Perioperative COX-2 and β-adrenergic blockade improves metastatic biomarkers in breast cancer patients in a phase-II randomized trial

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Abstract

Background—Translational studies suggest that excess perioperative release of catecholamines and prostaglandins may facilitate metastasis and reduce disease-free survival. This trial tested the combined perioperative blockade of these pathways in breast cancer patients.

Methods—In a randomized placebo-controlled biomarker trial, 38 early-stage breast cancer patients received 11 days of perioperative treatment with a beta-adrenergic antagonist (propranolol) and a cyclooxygenase-2 (COX-2) inhibitor (etodolac), beginning five days before surgery. Excised tumors and sequential blood samples were assessed for pro-metastatic biomarkers.

Results—Drugs were well tolerated with adverse event rates comparable to placebo. Transcriptome profiling of the primary tumor tested a priori hypotheses and indicated that drug

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treatment significantly (i) decreased epithelial-to-mesenchymal transition, (ii) reduced activity of pro-metastatic/proinflammatory transcription factors (GATA-1, GATA-2, early-growth-response-3/EGR3, signal transducer and activator of transcription-3/STAT-3), and (iii) decreased tumor-infiltrating monocytes while increasing tumor-infiltrating B cells. Drug treatment also significantly abrogated presurgical increases in serum interleukin-6 (IL-6) and C-reactive protein levels, abrogated perioperative declines in stimulated interleukin-12 and interferon-gamma production, abrogated postoperative mobilization of CD16⁻ "classical" monocytes, and enhanced expression of CD11a on circulating natural killer cells.

Conclusions—Perioperative inhibition of COX-2 and β-adrenergic signaling provides a safe and effective strategy for inhibiting multiple cellular and molecular pathways related to metastasis and disease recurrence in early-stage breast cancer.

Keywords

Breast cancer; COX-2 inhibition; β-adrenergic blockade

Introduction

The removal of a primary tumor, and the abolition of its potential immunosuppressive and metastasis-promoting effects (1), presents a window of opportunity to eliminate or control any remaining minimal residual disease. Unfortunately, the perioperative period and the excision of a primary tumor also trigger a variety of physiological processes that may potentially accelerate the progression of pre-existing micrometastases and promote the initiation of new metastases (2,3). As such, the perioperative period plays a critical role in determining long-term cancer outcomes, disproportionally to its short duration (4). Importantly, pre-clinical animal models of cancer suggest that pharmacological modification of perioperative physiology could be exploited to reduce the burden of residual disease (4).

Specifically, animal studies using syngeneic or human xenograft models of cancer have implicated peri-surgical high levels of catecholamines and prostaglandins in mediating many of the pro-metastatic effects of surgery and perioperative stress (2,4,5). Catecholamines and prostaglandins are released by tumor cells, stromal cells within the tumor microenvironment, and by host physiological systems as a result of physiological and psychological stress responses to coping with cancer, tissue damage, pain, and a variety of surgical impacts (6). These signaling pathways can act directly on tumor cells to enhance their proliferation, motility, invasive capacity, resistance to anoikis, secretion of angiogenic factors (5,7–9), and epithelial-to-mesenchymal transition (EMT) (7,10). Catecholamines and prostaglandins can also indirectly promote metastasis by suppressing cell-mediated immunity (2), increasing pro-metastatic cytokines (e.g., interleukin-8; IL-8) (11), and inducing inflammation, which is a hallmark of cancer progression (12).

During the last decade, we and others have found that pharmacologic inhibition of β-adrenoceptors and/or prostaglandin synthesis can reduce the pro-metastatic and immune-suppressive effects of stress and surgery (9,13–18). In these preclinical studies, the simultaneous administration of a β-blocker (propranolol) and a COX-2 inhibitor (etodolac) in combination (rather than each drug alone) has generally proven most effective, and

sometimes constitutes the only effective approach (19–21). The synergistic effect of combined treatment protocols may stem from the fact that catecholamines and prostaglandins are both elevated perioperatively and each can increase metastatic propensity through converging pathways (i.e., activation of the cAMP-Protein Kinase A signaling system). Simultaneous inhibition of COX-2 and β-adrenergic signaling has reduced postoperative metastasis (and in some cases improved overall survival) in multiple pre-clinical tumor models including breast, colon, lung, melanoma, and leukemia (19,20,22–24). Consistent with these pre-clinical studies, several pharmaco-epidemiological studies have also documented reductions in breast cancer progression or recurrence in patients who happened to be taking β-blockers at or before initial diagnosis (25,26). Long-term use of COX-inhibiting NSAIDS was also associated with reduced risk of colorectal cancer (27). To assess the potential biological impact of combined perioperative COX-2 and β-adrenergic inhibition in human breast cancer, we herein conducted a randomized placebo-controlled biomarker trial employing etodolac and propranolol. Primary outcome analyses tested whether this combined drug treatment would reduce proinflammatory and prometastatic transcriptome profiles in the malignant tissue.

Three specific transcriptome signatures were targeted a priori based on previous research implicating them in COX-2 and/or β-adrenergic influences on breast cancer progression and metastasis. (i) The primary tumor's EMT profile was assessed as mesenchymal polarization has been shown to promote intravasation and extravasation of epithelial tumor cells (28), and because both COX-2 activation and β-adrenergic signaling can promote EMT (COX-2 by inducing matrix metalloproteinase-1 (MMP-1) and MMP-2 (10) and inhibiting Smad signaling (29), and β-adrenergic signaling by upregulating SNAIL and TWIST transcription factors (7)). (ii) Transcriptome signatures of tumor-infiltrating leukocyte subpopulations were assessed based on data linking monocyte/macrophage infiltration to breast cancer metastasis and B lymphocyte infiltration to reduced progression. (iii) Pro-inflammatory and pro-metastatic transcription control pathways previously implicated in breast cancer progression were also assessed (nuclear factor-kappaB [NF-κB]/cRel, activator-protein-1 [AP-1], GATA family, STAT family, NRF-2, EGR family transcription factors and the glucocorticoid receptor; GR). Secondary outcome analyses addressed peripheral immune parameters, including serum and ex-vivo stimulated Th1 and inflammatory cytokines (IL-12, IFN-γ), NK cell activation markers, and circulating leukocyte populations (with a particular focus on monocytes due to their involvement in metastasis). This is the first clinical trial to test the efficacy of a combined perioperative treatment with a β-blocker and a COX-2 inhibitor in breast cancer patients.

Methods

Patients and inclusion/exclusion criteria

Thirty-eight women (age 33–70) diagnosed with stage I–III breast cancer were enrolled from three medical centers in Israel. Exclusion criteria included (i) any contraindication for the drugs, such as diabetes, asthma, cardiovascular disease, or low blood pressure, (ii) chronic use of any β-blocker or COX inhibitor, and, (iii) chronic autoimmune disease. The study protocol (ClinicalTrials.gov Identifier: NCT00502684) was approved by IRBs at each

study site, and written informed consent was obtained from patients before performing any study-related procedures.

Study design and drug treatment

This multicenter double-blind placebo-controlled randomized biomarker trial employed two equal-sized arms of drug- and placebo-treatment (Fig. 1 A and B). Patient randomization was stratified by age within each medical center (below or above 50). Drug/placebo was administered for 11 consecutive days, starting five days before resection of the primary tumor (Fig. 1 B). Oral BID etodolac (400 mg) was administered throughout the treatment period. Propranolol was administered orally using extended release formulations: 20 mg BID during the five days preceding surgery; 80 mg on the morning of surgery and on the evening and morning following surgery; and 20 mg BID thereafter during five postoperative days. Identical schedules were used for placebo and medication.

Endpoints and assessments

Excised tumor tissue was fixed in 4% formaldehyde and stored as a formalin-fixed-paraffin-embedded (FFPE) block. Five 5μm sections were used for gene expression profiling as described below. Four blood samples were obtained between 7–11 AM. The first was taken before medication initiation (T1); the second and third were taken on the mornings before and after surgery (T2 and T3, respectively), at least 1 hr after the morning medication dose; and the fourth was taken at least 2 days after treatment cessation (T4; median of 16 days post-medication) (Fig. 1B).

Gene expression profiling and bioinformatic analysis

Detailed methods and references for gene expression profiling and bioinformatic analysis are presented in the Supplementary Methods. Briefly, RNA was extracted from five 5μm FFPE sections of breast tumors, tested for sufficient mass, and subjected to genome-wide transcriptional profiling using Illumina Human HT-12 v4 Expression BeadChips (Illumina Inc., San Diego, California) with quantile normalization (30). Linear model analyses of log2-transformed expression values quantified the difference in average expression between groups (drug treatment vs. placebo) after controlling for tumor stage. A priori hypotheses regarding EMT polarization and tumor-associated leukocyte transcriptomes were tested using Transcript Origin Analyses to relate all genes showing   1.25-fold differential expression in this study to previously published reference transcriptome profiles derived from mesenchymal- vs. epithelial-polarized breast cancer cells (GSE13915) or isolated leukocyte subsets (GSE1133). A priori hypotheses regarding activity of breast cancer-relevant transcription control pathways were tested using TELiS bioinformatic analysis of transcription factor binding motifs (TFBMs) in the promoters of all genes showing   1.25-fold differential expression, using TRANSFAC position-specific weight matrices for inflammation-related pathways (NF-κB/cRel, AP-1), GATA family factors GATA1-GATA3, cytokine response factors STAT1 and STAT3, the oxidative stress response factor NRF-2, the neuroendocrine response factor GR, and EGR family transcription factors EGR1-EGR4/NGFIC, as previously described. Statistical testing of bioinformatics results was based on standard errors derived from bootstrap resampling of linear model residual vectors over all genes assayed (which accounts for any potential correlation across genes).

Blood collection, ELISA, flow-cytometry, and induced cytokine production

The Supplementary Methods detail the standard procedures used to assess serum IL-6, CRP, IL-10, and cortisol; ex-vivo lipopolysaccharide- (LPS) & phytohaemagglutinin- (PHA) stimulated production of interferon-gamma (IFN-γ) and IL-12; and flow cytometric analyses of NK cell activation markers and leukocyte subset prevalence.

Statistical Analysis

All analyses were two-sided and conducted based on a priori hypotheses. Our primary hypothesis was that drug treatment would reduce three progression-related transcriptome profiles in malignant tissue (EMT, tumor-associated monocyte/macrophage transcriptomes, and proinflammatory/prometastatic transcription factors). Our secondary hypothesis was that drug treatment would shift circulating immune parameters towards lower inflammatory and higher anti-metastatic immunity as indicated by serum and ex-vivo stimulated cytokine levels, NK cell activation markers, and circulating "classical" (CD14^{++}CD16^{-}) monocytes.

For tumor transcriptome analyses, the statistical significance of bioinformatic result ratios (Drugs/Placebo) was tested by Student's t-test. For blood-measures analyses, a planned contrast was used to compare the impact of drug treatment (average of T2 and T3) to untreated levels (average of T1 and T4) (i.e., Drugs [(T2+T3) − (T1+T4)] – Placebo [(T2+T3) − (T1+T4)]), and post-hoc comparisons were performed to assess group differences at specific time points. For serum cytokine levels and gene expression assessments, data were log transformed to stabilize variance. Blood sample data during treatment were expressed as a % of the average value at no-treatment time points (i.e., average of T1 and T4).

Results

Demographics, adverse events, and drug compliance

The two groups did not differ on any demographic or cancer-related characteristic assessed (Table 1 and 2). Two patients reported physical discomfort within the first 2 days of treatment (before hospitalization): one placebo-treated patient reported anxiety and showed increased heart rate and blood pressure; a second drug-treated patient reported nausea. Both self-withdrew without further medical examination, and no additional samples were collected from these patients. The other 36 women reported no adverse events and consumed at least 95% of their medication/placebo doses.

Tumor gene expression

Genome-wide transcriptional profiling of tumor tissues identified 163 genes showing >1.25 fold up-regulation in tumors from drug-treated patients vs. placebo-treated controls, and 141 genes were equivalently down-regulated. A priori-specified bioinformatic analyses, using previously published mesenchymal and epithelial breast cancer cell transcriptomes as reference points, showed that drug treatment reduced the extent of mesenchymal polarization (diagnosticity z-score: mean = −0.43 ± SE 0.09, p <.0001) but had no significant effect on epithelial-characteristic gene expression (+0.13 ± 0.11, p = .106; Fig. 2A). Similar a priori-specified tumor transcriptome analyses using isolated leukocyte

subpopulations as reference points (Fig. 2B) indicated that drug treatment reduced expression of CD14^{+} monocyte-related transcripts (-0.42 ± 0.15, p=.0036) and increased expression of genes characteristic of CD19^{+} B cells ($+0.45 \pm 0.17$, p=.0033). We also tested a priori hypotheses regarding specific transcription control pathways that are linked to pro-metastatic processes of inflammation, tissue invasion, and EMT. Promoter-based bioinformatic analyses (Fig. 2C) indicated down-regulated activity of GATA-1 ($\log_2$ fold difference in promoter binding site prevalence: mean = -0.48 ± 0.10, p<.0001), GATA-2 (-0.40 ± 10, p=.0001), STAT3 (-1.61 ± 0.66, p=.0154), EGR-3 (-0.70 ± 0.35, p=.048) and GRE (-0.85 ± 0.42, p=.043) in tumors from drug-treated patients.

Serum levels of soluble factors

As expected, given that psychological and physiological stress responses intensify in the lead-up to surgery (31), serum levels of IL-6 increased by $24\% \pm 12.1\%$ from T1 to T2 in the placebo-treated group and CRP levels similarly increased by $41.5\% \pm 20.5\%$ (Fig. 3A–B). However, this pattern was significantly reversed in the drug-treated group ($11.3\% \pm 5.5\%$ decline for IL-6, p=.0009; $10\% \pm 10.7\%$ decline for CRP, p=.034). On the morning after surgery (T3), both placebo- and drug-treated groups showed increases in IL-6 and CRP above pre-surgical levels (IL-6: $+573\% \pm 97\%$ and $+442\% \pm 70\%$ for placebo- and drug-treated groups, respectively; CRP: $+828\% \pm 285\%$ and $+635\% \pm 281\%$; all p <.001). A planned contrast of drug- vs. placebo-treated groups during treatment (average of T2 and T3) vs. off treatment (average of T1 and T4) showed a significant reduction in IL-6 for the drug-treated group (p =.011), and a marginally significant reduction in CRP. Drug treatment did not significantly affect serum cortisol or IL-10 concentrations at any time point (Fig. 3C–D).

Immune indices in blood samples

In the placebo-treated group, ex-vivo LPS- and PHA-stimulated production of IL-12 and IFN-γ decreased progressively from T1 to T3 (IL-12: $-38\% \pm 10\%$; IFN-γ: $-30.2\% \pm 9.1\%$; both p<.0001), as previously reported (31). Drug treatment blocked this decrease, resulting in higher levels of these cytokines at T2 and T3 (i.e., for the planned contrast described above, IL-12: $+50.8\% \pm 15.2\%$, p=.028; IFN-γ: $+31\% \pm 8.6\%$, p=.024) (Fig. 4A–B).

Drug treatment also blocked an influx of CD14^{++}CD16^{−} classical monocytes into circulation on the morning after surgery (T3; difference = $85\% \pm 15\%$, p=.032) (Fig. 4C), and increased expression of the activation marker CD11a on NK cells (CD3^{−}CD56^{+}CD16^{+}) during treatment (average of T2 and T3) vs. off treatment (average of T1 and T4; difference = $+16\% \pm 6.3\%$, p=.024) (Fig. 4D).

Discussion

These data show that perioperative administration of the β-adrenergic antagonist propranolol and the COX-2 inhibitor etodolac induces multiple favorable impacts on (i) primary tumor gene expression profiles (bioinformatic indications of reduced EMT; reduced activity of GATA-1, GATA-2, EGR3, GRE, and STAT3 transcription factors; and reduced tumor-associated monocytes and increased tumor-associated B cells) and on (ii) circulating

immune parameters (serum and ex vivo-induced cytokine levels, reduced classical monocyte influx, and increased NK cell activation markers). Each of these outcomes has previously been linked to reduced tumor progression in pre-clinical animal models and/or human clinical studies. This study was designed solely as a randomized controlled test of perioperative propranolol and etodolac effects on biomarker outcomes, and involved no long-term assessment of clinical outcomes. However, the favorable safety profile and favorable impacts on tumor transcriptome profiles and immune parameters provide a rationale for future clinical trials employing more robust sample sizes and long-term follow-up to assess impacts of perioperative COX-2 and β-adrenergic inhibition on clinical outcomes in early-stage breast cancer.

Tumor molecular characteristics

Drug treatment reduced tumor molecular biomarkers of EMT. This finding corresponds well with animal studies indicating that COX-2 inhibitors and β-adrenergic antagonists can inhibit EMT in human tumor xenografts (5,32). In metastatic breast cancer, a mesenchymal phenotype is more prevalent in circulating tumor cells than in the primary tumor (33), suggesting the clinical significance of EMT for metastasis. Moreover, the EMT profile of the primary tumor predicts long-term cancer outcomes, including overall survival (34), in several cancer types.

Drug treatment also decreased intra-tumoral molecular indicators of several pro-metastatic transcription control pathways, including GATA-1, GATA-2, and EGR3. GATA-1 exerts anti-apoptotic activity (35) and promotes EMT by down-regulating E-cadherin (5), and GATA-2 can inhibit the tumor suppressor gene, phosphatase and tensin homolog (PTEN) (36). Both factors promote breast cancer development and progression (37). EGR3 is induced by estrogen signaling (38), and has been linked to lymph node status, metastatic spread, and poor prognosis (39). Drug treatment also reduced indicated activity of the glucocorticoid receptor (GR), which can enhance tumor cell survival by inducing Bcl-xL (40), inducing anti-apoptotic signaling (41,42), suppressing p53 activity (43), and inducing chemoresistance (8).

Inflammatory indicators in the tumor and circulation

Previous studies have reported that psychological and surgery-related sympathetic nervous system stress responses can elevate levels of proinflammatory ligands, including IL-6 and CRP (44). In the current study, both IL-6 and CRP levels increased before surgery in the placebo group (from T1 to T2), but drug treatment reversed this effect and reduced levels of both inflammatory indicators at T2. IL-6 and CRP are associated with tumor progression and poor prognosis in multiple solid tumor types, including breast, lung, and prostate, and hematopoietic malignancies (37,45). IL-6 activates the janus-kinase-STAT signaling pathway, which is well known to promote tumor cell proliferation, survival, and invasion, as well as immunosuppression and inflammation. STAT3 and STAT5 are strongly associated with cancer progression (37). Here, both plasma IL-6 levels and indicators of tumor STAT3 activity were reduced by the drug treatment. Drug treatment also effectively blocked a marked postoperative (T3) mobilization of "classical" pro-inflammatory $CD14^{++}CD16^{-}$ monocytes. Thus, combined perioperative β-blockade and COX-2 inhibition may inhibit

stress-induced inflammatory and metastatic processes through multiple cellular and molecular pathways.

Immune status in the tumor and circulation

Transcriptome profiling of the primary tumor also indicated potential effects of the combined perioperative drug treatment in increasing tumor-infiltrating B cells and decreasing tumor-associated monocyte/macrophages. Tumor-infiltrating B cells comprise up to 60% of the tumor-associated lymphocytes (46,47), and predict increased survival rates in breast cancer (47,48). Monocyte recruitment by tumors was shown in several animal models to be enhanced by β-adrenergic signaling, and to promote cancer progression (5). In human cancers, tumor-infiltrating monocytes, which often transform into M2-macrophages, are correlated with decreased survival in many solid tumors including breast, thyroid, and bladder cancers (49,50). Thus, the profile of immune cell alterations reflected in whole-tumor transcriptome profiling indicates favorable effects of this drug regimen on local immune cell mediators of disease progression.

Consistent with previous reports in animal models, (31) stress and surgery also decreased LPS- & PHA-induced production of IFN-γ and IL-12 by circulating leukocytes. However, this suppression was abrogated by drug treatment. These Th1 cytokines are prominent activators of anti-tumor CTL and NK cells (2). The drug treatment also increased expression of CD11a (lymphocyte function-associated antigen-1; LFA-1) on circulating NK cells. This membrane glycoprotein is a marker of NK cell activation and interacts with intercellular adhesion molecule-1 (ICAM-1) and other tumor ligands to promote tumor lysis by NK cells (51). In a pre-clinical model, where a spontaneously metastasizing orthotopic primary tumor was excised, surgery reduced CD11a expression on NK-cells and our perioperative drug treatment (propranolol and etodolac) blocked that effect and improved long-term survival rates (20).

Perioperative significance of the treatment and safety concerns

Combined administration of propranolol and etodolac had favorable impacts on multiple biomarkers assessed before, during, and following surgery. Given that both catecholamines and prostaglandins are abundant throughout the perioperative period, and that micrometastases and residual disease may exist before and following surgery, these data suggest that treatment throughout the entire perioperative period is optimal.

The safety of this drug regimen is discussed in greater detail in the Supplementary Methods. Briefly, for patients without contraindications, the safety profiles of propranolol and etodolac are well established, especially for the short duration employed here (52,53). Concerns and variable findings have been reported regarding the perioperative use of β1-selective antagonists (54), but no evidence indicates a risk associated with use of non-selective β-adrenergic antagonists such as propranolol. Tissue healing was shown in animal studies not to be adversely affected by either drug or by their combined use (55). In the current study and in a previous study in cancer patients (56), we observed no serious or moderate adverse events associated with this treatment regimen. This favorable safety profile is important in evaluating the overall cost-benefit ratio for the present drug regimen,

especially as both chronic and perioperative use of COX inhibitors or β-adrenergic blockers have been associated with improved cancer outcomes (4,7,57). Thus, any hypothetical long-term risk associated with the combined use of propranolol and etodolac appears empirically unlikely, and should be weighed against the positive outcomes reported by translational, epidemiological, and clinical studies, as well as by the favorable profile of molecular and cellular biomarkers observed in this trial.

Limitations

This study was designed solely as a randomized controlled proof-of-concept test of perioperative propranolol and etodolac effects on metastasis-related biomarkers in early-stage breast cancer. This study was powered only to detect those biomarker outcomes (based on effect sizes previously observed in pre-clinical studies) and it provides no information about long-term clinical outcomes (e.g., effects on relapse-free or overall survival). The generality of these results also needs to be examined in future studies beyond the present single-nation context, and possibly examining alternative treatment durations and regimens (including the use of each agent alone in addition to combined use) and selective targeting of high-risk disease settings (e.g., ER-/PR-/her2- breast cancer) and other cancer types. It is also important to note that the biomarkers examined in this trial were selected a priori as outcomes (based on previous clinical and preclinical research) and are not intended to provide a prognostic biomarker for clinical disease progression.

Summary

Data from this first clinical trial of perioperative treatment with a COX-2 inhibitor and a β-adrenergic antagonist in early stage breast cancer finds a favorable safety profile and favorable impact on multiple tumor and circulating biomarkers associated with cancer progression and metastasis. These findings provide a biological rationale for future clinical trials to assess the impact of this easily implemented, safe, and inexpensive treatment regimen on long-term clinical outcomes (e.g., overall and recurrence-free survival).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Translational relevance

The clinical trial reported here supports the safety and efficacy of pharmacologically inhibiting β-adrenergic and COX-2 pathways during the perioperative period in early-stage breast cancer. Preclinical studies have shown that simultaneous blockade of these two pathways improves long-term survival rates in several models of primary tumor excision. Moreover, this treatment is inexpensive and clinically feasible for patients without contraindications for the medications used (~50% of patients). Given the positive biomarker indications reported in the present proof-of-concept trial, future studies assessing clinical impact on disease progress/recurrence and overall survival are justified. This treatment may also be beneficial in a variety of cancer types and does not contraindicate other cancer therapies. As such, brief perioperative inhibition of β-adrenergic and COX-2 signaling may provide a novel strategy for improving long-term cancer outcomes.

C O N S O R T

TRANSPARENT REPORTING of TRIALS

Enrollment

Assessed for eligibility (n=99)

Excluded (n=61)
- Not meeting inclusion criteria (n=52)
- Declined to participate (n=9)
- Other reasons (n=0)

Randomized (n=38)

Allocation

Allocated to drug intervention (n=19)
- Received allocated intervention (n=19)
- Did not receive allocated intervention (n=0)

Allocated to placebo intervention (n=19)
- Received allocated intervention (n=19)
- Did not receive allocated intervention (n=0)

Discontinued intervention

Discontinued intervention (n=1). Nausea on 2nd treatment day. Self-withdrew

Discontinued intervention (n=1). Anxiety & increased heart rate on 2nd treatment day. Self-withdrew

Analysis

Analysed (n=18)

Blood biomarkers (n=18)

Tumor analysis (n=10). All tumors with assured sufficient RNA mass

Analysed (n=18)

Blood biomarkers (n=18)

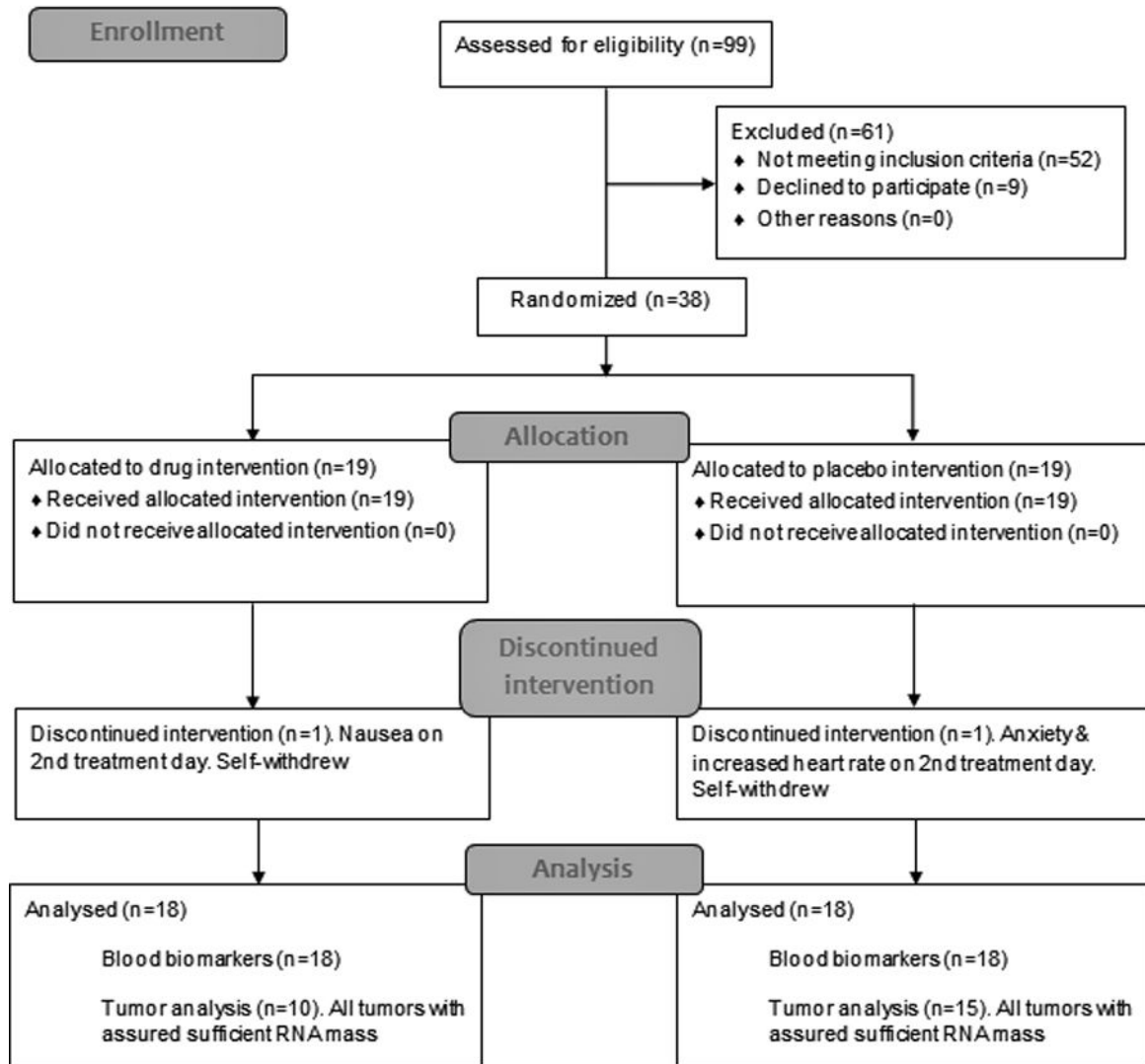Tumor analysis (n=15). All tumors with assured sufficient RNA mass

Fig. 1A.
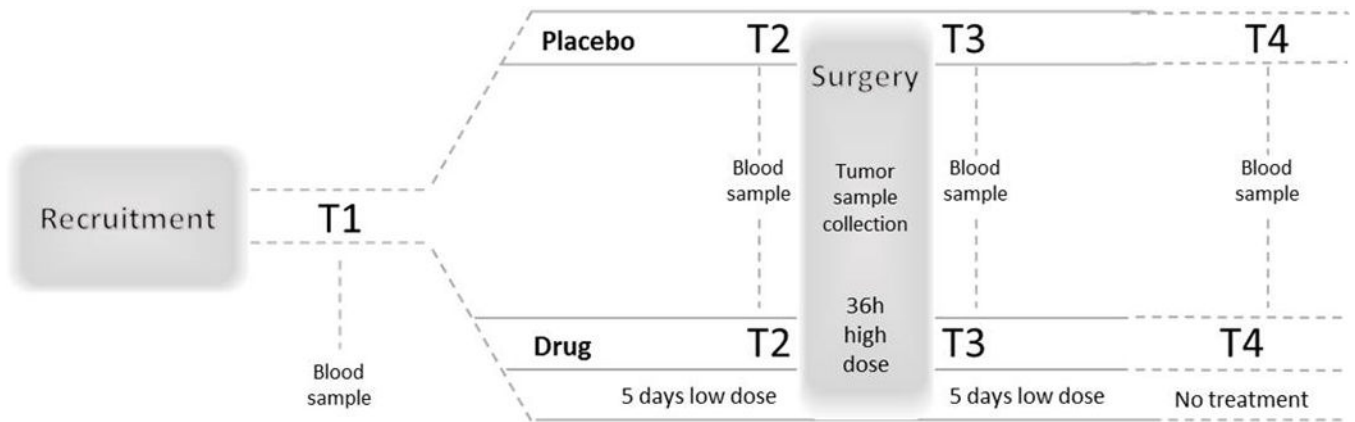CONSORT diagram of clinical trial enrollment and treatment.

Fig. 1B. Schematic presentation of the design and time schedule of the study
A double-blind placebo-controlled biomarker trial was conducted in early stage breast cancer patients, treating patients with placebo or with propranolol and etodolac for 11 consecutive days, starting 5 days before surgery. Propranolol doses were increased on the day of surgery. Of the 38 patients recruited, one from each group self-withdraw before surgery. Blood samples were collected before drug initiation (T1), on the morning before surgery (T2), on the morning after surgery (T3), and several days after cessation of drug treatment (T4). Tumor tissue was collected during surgery.
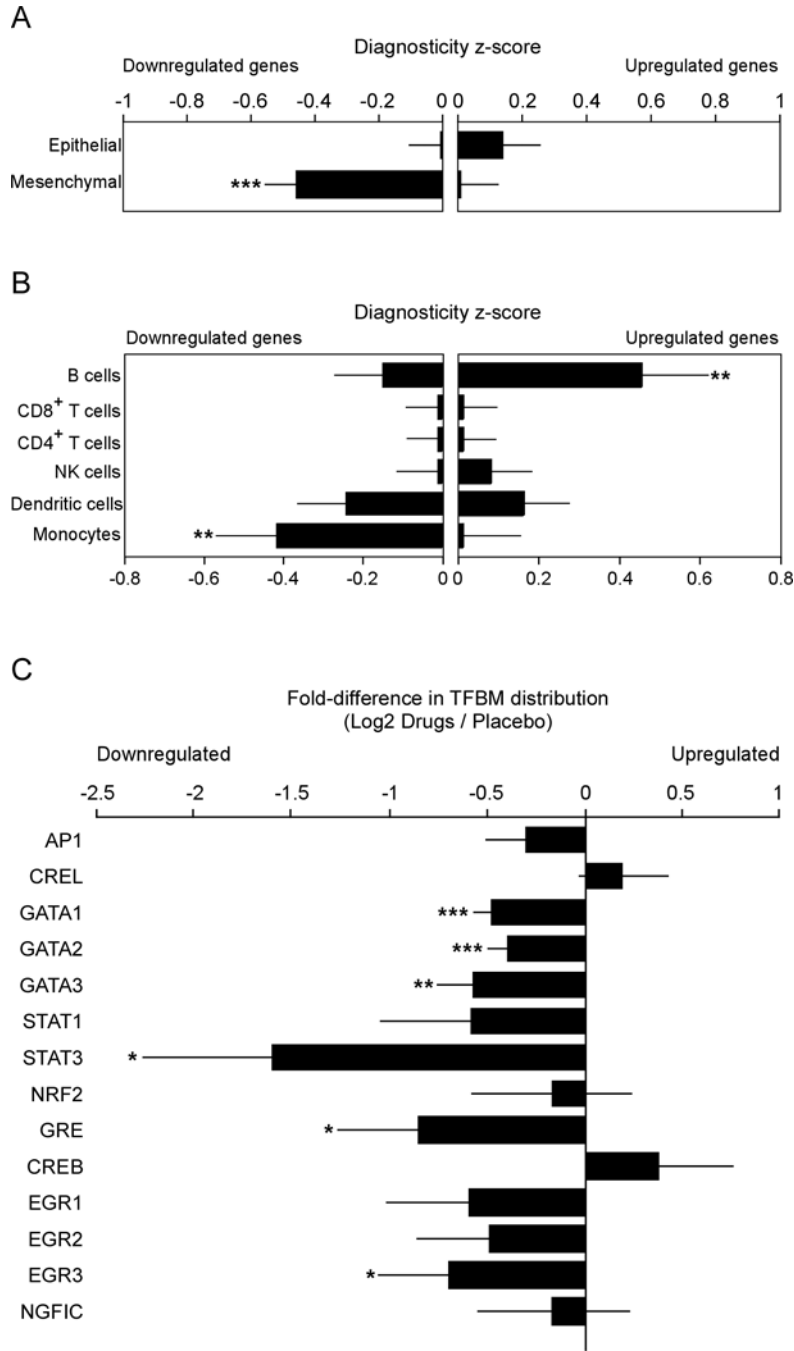
Fig. 2. Effect of drug treatment on primary tumor transcriptome indicators of EMT, tumor-associated leukocytes, and pro-metastatic transcription factors

Twenty-five tumors yielded RNA of sufficient quality for transcriptome profiling (10 drug-treated and 15 placebo). (A) Effects of drug treatment on primary tumor EMT gene expression were quantified by Transcript Origin Analysis (58) of 163 genes showing > 1.25-fold up-regulation and 141 genes showing equivalent down-regulation in tumors from drug-treated patients vs. controls, using reference transcriptome profiles derived from mesenchymal- vs. epithelial-polarized breast cancer cells (59). (B) Transcript Origin Analysis also assessed the effects of drug treatment on expression of genes derived from

monocytes, dendritic cells, CD4+ and CD8+ T cells, B cells and NK cells, using reference data derived from isolated samples of each cell type (22). (C) Effect of drug treatment on transcription control pathways as indicated by bioinformatics analysis of transcription factor-binding motifs in promoters of differentially expressed genes. Data is presented as mean ± SEM. Group differences are indicated by * ($p < 0.05$), ** ($p < 0.01$), or *** ($p < 0.001$).

Fig. 3. Effect of drug treatment on circulating levels of IL-6, CRP, IL-10 and cortisol levels (n=18 per group)

Serum levels of IL-6 (A), C-reactive protein (B), cortisol (C), and IL-10 (D) were assessed by commercial enzyme-linked immunosorbent assay (high-sensitivity ELISA kits for IL-6 and IL-10). Data represent mean ± SEM. Group differences at a specific time point are indicated by * ($p<.05$), *** ($p<.001$). A significant contrast between drug and placebo conditions during treatment (T2+T3) [vs off treatment (T1+T4)] is indicated by #.

Fig. 4. Effect of drug treatment on ex vivo stimulated production of IL-12 and IFN-γ, on numbers of circulating CD16⁻ monocytes, and on CD11a (LFA-1) expression levels on NK cells (n=18 per group)
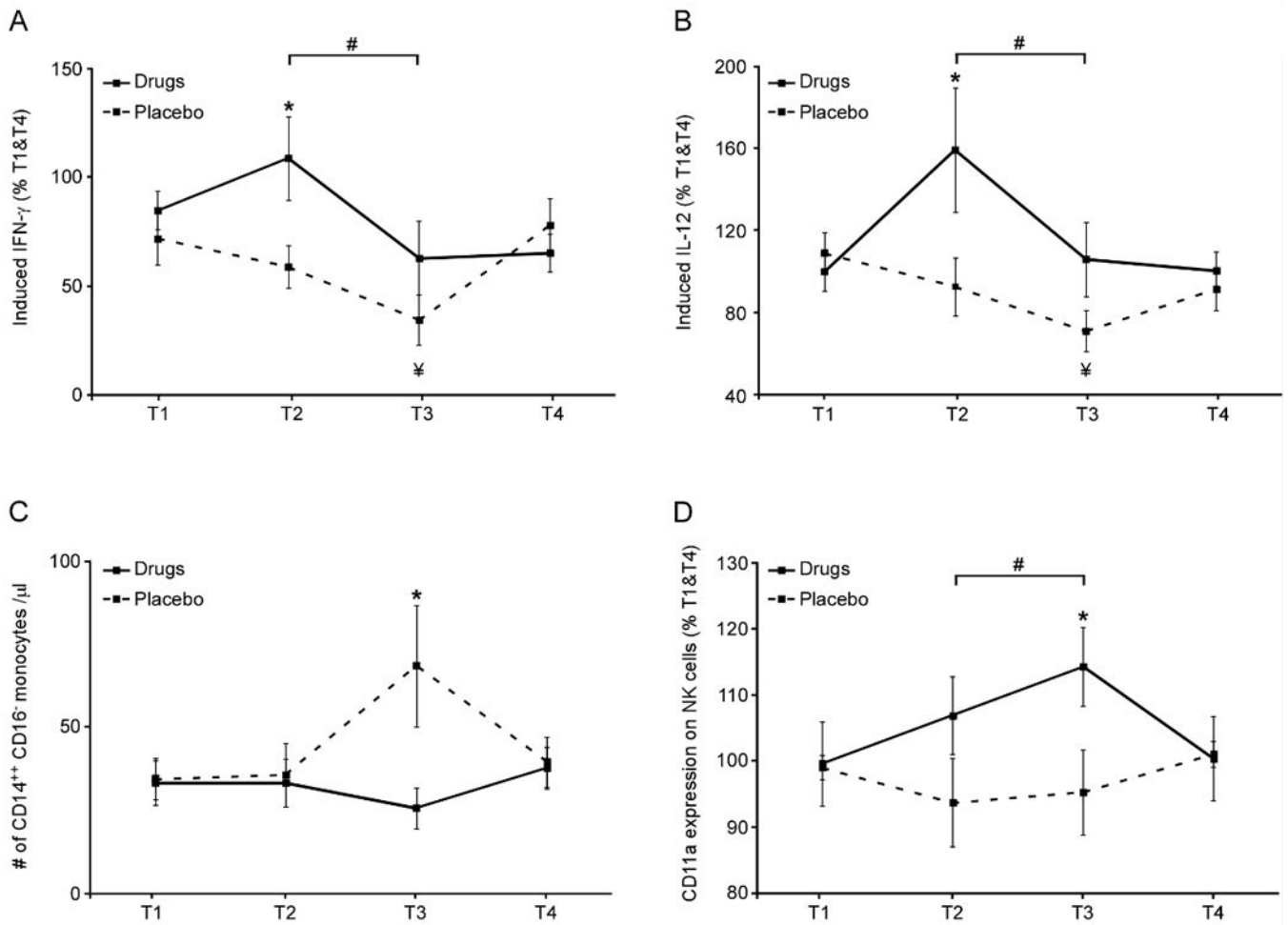
Venipuncture blood samples were assayed for: (A and B) Induced cytokine levels following 21-hrs LPS & PHA-stimulation, assessed in culture supernatant by enzyme-linked immunosorbent assay (ELISA); (C) circulating frequency of CD14^{++}CD16$^-$ "classical" monocytes, and (D) expression levels of the activation marker CD11a on NK cells (CD3$^-$CD56$^+$CD16$^+$ lymphocytes), assessed by flow cytometry. Data represent mean ± SEM. Group differences at a specific time point are indicated by * ($p < 0.05$). A significant contrast between drug and placebo treatments at T2+T3 [vs off treatment (T1+T4)] is indicated by #. A significant decrease from T1 to T3 within the placebo group is indicated by ¥.

Table 1

Baseline Patient Demographic and Clinical Characteristics (36 patients providing blood samples)

	Control Group n=18		Treatment Group n=18		p
Age Mean (MIN, MAX)	55.2 (33, 70)		55.3 (41, 70)		.97
BMI Mean (MIN, MAX)	25.7 (20.3, 32.0)		26.3 (19.4, 36.5)		.73
Weight Mean (MIN, MAX)	68.1 (52, 86)		69.5 (50, 103)		.75
Smoking – Present	NO	15	NO	9	.06
	YES (<5 cigarette per day)	1	YES (<5 cigarette per day)	2	
	YES (>5 cigarette per day)	1	YES (>5 cigarette per day)	6	
	NA	1	NA	1	
T Staging	Tis	2	Tis	0	.33
	T1	9	T1	13	
	T2	5	T2	3	
	T3	0	T3	0	
	NA	2	NA	2	
Histological Grade (HG)	HG1	5	HG1	3	.74
	HG2	6	HG2	10	
	HG2/3	1	HG2/3	1	
	HG3	2	HG3	1	
	DCIS/LCIS	4	DCIS/LCIS	3	
Surgical Resection	Lumpectomy	13	Lumpectomy	15	.56
	Mastectomy	2	Mastectomy	2	
	Other [a,b,c]	3	Other [d]	1	
Metastatic Spread	No	18	No	16	.34
	NA	0	NA	1	
			Axillary metastasis	1	
ER Status	Negative	2	Negative	1	.83
	Positive	16	Positive	17	
PR Status	Negative	6	Negative	5	.93
	Positive	12	Positive	13	
HER2/neu status	Negative	9	Negative	8	.76
	NA	4	NA	3	
	Positive	5	Positive	7	
Tumor Max. Diameter	1.6 cm		1 cm		.11
Carcinoma	Invasive	13	Invasive	15	.56
	Non-invasive	3	Non-invasive	1	
	NA	2	NA	2	

[a] Mastectomy +Immediate reconstruction with silicone,

[b] Lumpectomy (double- Lt&Rt),

[c] Lumpectomy (+Intraoperative radiation),

[d] Mastectomy with axillary sentinel lymph node excision

NA - not available;

DCIS – Ductal Carcinoma In Situ;

LCIS – Lobular Carcinoma In Situ

Table 2

Baseline Patient Demographic and Clinical Characteristics for Transcriptome Profiling[*]

	Control Group n=15		Treatment Group n=10		p
Age Mean (MIN, MAX)	57 (43,70)		57.3 (46, 70)		.93
BMI Mean (MIN, MAX)	25.1 (20.3, 32.0)		26.4 (19.4, 34.5)		.48
Weight Mean (MIN, MAX)	66.1 (52, 86)		67.7 (50, 94)		.76
Smoking – Present	NO	13	NO	5	.08
	YES (<5 cigarette per day)	1	YES (<5 cigarette per day)	2	
	YES (>5 cigarette per day)	0	YES (>5 cigarette per day)	2	
	NA	1	NA	1	
T Staging	Tis	2	Tis	0	.40
	T1	8	T1	9	
	T2	4	T2	1	
	T3	1	T3	0	
Histological Grade(HG)	HG1	5	HG1	2	.79
	HG2	6	HG2	6	
	HG2/3	1	HG2/3	0	
	HG3	1	HG3	1	
	DCIS/LCIS	2	DCIS/LCIS	1	
Surgical Resection	Lumpectomy	11	Lumpectomy	9	.31
	Mastectomy	1	Mastectomy	1	
	Other [a,b,c]	3	Other	0	
Metastatic Spread	No	15	No	10	1
ER Status	Negative	0	Negative	1	.45
	Positive	15	Positive	9	
PR Status	Negative	3	Negative	3	.84
	Positive	12	Positive	7	
HER2/neu status	Negative	8	Negative	6	.30
	NA	3	NA	0	
	Positive	4	Positive	4	
Tumor Max. Diameter	1.8 cm		1.2 cm		.32
Carcinoma	Invasive	12	Invasive	8	.93
	Non-invasive	2	Non-invasive	1	
	NA	1	NA	1	

[*] Twenty-five tumors yielded RNA quality assured for sufficient mas

[a] Mastectomy + Immediate reconstruction with silicone,

[b] Lumpectomy (double- Lt&Rt),

[c]Lumpectomy (+Intraoperative radiation)

NA - not available;

DCIS – Ductal Carcinoma In Situ;

LCIS – Lobular Carcinoma In Situ