A Randomized, Double-Blind, Phase II Study of Two Doses of Pemetrexed as First-Line Chemotherapy for Advanced Breast Cancer

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Abstract

Purpose: Pemetrexed has shown varied response rates in advanced breast cancer. This randomized, double-blind, phase II study was conducted to assess the efficacy and safety of two doses of pemetrexed in a homogeneous population. A secondary objective was to identify molecular biomarkers correlating with response and toxicity.

Experimental Design: Patients with newly diagnosed metastatic breast cancer or locally recurrent breast cancer received 600 mg/m^2 (P600 arm) or 900 mg/m^2 (P900 arm) of pemetrexed on day 1 of a 21-day cycle. All patients received folic acid and vitamin B_{12} supplementation.

Results: The P600 (47 patients) and P900 (45 patients) arms had response rates of 17.0% (95% confidence interval, 7.7-30.8%) and 15.6% (95% confidence interval, 6.5-29.5%) with ~50% stable disease per arm, median progression-free survival of 4.2 and 4.1 months, and median times to tumor progression of 4.2 and 4.6 months, respectively. Both arms exhibited minimal toxicity (grade 3/4 neutropenia <20%, leukopenia <9%, and other toxicities <5%). Tumor samples from 49 patients were assessed for the expression levels of 12 pemetrexed-related genes. Folylpolyglutamate synthetase and thymidine phosphorylase correlated with efficacy. Best response rates and median time to tumor progression for high versus low thymidine phosphorylase expression were 27.6% versus 6.3% (P = 0.023) and 5.4 versus 1.9 months (P = 0.076), and for folylpolyglutamate synthetase were 37.5% versus 10.0% (P = 0.115) and 8.6 versus 3.0 months (P = 0.019), respectively. γ-Glutamyl hydrolase expression correlated with grade 3/4 toxicities: 78.6% for high versus 27.3% for low γ-glutamyl hydrolase (P = 0.024).

Conclusion: The two pemetrexed doses yielded similar efficacy and safety profiles. Exploratory biomarker analysis identified efficacy and toxicity correlations and warrants further evaluation.

Patients with advanced breast cancer who experience tumor progression after anthracycline- and taxane-based regimens were considered unlikely to benefit from other chemotherapeutics (1). However, the development of novel agents like gemcitabine (2) and capecitabine (3) has yielded palliative improvement and survival gains. Additional development of novel, safe, and effective agents is greatly needed to further improve the palliation and survival of these patients.

Several phase II studies have established the single-agent activity of 600 mg/m^2 of pemetrexed, and later, 500 mg/m^2 of pemetrexed, in patients with untreated (4), minimally pretreated (one to two prior chemotherapies; refs. 5, 6), or heavily pretreated (three to five prior chemotherapies; refs. 7, 8) advanced breast cancer. Response rates ranged from 8% to 31%, with higher rates in the 600 mg/m^2 studies with untreated or minimally pretreated patients. Although both doses yielded an acceptable safety profile, toxicities in the later 600 mg/m^2 studies were significantly reduced with folic acid and vitamin B_{12} supplementation before and during treatment. In view of the heterogeneous patient population in some of these studies, the change in standard pemetrexed dosing, and the later use of supplemental folic acid/vitamin B_{12}, additional evaluation of pemetrexed dosing is needed.

Due to the higher response rates with 600 mg/m^2 of pemetrexed, the standard dose of pemetrexed in this study was fixed at 600 mg/m^2. A recent study established the maximum tolerated dose of pemetrexed with folic acid as 1,200 mg/m^2 in lightly pretreated patients and 925 mg/m^2 in heavily pretreated patients (9). These data suggest that 925 mg/m^2 of pemetrexed with vitamin supplementation should be well

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Received 9/26/06; revised 2/7/07; accepted 3/22/07.

Grant support: Eli Lilly and Company.

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Note: This abstract was presented at the 2006 ASCO Meeting, Atlanta, Georgia.

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doi:10.1158/1078-0432.CCR-06-2377

tolerated in relatively healthy, minimally pretreated patients with advanced breast cancer. To explore a possible dose-response relationship, 900 mg/m^2 of pemetrexed was chosen as the high dose in this study. Antitumor activity, time-to-event efficacy variables, and the toxicity profile were evaluated for both doses.

Given the heterogeneity of breast cancer and the variety of drugs available, clinical studies today need to include biomarker analyses to identify molecular biomarkers that correlate with response and toxicity, in addition to investigating standard efficacy and toxicity variables. Ultimately, the goal is to identify patients most likely to respond to particular therapies and then to construct an individualized treatment concept for each patient.

The mechanism of action of pemetrexed is well documented (10). Cells expressing high levels of thymidylate synthase (TS) or dihydrofolate reductase (DHFR) *in vitro* exhibit reduced sensitivities to pemetrexed (4, 11, 12). Likewise, pretreatment intratumoral TS expression levels are correlated with clinical outcome in patients treated with TS inhibitor 5-fluorouracil (13, 14). Other molecules involved with folate and antifolate metabolism, transport, and mechanism of action may similarly affect pemetrexed efficacy and toxicity. These include dihydropyrimidine dehydrogenase (DPD), folylpolyglutamate synthetase (FPGS), γ-glutamyl hydrolase (GGH), methylene tetrahydrofolate reductase (MTHFR), thymidine phosphorylase (TP), folate receptor α (FRα), and reduced folate carrier 1 (RFC1). Also, protein kinase Cβ (PKCβ) and vascular endothelial cell growth factor (VEGF) are two factors that may help elucidate the role of angiogenesis in this disease. This study evaluated the pretreatment tumor mRNA expression levels of each of these molecules and then correlated expression with efficacy and toxicity results.

Materials and Methods

Patient selection. Women ≥18 years with newly diagnosed metastatic breast cancer or relapsed breast cancer (local or distant) following adequate primary therapy were eligible. Patients with relapsed breast cancer were disease-free for ≥1 year and not amenable to curative local therapy. Patients may have received prior adjuvant/neoadjuvant chemotherapy, hormonal therapy, or immunotherapy; however, prior chemotherapy or immunotherapy for metastatic disease was not allowed. Other inclusion criteria were measurable disease according to Response Evaluation Criteria in Solid Tumors, Eastern Cooperative Oncology Group performance status of 0 to 2; estimated life expectancy of ≥6 months; and adequate marrow, hepatic, and renal function. Exclusion criteria included leptomeningeal metastases, serious concomitant systemic disorder, inability to interrupt nonsteroidal anti-inflammatory drugs for 2 days before and after pemetrexed administration, and unwillingness to take folic acid/vitamin B_{12} supplementation. Institutional ethics review boards approved the protocol, and the trial was conducted according to Good Clinical Practice guidelines and the Declaration of Helsinki. All patients provided written informed consent before treatment.

Study design. This was a multicenter, randomized, parallel, double-blind phase II study of two doses of pemetrexed. Patients were randomly assigned to 600 mg/m^2 (P600) or 900 mg/m^2 (P900) doses of pemetrexed. Randomization was balanced for performance status, prior chemotherapy, visceral (hepatic and/or pulmonary) involvement, and investigational site (probability factor, 0.75; ref. 15).

Treatment plan. Pemetrexed was administered i.v. on day 1 of a 21-day cycle. Treatment continued until progressive disease (PD) or investigator/patient decision to discontinue treatment. Patients took oral folic acid (350-1,000 μg) daily beginning 1 to 2 weeks before the first pemetrexed dose until 3 weeks after the final dose. Vitamin B_{12} (1,000 μg i.m. injection) was administered 1 to 2 weeks before the first pemetrexed dose and every 9 weeks thereafter until 3 weeks after the final dose. Dexamethasone (4 mg or equivalent) was administered orally twice daily beginning the day before and ending the day after each pemetrexed dose.

Pemetrexed doses were delayed (until resolution or return to baseline) and modified for either absolute neutrophil count $<0.5 \times 10^9$/L and a platelet count of $\geq 50 \times 10^9$/L (25% dose reduction) or a platelet count of $<50 \times 10^9$/L (50% reduction). Similarly, treatment was delayed for grade 3/4 nonhematologic toxicities (except for grade 3 transaminase elevation, nausea, and vomiting) or calculated creatinine clearance <45 mL/min. When nonhematologic toxicities resolved, therapy resumed at 50% of the previous level for grade 3/4 mucositis, and 75% of the previous level for grade 4 transaminase elevation, grade 3/4 diarrhea or diarrhea requiring hospitalization, and any other grade 3/4 nonhematologic toxicity deemed appropriate. Dose re-escalation was not allowed. Any patient requiring a third dose reduction or ≥42 days in between treatments was discontinued from the study.

Baseline and treatment assessments. Baseline tumor measurements were taken within 4 weeks before enrollment via computerized tomography, magnetic resonance imaging, or X-ray of clearly defined pulmonary lesions. The same baseline tumor assessment method was repeated before every other cycle. Tumor response was confirmed at least 4 weeks after first evidence and every other cycle thereafter. Objective tumor response was rated using Response Evaluation Criteria in Solid Tumors guidelines (16). Body surface area calculations, body weight, and clinical laboratory tests (hematology, blood chemistry, and creatinine clearance) were completed before each cycle. Toxicities were rated before each cycle using National Cancer Institute-Common Toxicity Criteria, version 2. When possible, primary tumor tissue samples were collected before treatment.

Time to tumor progression (TtTP) was measured from the time of randomization to the first observation of PD, progression-free survival from randomization to the first observation of PD or death, duration of response from the first observation of response to the first observation of PD or death, time to treatment failure (TtTF) from randomization to the first observation of PD, death, or early discontinuation of therapy, and overall survival from randomization to the time of death.

Statistical considerations. Enrolled patients who received one pemetrexed dose were evaluable for safety, and those who also had measurable disease were evaluable for efficacy. The response rate and its 95% exact binomial confidence interval (CI; ref. 17) were assessed for each arm. A one-sample two-sided binomial exact test was conducted for each arm to test whether the null hypothesis of response rate was equal to 0.2 against the alternative hypothesis that the response rate was not equal to 0.4; the preplanned sample size and power for this test was 41 patients per arm with 82% power. The distribution of time-to-event end points was estimated using the Kaplan-Meier method (18). All hypotheses, one- or two-sided, were tested at an α level of 0.05. All 95% CIs were two-sided. Calculation of *P* values, point estimates, CIs, and least squares means were done using SAS version 8 (SAS Institute, Inc., 1999).

Molecular biomarker analysis. Gene expression levels were determined using reverse transcriptase-PCR (Response Genetics, Inc.) for formalin-fixed, paraffin-preserved tumor tissue (19). For each gene, the assay was considered validated by achieving consistency between different preparations from the same tumor block. Gene expression levels were reported as the difference in reverse transcriptase-PCR cycle times (δ CT) of an endogenous reference (β-actin) and each of the 12 genes: *DHFR*, *DPD*, *FPGS*, *FRα*, *GARFT*, *GGH*, *MTHFR*, *PKCβ*, *RFC1*, *TP*, *TS*, and *VEGF*.

Associations between clinical outcomes (best tumor response, TtTP, incidence of grade 3/4 toxicities) and marker expression values were analyzed by fitting low- versus high-expression subgroups as a factor in the appropriate analysis. Each marker was analyzed separately, no other

covariates were included in the model. For each gene, the grouping that maximized the association with each clinical outcome was found by splitting the distribution of δ CT values at each value within the central 50% and selecting the threshold providing the maximum Wald χ^2 test statistic for the expression groups effect. Because a lower δ CT value reflects a higher relative gene expression, patients with a δ CT value at or above the threshold were classified as having low relative gene expression levels, whereas patients with a δ CT value below the threshold were classified as having high relative gene expression levels. Because the point best discriminating between groups was identified for each gene, significance tests were based on the asymptotic distribution of a maximum χ^2 value (20).

Results

Patient characteristics

Between May 2003 and March 2004, 15 investigational sites in Argentina, Belgium, Germany, Romania, Spain, and the United Kingdom enrolled 105 patients into this study. Thirteen patients did not meet the inclusion/exclusion criteria and did not receive the study drug. The remaining 92 patients were randomly assigned to the P600 arm (47 patients) or the P900 arm (45 patients; Fig. 1). Baseline characteristics are summarized in Table 1. The two arms were similar, although there were numerically more hormonal receptor–positive patients on the P900 arm (Fisher's exact test $P = 0.1582$ for estrogen receptor status and $P = 0.2096$ for progesterone receptor status).

Treatment

Patients received 285 pemetrexed doses on the P600 arm and 270 doses on the P900 arm. For each patient, the P600 arm administered a mean of 6.1 cycles (median, 6; range, 1-29), and the P900 arm administered a mean of 6.0 cycles (median, 5; range, 1-18). Both arms received a high percentage of the planned dose (P600 = 98.2%; P900 = 95.7%). Neither treatment arm had any dose omissions. One patient in the P600 arm received a reduced dose (alanine aminotransferase elevation). Six patients in the P900 arm received reduced doses: two patients due to platelet count decrease, and one patient each due to alanine aminotransferase elevation, weight loss, cellulitis, and fatigue/dyspnea. Both arms had a similar number of cycle delays (P600 = 12; P900 = 11) for clinically significant reasons.

Clinical results

Efficacy. Seventeen percent of the P600 patients were responders (all partial responses; Table 2). This was not statistically different from the P900 arm, with 15.6% responders (two complete responses and five partial responses). Both arms reported stable disease (SD) for about half of the patients. The median duration of SD was 6.5 months (95% CI, 4.6-12.1) for the P600 arm and 8.2 months (95% CI, 5.2-11.5) for the P900 arm. Of the 23 (P600) and 24 (P900) patients with SD, 9 (39.1%) and 13 (54.2%) had SD for ≥6 months, respectively. Defining clinical benefit as responders plus patients with SD >6

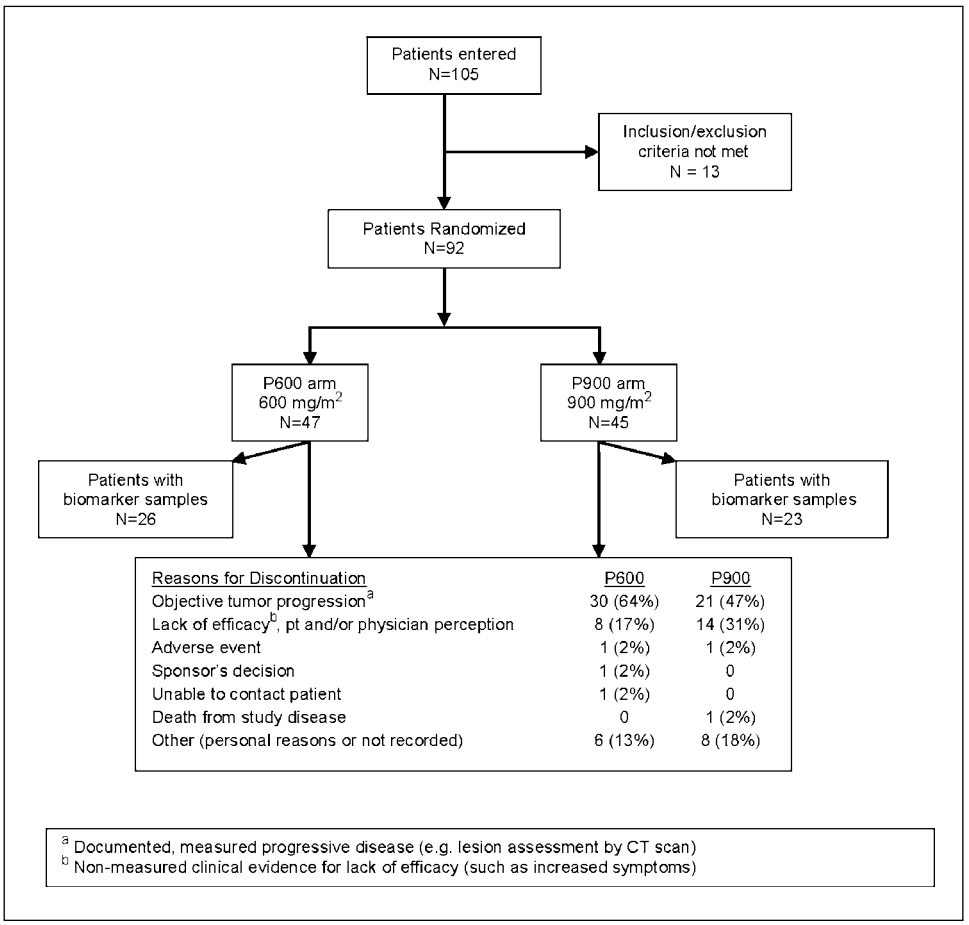

Fig. 1. Disposition of all patients entered into the study.

Table 1. Patient demographics and baseline characteristics (comparison between arms)

Variable	P600 (*n* = 47)	P900 (*n* = 45)
Median age (range)	56 (35-81)	61 (33-78)
Disease stage, *n* (%)		
Locally advanced (M_0)	1 (2.1)	4 (8.9)
Metastatic (M_1)	46 (97.9)	41 (91.1)
Number of disease sites, *n* (%)		
1	2 (4.3)	3 (6.7)
2	9 (19.1)	7 (15.6)
≥3	36 (76.6)	35 (77.8)
Site of disease, *n* (%)		
Visceral	31 (66.0)	29 (64.4)
Lung	14 (29.8)	17 (37.8)
Liver	19 (40.4)	17 (37.8)
Other	2 (4.3)	0
Nonvisceral	38 (80.9)	37 (82.2)
ECOG performance status, *n* (%)		
0	25 (53.2)	25 (55.6)
1	18 (38.3)	20 (44.4)
2	3 (6.4)	0
Unknown	1 (2.1)	0
Prior treatment		
Surgery	38 (80.9)	41 (91.1)
Chemotherapy (adjuvant or neoadjuvant)	28 (59.6)	29 (64.4)
Radiotherapy	34 (72.3)	32 (71.1)
Hormonal*	23 (48.9)	27 (60.0)
ER/PR		
+/+	12 (25.5)	18 (40.0)
-/-	18 (38.3)	9 (20.0)
+/- or -/+	7 (14.9)	7 (15.6)
Unknown	10 (21.3)	11 (24.4)

Abbreviations: ECOG, Eastern Cooperative Oncology Group; ER, estrogen receptor; PR, progesterone receptor.
*All patients who received prior hormonal therapy did so in an adjuvant setting, except one patient in each treatment arm. Three patients in each arm received prior hormonal therapy in a locally advanced setting.

months, the clinical benefit of the P600 arm was 36.2% and for the P900 arm it was 44.4%. The 35 chemo-naïve patients (both arms) had a similar distribution of responses as the whole population (data not shown). Secondary efficacy assessments showed similar treatment effects on both arms (Table 3).

Safety. Both arms yielded minimal toxicity. Of the serious drug-related toxicities that did occur, most were hematologic (Table 4). Grade 3/4 nonhematologic toxicities were infrequent, and no incidents of grade 3/4 rash/desquamation or mucositis (stomatitis, pharyngitis) occurred. Additionally, only one patient had grade 2 alopecia, and seven patients reported grade 1 alopecia. There were no treatment-related deaths. Two patients discontinued treatment due to a renal-associated adverse event. One patient in the P600 arm with a baseline creatinine level of 1.1 mg/dL experienced grade 1 creatinine increase to 1.5 mg/dL at cycle 8, day 23. Creatinine was still elevated (1.3 mg/dL) at cycle 8, day 32, and the patient discontinued from the study. The elevated creatinine persisted for more than 3 months after discontinuation. The second patient (P900) experienced decreased creatinine clearance (≤45 mL/min) which began at cycle 4 and persisted throughout the study. At cycle 10, the patient developed grade 3 renal failure and was discontinued from the study. The renal failure resolved 13 days later. The patient also experienced grade 3 anemia during cycle 9, grade 4 anemia during cycle 10, and grade 3 thrombocytopenia 3 days after the onset of renal failure.

Biomarker results

Efficacy. Tumor samples from 49 patients were used for biomarker expression analyses. This included 26 samples from P600 patients (5 responders, 11 SD, 9 PD, 1 unknown) and 23 samples from P900 patients (4 responders, 9 SD, 9 PD, 1 unknown). The FRα expression was out of range for most samples; thus, the results were inconclusive and not reported here. For the other 11 markers, most tumor samples produced mRNA expression results within range of the reverse transcriptase-PCR detection limits.

When biomarker expression values were analyzed for association with efficacy measures (Table 5), only TP was significantly associated with best tumor response ($P = 0.023$). As detailed in Table 6, 23 of 29 patients (79.3%) with high TP expression had a clinical benefit [8 (27.6%) responders, 15 (51.7%) SD]. In contrast, 5 of 16 patients (31.3%) with low TP expression had a clinical benefit [1 (6.3%) responder, 4 (25.0%) SD]. FPGS expression was weakly associated with best tumor response ($P = 0.115$; Table 5).

Only FPGS was significantly associated with TtTP ($P = 0.019$; Table 5). The FPGS high-expression subgroup (21 of 48 patients; 43.8%) had a median TtTP greater (8.6 months) than the low-expression subgroup (3.0 months; Table 6). In contrast, TP expression was weakly associated with TtTP ($P = 0.076$).

Safety. No significant associations were detected between individual toxicities or classes of toxicities and gene expression levels. Only GGH correlated with the occurrence of grade 3/4 toxicities in general. Eleven of 14 (78.6%) patients with high

Table 2. Comparison of best study response between treatment arms

Best study response	P600 arm (*n* = 47)	P900 arm (*n* = 45)	Difference between treatment arms
CR, *n* (%)	0	2 (4.4)	
PR, *n* (%)	8 (17.0)	5 (11.1)	
SD, *n* (%)	23 (48.9)	24 (53.3)	
PD, *n* (%)	14 (29.8)	13 (28.9)	
Unknown, *n* (%)	2 (4.3)	1 (2.2)	
Responders (CR + PR), *n* (%)	8 (17.0)	7 (15.6)	
95% CI	7.7-30.8	6.5-29.5	
*P**	0.749	0.823	0.657
P†			0.885

Abbreviations: CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.
*The between-group *P* value was derived with the Fisher's exact test based on response category (CR, PR, SD, PD, unknown). The within-group *P* value is for the one-sided test of the null hypothesis of response rate ≤0.2.
† Mantel-Haenszel test of column mean score difference in tumor response (based on CR, PR, SD, PD, unknown) between the two arms.

Table 3. Secondary efficacy variable results

Variable, P600 arm/P900 arm (censoring rate, %)	P600 (n = 47) median, months (95% CI)	P900 (n = 45) median, months (95% CI)	HR (95% CI)	Log-rank P
TtTP (27.7/26.7)	4.2 (2.7-5.6)	4.6 (3.0-7.2)	0.80 (0.49-1.30)	0.364
PFS (27.7/24.4)	4.2 (2.7-5.6)	4.1 (3.0-6.2)	0.82 (0.50-1.34)	0.434
DR* (50.0/14.3)	5.4 (2.9-?)	7.4 (2.8-14.0)	1.64 (0.45-5.89)	0.447
TtTF (6.4/0.0)	4.1 (2.7-4.5)	3.3 (2.7-3.9)	1.04 (0.68-1.59)	0.849
OS* (61.7/66.7)	(15.1-?)	21.4 (17.5-22.2)	0.87 (0.44-1.72)	0.687

Abbreviations: PFS, progression-free survival; DR, duration of response; TtTF, time to treatment failure; OS, overall survival.
*The high censoring rate and partial confidence intervals precluded meaningful interpretation of the results.

GGH expression experienced a grade 3/4 toxicity, whereas 9 of 33 (27.3%) patients with low GGH expression experienced a grade 3/4 toxicity (P = 0.024).

Discussion

A randomized, phase II study of pemetrexed as first-line chemotherapy in vitamin-supplemented, advanced breast cancer patients found similar antitumor activity at 600 and 900 mg/m^2 of pemetrexed as assessed by response rate (17.0% and 15.6%) and by median TtTF, time to treatment failure, and progression-free survival. Additionally, when patients with SD for >6 months were included with responders as receiving clinical benefit, the arms were again comparable: 36.2% clinical benefit (P600) and 44.4% clinical benefit (P900). The safety profile for the two arms was also similar, both showing minimal toxicity. Vitamin supplementation likely eliminated the incidence of grade 3/4 mucositis, which occurred in studies without supplementation, and reduced the frequency of severe hematologic toxicities (21). Additionally, prophylactic dexamethasone likely eliminated grade 3/4 rash/desquamation compared with studies without dexamethasone pretreatment (5). As with other pemetrexed studies, the incidence of alopecia was low, an important patient advantage over some chemotherapies. Two patients discontinued from the study due to renal adverse events: one modest, one severe. Accumulated data suggests that pemetrexed does not have significant (>5%) nephrotoxicity (Pemetrexed Core Data Sheet, September 21, 2005; Eli Lilly and Company); however, there have been sporadic reports of elevated creatinine and renal toxicity (22). The mechanism of such toxicity remains unknown.

The efficacy in this trial confirms and extends the results from previous pemetrexed phase II trials. The response rate was comparable to the 21% observed when 600 mg/m^2 of pemetrexed was given to patients with locally advanced or metastatic breast cancer, most of whom had one prior chemotherapy regimen (6). As expected, the vitamin supplementation included in this study greatly improved the pemetrexed safety profile over that observed by Martin et al. (6). The response rate in this trial exceeded the reports of two trials which used 500 mg/m^2 of pemetrexed (9% and 8%; refs. 7, 8). Because elevating pemetrexed to 900 mg/m^2 did not

Table 4. Summary of all drug-related Common Toxicity Criteria grade 3 and 4 toxicities

Toxicity	P600 arm (n = 47), n (%)		P900 arm (n = 45), n (%)	
	Grade 3	Grade 4	Grade 3	Grade 4
Hematologic				
Neutropenia	7 (14.9)	2 (4.3)	4 (8.9)	2 (4.4)
Leukopenia	3 (6.4)	0	2 (4.4)	2 (4.4)
Anemia	2 (4.3)	0	1 (2.2)	1 (2.2)
Thrombocytopenia	0	0	2 (4.4)	0
Nonhematologic				
Alanine aminotransferase	1 (2.1)	0	2 (4.4)	0
Aspartate aminotransferase	1 (2.1)	0	0	0
Alkaline phosphatase	1 (2.1)	0	0	0
Hypocalcemia	1 (2.1)	0	1 (2.2)	0
Bilirubin	1 (2.1)	0	0	0
Fatigue	1 (2.1)	0	1 (2.2)	1 (2.2)
Diarrhea	2 (4.3)	0	0	0
Anorexia	1 (2.1)	0	0	0
Nausea	0	0	1 (2.2)	0
Febrile neutropenia	0	0	1 (2.2)	0
Infection without neutropenia	0	0	1 (2.2)	0
Renal failure	0	0	1 (2.2)*	0
Other neurology	0	0	1 (2.2)	0

*This patient discontinued from the study due to renal failure. Another patient also discontinued from the study due to renal function–associated reasons, which was a grade 1 creatinine increase that persisted ≥9 d.

Table 5. Association of biomarker expression with efficacy variables: best response rate and TtTP

Marker	Response rate, *P**	TtTP, *P**
DHFR	0.575	0.446
DPD	0.707	1.000
FPGS	0.115	0.019
GARFT	0.553	0.489
GGH	0.704	0.711
RFC1	0.747	0.678
TP	0.023	0.076
TS	0.627	0.836
MTHFR	0.823	0.235
PKCβ	0.777	0.503
VEGF	0.963	0.489

*Asymptotic probability of the observed maximum χ^2 statistic under the null hypothesis of no association between best study response or TtTP and marker expression level, limiting the search to the central 50% of values. Calculated with the formula of Miller and Siegmund (22).

increase the response rate in this trial, the lower response rates in these other trials is likely not due to the lower dose, but rather to the heavily pretreated patients. This is supported by the 31% response rate observed when 500 mg/m^2 of pemetrexed was given to chemo-naïve patients (4).

Our study indicated that high TP expression was significantly associated with positive tumor response (P = 0.023) and the trend toward improved TtTP. Similarly, high FPGS expression showed a trend in its association with positive tumor response and was significantly associated with improved TtTP (P = 0.019).

The association found between high mRNA expression levels of TP and pemetrexed efficacy has precedence in multiple preclinical studies. TP catalyzes the reversible phosphorolysis of thymidine to thymine and is involved in the activation of fluropyrimidine. High TP expression in tumor cells has been associated with a greater responsiveness to TS inhibitors (23). Folate-based TS inhibitors may be more effective in tumors with high TP because of the increased rate of degradation of thymidine (24). Other studies have found a seemingly conflicting result: high TP expression was correlated with poor prognosis in some cancers (25–29). This might be due to its angiogenic properties, as TP is also referred to as platelet-derived endothelial cell growth factor.

FPGS converts pemetrexed to a polyglutamated form facilitating its intracellular bioaccumulation and preferential use as a substrate for some folate-dependent enzymes, thus increasing pemetrexed's potency. Recent preclinical studies have correlated low FPGS expression with antifolate resistance (30–33). Our study suggests that the converse is true as well: high FPGS expression is associated with antifolate efficacy. Alternatively, because this study did not include a nonpemetrexed arm, FPGS may be a positive prognosticator for TtTP in general, rather than for pemetrexed specifically. A similar conclusion can be drawn for TP.

Table 6. Association of high versus low TP and FPGS expression subgroups with response rate and TtTP

Response category	High-expression subgroup* (δ CT below threshold), *n* (%)	Low-expression subgroup* (δ CT at or above threshold), *n* (%)	OR† (95% CI)
Association of high versus low TP expression with response‡			
CR	1 (3.4)	0	8.65 (2.22-33.68)
PR	7 (24.1)	1 (6.3)	
SD	15 (51.7)	4 (25.0)	
PD	6 (20.7)	11 (68.8)	
Association of high versus low FPGS expression with response§			
CR	1 (6.3)	0	4.78 (1.38-16.50)
PR	5 (31.3)	3 (10.0)	
SD	7 (43.8)	13 (43.3)	
PD	3 (18.8)	14 (46.7)	

Gene	High expression, median TtTP (95% CI) [*n*]	Low expression, median TtTP (95% CI) [*n*]	*P*, HR (95% CI)
Association of high versus low FPGS and TP expression subgroups with TtTP			
FPGS‖	8.6 (4.1-10.5) [21]	3.0 (1.7-4.1) [27]	0.019, 0.3 (0.1-0.6)
TP¶	5.4 (3.5-9.0) [30]	1.9 (1.6-5.6) [16]	0.076, 0.4 (0.2-0.8)

Abbreviations: OR, odds ratio; HR, hazard ratio; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; CT, reverse transcriptase-PCR threshold cycle value; δ CT, the difference between the marker of interest CT and the endogenous β-actin CT within each patient.

*The threshold δ CT value provided the best association between high- and low-expression subgroups of patients and best study response. Patients with a δ CT value at or above the threshold were classified as having low relative gene expression levels; patients with a δ CT value below the threshold were classified as having high relative gene expression levels.

† Overall OR of better clinical outcome from logistic regression analysis.

‡ Of the 49 tumor samples, four yielded TP mRNA expression levels that were out of range or not detected. The threshold for the 45 samples used was 5.485.

§ Of the 49 tumor samples, three yielded FPGS mRNA expression levels that were out of range or not detected. The threshold for the 46 samples used was 5.742.

‖ Forty-eight of the 49 tumor samples were used for this analysis; the threshold was 5.841.

¶ Forty-six of the 49 tumor samples were used for this analysis; the threshold was 5.485.

The present study did not find a correlation between efficacy and TS expression as noted in the Gomez et al. trial (4). This may have been due to different patient populations such as disease characteristics (metastatic versus nonmetastatic) and extent and type of prior therapy, or due to differences in sample collection and handling methodology. As future studies examine biomarkers, it will be important to control these variables and provide patient and sample numbers necessary for a sufficiently powered study.

The expression level of each biomarker was also compared with safety data to determine if any marker was predictive of the toxicity of pemetrexed. The association between high GGH expression and greater risk for experiencing grade 3/4 toxicity was an unexpected result. Given that this enzyme functions to cleave intracellular polyglutamate derivatives, thus breaking down the active form of pemetrexed, one might have predicted that the high-expression subgroup would have had fewer grade 3/4 toxicities. High GGH expression has been shown in cells resistant to antifolates (34), although other research has shown that it alone is insufficient to produce clinical antifolate resistance (35). Importantly, one must note that the biomarkers were measured in tumor tissue, and normal tissue expression might differ markedly. Because it is the normal tissue's response to the drug that typically causes safety concerns, future studies will have to examine if this association exists when GGH is measured in normal tissue and in studies with greater patient and sample numbers.

As in all studies analyzing biomarkers, the results presented for this study have their limitations. The tissue preservation technique, the lag between obtaining the tissue biopsy and the beginning of treatment, and possible intervening treatments during this interval, all could reduce the association between assay results and clinical outcomes. Given that the analyses presented describe the empirical support for the presence or absence of this association, if an association is found, it exists given all of these caveats. If there isn't a significant result, each is a possible reason for the lack of association, along with the possibility of the absence of *in vivo* association. Another limitation of biomarker studies is that all genes potentially affecting the clinical effectiveness of the drug are usually not analyzed. Despite these limitations, biomarker analysis could provide important insights. Future studies will undoubtedly continue to refine the methodology, scope, and interpretation of this type of analysis.

Pemetrexed yielded moderate antitumor activity (response rate 16%, clinical benefit ~40%) and minimal toxicity as the first line of treatment in patients with advanced breast cancer. Elevating the dose to 900 mg/m^2 was insufficient to increase the response rate. Considering the low toxicity profile observed with vitamin supplementation, further increases in the dose of pemetrexed might yield improved efficacy. Our results suggest that pemetrexed responsiveness and toxicity is associated with particular expression patterns of folate pathway genes. Future trials should continue to examine gene expression levels important to the folate pathway and breast cancer in order to identify a multigene profile predictive of pemetrexed efficacy and toxicity. This would enable us to prospectively identify patients either sensitive or relatively resistant to pemetrexed, and thus, better target appropriate treatments.

Acknowledgments

The authors thank all the patients, investigators, and institutions involved in this study and Mary Dugan Wood, Pete Fairfield, Ghulam Kalimi, and Lori Anderson for writing and editorial assistance.

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Clin Cancer Res 2007;13:3652-3659.