toxicity, especially diarrhea (38). This, together with economic implications of the rising cost of cancer care, highlights the potential impact of our results. Clinical trials comparing the standard 1-year approach to shorter and intermittent dosing schedules should be explored. If as effective as standard therapy, these alternative schedules would significantly lower the cost and toxicity of treatment.

Our molecular studies show that the combination of L and T reduces p-HER more effectively than either agent alone.

In addition, the L + T combination inhibits downstream signaling through 2 important cell proliferation and survival pathways mediated through AKT and MAPK, thus explaining the increased antitumor activity of the combination. Our finding of a significant reduction in

proliferation and increased apoptosis in the L + T-treated tumors over those treated with a single agent also supports this conclusion.

In addition to direct potent inhibition of HER signaling, the combination may be working by other mechanisms. There are data showing that L stabilizes different HER dimers, which are then internalized after T binding to HER2 (19). In addition, there is mounting evidence that both agents are active against tumor-initiating cells, which may explain tumor xenograft elimination in some animals in our study (39, 40)

Our data, derived from experimental models, may not represent the totality of HER2-overexpressing breast cancers in patients. Both our models are ER-positive, although established BT-474 xenografts are not estrogen dependent (21). However, our findings have important and compelling clinical implications.

In addition to lending support for clinical trials studying the combination of L and T, our findings strongly argue for timely clinical testing of intermittent dosing of this com-

bination. As personalized cancer therapy becomes the new standard of care in oncology, it is a high priority to combine optimal therapeutic strategies with minimal toxicity and cost.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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