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Phase II trial of gemcitabine and genistein in metastatic breast cancer patients, with biomarker assays

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PRECIS

Metastatic breast cancer remains an incurable disease. Single agent conventional chemotherapy such as gemcitabine yields response rates in the 20-25 % response rates, with progression free survival durations of ~6 months. There is public and scientific interest in soy isoflavones such as genistein as a breast cancer prevention or treatment agent. Recently, genistein has been shown to induce breast cancer growth inhibition and apoptosis in preclinical studies, in part by inhibiting Akt and NF-κB signal pathways. When soy isoflavones are administered to humans at a well tolerated dose, NF-κB signaling is inhibited in human peripheral lymphocytes, demonstrating bioactivity at these doses. Moreover, combinations of gemcitabine and genistein have demonstrated at least additive inhibitory effects in vitro in carcinoma cells. Although there is significant lay and scientific interest in genistein's effects in human breast cancer, there are no peer-reviewed published data regarding the in vivo effects of genistein in patients with breast cancer. Thus, the major rationale is that the addition of genistein may increase the effectiveness without added toxicity. Therefore, we propose to combine gemcitabine 1,000 mg/m^2 IV days 1 and 8, with genistein 100mg PO BID days 1-21, with cycles q21 days, for first or second line treatment of patients with metastatic breast cancer. Prior to cycle 1, day 1, genistein will be administered alone for seven days for the collection of baseline plasma levels, and for the collection of accessible tumor tissue for molecular biomarker studies. Along with standard dose modification guidelines for toxicity, prospective safety monitoring and stopping rules are incorporated into the study design insure the safety of this protocol. The primary objective is to estimate the objective response rate. Secondary clinical objectives are to obtain data on the quality of the responses, overall survival, and the toxicity and tolerance to this combination. Secondary translational objectives are to assess plasma genistein levels and explore associations with responses, and to explore the effect of genistein alone on tumor biomarkers in order to better understand its mechanisms.

1.0 OBJECTIVES

1.1 Primary Objective

1.1.1 To estimate the objective response rate in patients with metastatic breast cancer treated with the combination of gemcitabine and genistein as first or second line therapy.

1.2 Secondary Objectives

1.2.1 To obtain data on duration of response, time to disease progression, and duration of survival.

1.2.2 To estimate the quantitative and qualitative toxicities of this regimen.

1.2.3 To assay the plasma genistein levels and explore associations with responses.

1.2.4 Explore the in vivo effects of genistein in human breast cancer tissue biomarkers (Ki67, TUNEL, phosphorylated-Akt, and NF-κB), and by cDNA microarray analysis.

2.0 BACKGROUND

2.1 Metastatic Breast Adenocarcinoma

Approximately 190,000 new cases of breast cancer are diagnosed each year in the USA (1). Although early stage disease is potentially curable through a multimodality approach involving surgery, chemotherapy and radiation, metastatic breast cancer remains a largely incurable disease accounting for 41,000 deaths each year (1, 2). For the vast majority of these patients, metastatic breast cancer is an incurable disease with a median survival of only 2 to 3 years after diagnosis (2). Long-term remissions with combination chemotherapy have been reported to occur in only 2-3% of patients (3). Historically, the main objective of treatment in the metastatic setting was the improvement in the quality of life. Recently, several trials have demonstrated a prolongation in median survival of women with metastatic breast cancer with effective systemic therapy (4, 5). These trials demonstrate the importance of introducing novel active agents or combinations of agents that could potentially improve survival as well as palliate symptoms.

2.2 Gemcitabine

Gemcitabine (2,2'-difluorodeoxcytidine, dFdC) is a nucleoside analog initially synthesized as a potential anti-viral drug and had excellent activity against both RNA and DNA viruses using cell culture assays. Despite early encouraging *in vitro* results, the *in vivo* therapeutic index was disappointingly low and the drug was not developed further. Concurrent evaluation of the cytotoxic activity of the compound demonstrated that gemcitabine was a potent and specific deoxycytidine analog with an acceptable *in vivo* therapeutic index. The mechanism of action and metabolism of gemcitabine have been well characterized. Gemcitabine inhibits the synthesis of DNA by at least two mechanisms. Gemcitabine is phosphorylated by deoxycytidine kinase to dFdC-5'-monophosphate (dFdCMP). Difluorodeoxyuridine is a product of gemcitabine deaminations and is inactive. dFdCMP is further metabolized to dFdC5'-diphosphate (dFdCDP) and dFdC-5'triphosphate (dFdCTP), which when incorporated into DNA, results in masked chain termination. In comparison to Ara-C incorporation into DNA, dFdCTP is less readily excised from DNA by DNA exonuclease. Thus, dFdCTP accumulates intracellularly to a greater degree than Ara-C-CTP, which may account, at least in part, for its different spectrum of pre-clinical and clinical activity. In addition, dFdCDP inhibits ribonucleotide reductase resulting in reduced formation of deoxyribonucleotides (6). Other intracellular effects of gemcitabine include stimulation of deoxycytidine kinase and inhibition of deoxycytidine monophosphate deaminase (7).

Of particular importance is the fact that the therapeutic effects of gemcitabine at the maximum tolerated dose level are dependent on the administration schedule. The drug was injected intraperitoneally to mice in various schedules at equitoxic maximum tolerated dose levels, resulting in a reversible weight loss that varied between 5% and 15%. Generally, it was found that treatment with 120 mg/kg gemcitabine, injected four times at 3-day intervals, was more effective than the schedules of daily (five times 2.5 to 3.5 mg/kg) or weekly (two times 240 mg/kg) injections (8). Gemcitabine, but not cytosine arabinoside (Ara-C), had a broad spectrum of anti-tumor activity against seven different types of murine solid tumors. The activity of gemcitabine was also schedule dependent (9). Additional experiments were performed on normal mice bearing the colon 26-10 murine colon carcinoma. The effect of a continuous intravenous infusion system was investigated by giving two injections of 15 mg/kg gemcitabine over 24 hours at a 7-day interval. Interestingly, the efficacy of treatment increased dramatically with this infusion schedule, producing complete remissions in most tumors (8).

Other investigators have shown that the 3-day interval schedule also was active in human pancreas and lung carcinoma xenografts (8). Gemcitabine was tested against 12 human carcinoma xenografts. When given on an every 3 day x 4 schedule, the following percent inhibitions (at maximally tolerated doses (MTD); MTD/2) in tumor growth were seen: MX-1 mammary (93%; 80%), CX-1 colon (92%; 82%), HC-1 colon (96%; 92%), GC3 colon (98%; 94%), VRC5 colon (99%; 100%), LX-1 lung (76%; 61%), CALU-6 lung (75%; 38%), NCI-H460 lung (45%; 46%), HS766T pancreatic (73%; not tested), PaCa-2 pancreatic (69%; 40%), PANC-1 pancreatic (70%; 60%), and BxPC-3 pancreatic (9%; 19%). In contrast, only the LX-1 lung carcinoma xenograft was responsive to Ara-C treatment, which inhibited tumor growth by a marginal 62%. Thus, like its activity against murine solid tumors, gemcitabine has excellent anti-tumor activity against a broad spectrum of human solid tumors (9).

Following the demonstration of broad-spectrum cytotoxic activity in pre-clinical models, Phase I human studies for gemcitabine began in September 1987 (10). Doses ranging from 10 to 1,000 mg/m^2 were administered over 30 minutes weekly times 3 weeks every 4 weeks. The maximum-tolerated dose was 790 mg/m^2. The dose-limiting toxicity was myelosuppression, with thrombocytopenia and anemia quantitatively more important than granulocytopenia. Non-hematologic toxicity was minimal. The maximum dFdC plasma concentration, reached after 15 minutes of infusion, was proportional to the total dose administered. Elimination, due mainly to deamination, was rapid (terminal half-life (t1/2), 8.0 minutes) and dose independent. The deamination product 2', 2'-difluorodeoxyuridine (dFdU) was eliminated with biphasic kinetics characterized by a long terminal phase ($t_{1/2}$, 14 hours); it was the sole metabolite detected in urine. The plasma and cellular pharmacology of gemcitabine was studied during a Phase I trial. The steady-state concentration of dFdC in plasma was directly proportional to the dFdC dose, which ranged between 53 and 1,000 mg/m^2 per 30 min. The cellular pharmacokinetics of an active metabolite, dFdC 5'-triphosphate (dFdCTP) was determined in mononuclear cells of 22 patients by anion-exchange high-pressure liquid chromatography. The rate of dFdCTP accumulation and the peak cellular concentration were highest at a dose rate of 350 mg/m^2 per 30 min, during which steady-state dFdC levels of 15-20 μM were in plasma. A comparison of patients infused with 800 mg/m^2 over 60 min. with those receiving the same dose over 30 min. demonstrated that the dFdC steady-state concentrations were proportional to the dose rate, but that cellular dFdCTP accumulation rates were similar at each dose rate. At the lower dose rate, the AUC for dFdCTP accumulation was 4-fold that observed at the higher dose rate. Consistent with these observations, the accumulation of dFdCTP by mononuclear cells incubated *in vitro* was maximal at 10-15 μM dFdC. These studies suggest that the ability of mononuclear cells to use dFdC for triphosphate formation is saturable (11).

A Phase I study utilizing twice-weekly injections was conducted in 50 eligible and evaluable patients. Twenty-nine patients received drug by 30-minute infusion at doses of 5-90 mg/m^2, and 22 patients by 5-minute bolus at 30-150 mg/m^2. The primary dose limiting toxicities were marrow suppression and flu-like symptomatology. Thrombocytopenia was dose limiting at 75 mg/m^2 on the infusion schedule and 150 mg/m^2 on the 5-minute schedule. Flu-like symptoms with fever rigors and malaise occurred the day of injection in many patients (12). In another Phase I study, patients received gemcitabine at therapeutically active doses ($\geq$ 875 mg/m^2/week x 3 every 28 days) (13). Except for one patient, all were given gemcitabine doses exceeding 1,000 mg/m^2 (1 patient at 875, 3 at 1,095 and 11 at 1,370

mg/m^2) for a total of 50 delivered courses. Dose-limiting hematologic toxicity was found at 1,370 mg/m^2/wk as underscored by the higher number of toxic treatment delays requiring subsequent dose attenuation in 6 of 11 patients. Toxicity was mild and easily managed. WHO grade 2-3 toxicity included leukopenia (53%), thrombocytopenia (20%), anemia (53%), AST/ALT rises (27%), emesis (40%) and fever (grade 2 only) (60%). An integrated database of toxicity was also analyzed according to starting dose (800, 1000 or 1250 mg/m^2) in a subset of 665 chemo-naive patients to see whether an increased dose resulted in increased toxicity. In general, only small, clinically insignificant differences in toxicity were seen between the three dose groups. Although neutropenia appeared to increase as starting dose increased (grade 3 or 4, 19.4%, 23.2%, 28.3%, respectively), this was not associated with an increased incidence of infection. In some cases, toxicity decreased with increasing dose but this may have been because of imbalances between the patient groups. These findings indicate that not only is gemcitabine well tolerated, but also the use of higher doses may be possible (13)

2.2 Gemcitabine In Breast Cancer

Table 1. Phase II trials of single agent gemcitabine in advanced breast cancer

Author	Year	N	Resp. Eval. Pts.	% prior chemo	% prior chemo. for met. disease	No. prior chemo. treatments for met. disease	% with prior anthra.	mg/m^2 dose and schedule	mg/m^2/week	Response rate	% grade 4 neutropenia	% FN (3)
Carmichael	1995	44	40	65%	43%	0 - 1	39%	800 q wk x 3, q 4 wks	600	25%	7%	2%
Brodowicz(1)	1998	24		100%		2 - 3	100%	1250 q wk x 3, q 4 wks	937.5	12%		
Possinger	1999	42	42	24%	0%	0		1000 q wk x 3, q 4 wks	750	14%	5%	0%
Spielman(2)	2001	47	41	100%	100%	1 - 2	100%	1200 q wk x 3, q 4 wks	900	29%	4%	0%
Blackstein	2002	39	35	49%	0%	0		1200 q wk x 3, q 4 wks	900	37%	0%	0%
Proposed trial						**0-1**		1200 q wk x 2, q 3 weeks	**800**			

1. Abstract
2. All patients had a prior response to the anthracycline for metastatic disease
3. Febrile neutropenia

Five Phase II studies have evaluated the activity of gemcitabine in metastatic breast cancer (Table 1). In the first study, Carmichael et al. reported 44 patients with advanced disease were given a dose of 800 mg/m^2 on days 1, 8, and 15 every 28 days (14). Twenty-six patients had received prior chemotherapy either in the adjuvant (7 patients) or metastatic setting (19 patients). The mean administered dose of gemcitabine was 725 mg/m^2/injection, or 543 mg/m^2/week. The response rate was 25%. In the second study Brodowicz preliminarily reported a response rate of only 12%, but all patients had prior chemotherapy and gemcitabine was third or fourth line chemotherapy for metastatic disease (15). In the fourth study, only patients with previous metastatic disease responsive to anthracycline-based chemotherapy were enrolled (16). Forty-seven patients were treated with gemcitabine at a dose of 1,200 mg/m^2 on days 1,8 and 15 every 28 days, with a delivered dose of 754 mg/m^2/week. The median time to progression of the responders and the median survival of all the patients were 8.1 and 18.6 months, respectively. The response rate was 29% of which 11% were complete responses. In the fifth study,

Blackstein reported 39 patients with advanced breast cancer of whom 21 had received prior adjuvant chemotherapy were treated with gemcitabine 1,200 mg/m^2 on days 1, 8 and 15 every 28 days (17), with a delivered dose of 1,053 mg/m^2/week. The response rate in this study was 37% of which 4 were complete responses. In comparison, the response rates in anthracycline-resistant disease reported with paclitaxel (18) and docetaxel (19) were 6-29% and 30-41%, respectively. In conclusion gemcitabine administered at a dose of 800-1,250 mg/m^2 on days 1 and 8 every 21 days, or days 1,8, and 15 every 28 days is tolerable and active in metastatic breast cancer. Thus, gemcitabine appears to be a very reasonable single agent for patients with metastatic breast disease.

The main toxicity of gemcitabine is myelosuppression. However, in all these studies, in which gemcitabine is administered at an intended dose of 600 – 900 mg/m^2/wk, the incidence of grade 4 neutropenia was $\leq$ 7%, with rare instances of febrile neutropenia (Table 1). Therefore, it is reasonable to propose gemcitabine at a dose of 1,000 mg/m^2 on days 1 and 8 every 21 days, which would yield a dose of 800 mg/m^2/wk, which is well within the dose range of achieving clinical responses dosing that produces a very low incidence of neutropenia or febrile neutropenia.

2.3 Aberrant Akt and NF-κB signaling in human breast cancer

2.3.1 Akt pathway in breast cancer

Akt is an oncogenic serine-threonine kinase that is significantly activated by the estrogen receptor (ER) and breast cancer related receptor tyrosine kinases (RTKs) such as EGFR, Her-2, insulin receptor (IR) and the insulin like growth factor-1 receptor (IGF-1R). We and others have shown that Akt1 is overexpressed or activated in the majority of breast cancer cell lines and tumors (20, 21). Roth et al. has reported that Akt1 and Akt3 is overexpressed in most breast cancer cell lines and in the majority of primary breast cancer samples, especially in ER negative tumors (20). Thirty-eight percent of breast cancers have elevated Akt1 kinase activity or expression of phosphorylated Akt (pAkt) by immunohistochemistry (IHC) (21). Ahmad et al. has reported that Akt1 levels are elevated in a panel of human breast cancer cell lines, and that MCF-7 cells stably overexpressing WT-Akt1 exhibited increased anchorage independent growth in response to serum, IGF-1, or estrogen (22). Heregulin and amplified Her-2 may both activate Akt in human breast cancer cell lines (23, 24). Recent *in vitro* data also indicate that Akt may augment ERα signaling by specific phosphorylation of ERα at Ser167. Stable overexpression of Akt1 in MCF-7 cells confers ligand independent activation and increased transactivation by ERα, and decreases growth inhibition and apoptosis in response to tamoxifen. Bcl-2 is also significantly induced in these cells (25). A recent multivariate analysis of a cohort of tamoxifen and/or goserelin treated breast cancer showed that expression of pAkt in tumors was an independent predictor of distant metastatic relapse (26). These data together suggest that Akt plays a mechanistic or predictive role in tamoxifen resistance, and that inhibition of Akt may be therapeutic in breast cancer.

2.3.2 NF-κB pathway in breast cancer

The nuclear factor kappa B (NF-κB) transcription factor is often activated in tumor cells. Growth factors such as the epidermal growth factor (EGF), cytokines and mitogens activate the phosphatidyinositol 3-kinase, protein kinase C and subsequently NF-κB (27). Activation of NF-κB by Akt may explain the pro-transforming effects of Akt. When the pathway is unstimulated NF-κB is sequestered in the cytosol of cells by the inhibitory I-κB protein. NF-κB activity is induced by Akt via activation of a kinase, IKK, a direct Akt substrate, which then phosophorylates I-κB at two conserved serines in the N-terminal domain, leading to its ubiquitin mediated degradation (28, 29). This releases NF-κB to translocate into the nucleus where it transactivates promoters of genes involved with cell cycle progression, such as cyclin D1 (30) or survival function such as cellular inhibitors of apoptosis (CIAPs) (31).

NF-κB is constitutively elevated in human breast cancer cell lines compared to untransformed breast cells, and its inhibition causes apoptosis (32, 33). Her-2 activation of NF-κB also occurs through Akt–

IKK mediated degradation of IKB (34). NF-κB expression is also elevated in the majority of human breast cancer tissues (33, 35). Furthermore NF-κB has been shown to be low in the cytosol but elevated in the nucleus of breast cancer cells indicating that NF-κB is not only overexpressed but also is functional (35). The role of NF-κB in anti-apoptosis (36, 37), invasion (38), cell cycle regulation (39) and growth of cells has been clearly demonstrated in breast cancer. In breast cancer cells NF-κB has been shown to upregulate secretion of urokinase-type plasminogen activator (38) and transcription of matrix metalloproteinase (40) both of which are promote cell invasion and metastasis. NF-κB has also been shown to disturb the cell cycle regulation in breast cancer cells through increased cyclin D1 transcription and retinoblastoma phosphorylation (39). Hence, NF-κB is a molecular target in breast and other cancers, in its own right, as numerous drugs are being developed which antagonize NF-κB activity (41).

NF-κB also appears to have a role in chemoresistance. Inhibition of NF-κB via adenoviral delivery of I-κB (42), or inhibition of proteasome-mediated degradation of I-κB increases apoptosis and chemosensitizes cancer cell lines (43). Introduction of the I-κB vector into breast cancer cell lines sensitizes these cells to the effects of paclitaxel (44). In a preclinical model, non-small cell lung cancer cells treated with a plasmid expressing I-κB resulted in increased apoptosis with gemcitabine as compared to the cells not treated with the vector (45). On the other hand, introduction of the NF-κB gene into cell lines lacking the NF-κB overexpression resulted in resistance to gemcitabine (46). Thus, inhibition of NF-κB activity may sensitize cancer cells to gemcitabine chemotherapy.

2.4 Genistein

Flavonoids compromise the most common group of plant polyphenols. More than 5,000 different flavonoids have been described and classified based on chemical structure into six subgroups (47). Genistein (4,5,7-trihydroxyisoflavone) is a naturally occurring flavonoid of the isoflavone subgroup present in soybeans (47). Genistein containing nutritional supplements have become popular in the public domain, with claims for potential breast cancer prevention or treatment, such that patients will seek administration of these without prospective vigorous clinical trial data. However, there is accumulating preclinical data that indicate that genistein has potential for the treatment or prevention of breast cancer, and suggest that careful clinical studies are warranted.

Genistein has been shown to elicit molecular changes that result in inhibition of cell growth and induction of apoptosis in different cancer cell lines. In the squamous cell cancer cell lines, genistein can induce cell cycle arrest through upregulation of $p21^{waf1}$ and downregulation of cyclin B1 (48). Genistein also induces apoptosis via upregulation of bax and downregulation of Bcl-2 gene expression (48). Furthermore, it downregulates the expression of matrix metalloproteinase 2 and 9 (48). In testicular cancer cell lines, genistein can induce apoptosis through activation of caspase-3 protease (49). In colon cancer cell lines, genistein can induce apoptosis through topo-isomerase II-mediated DNA breakage (50). In the breast cancer cell lines, genistein has been shown to block cells at the G2M phase (51). Genistein also can induce apoptosis through modulation of ERK phosphorylation and c-fos expression in breast cancer cell lines (52).

Recent evidence indicates that genistein is also a potent inhibitor of Akt and NF-κB pathways. Our laboratory has shown that genistein may facilitate apoptosis in cancer cells by inhibiting several critical intracellular survival pathways, including the inhibition of the PI3-kinase – Akt dependent signaling and NF-κB activation (48, 53). Genistein has also been shown to inhibit NF-κB activation in prostate cancer (53) and head and neck squamous cell cancer cell lines (48).

Several clinical studies have been done with soy products or supplements. We have shown that Novasoy, which contains ~40% genistein, inhibits NF-κB activation in humans without toxicity. In a clinical study involving six healthy male volunteers, 50 mg of genistein administered twice daily for three weeks resulted in inhibition of NF-κB activation by TNF-α in peripheral lymphocytes. The

volunteers experienced no side effects (54). Moreover, a phase II clinical study at Wayne State University has been completed in which 41 patients with prostate cancer received Novasoy at 100 mg BID for six months. Overall a decrease in PSA was noted, without any side effects (Hussein et al., manuscript submitted) (55). Recently, a limited, preliminary phase I clinical study with pharmacokinetics was reported in male volunteers with a soy supplement similar in content to Novasoy. Fifteen healthy volunteers were given single doses at 1, 2, 4, 8 or 16 mg/kg, with three patients at each level. At 1 and 2 mg/kg doses levels (equivalent to 70 and 140mg for a 70kg man), no clinical or laboratory toxicity was observed. At 4, 8 and 16 mg/kg (~280, 560, and 1120 mg doses, respectively), there were 7 cases of grade 1 - 2 adverse events, including 3 episodes of hypophosphatemia judged to be "clinically insignificant," one pedal edema, one loss of appetite, one abdominal tenderness, and one leukopenia (grade 2 at 16 mg/kg). Mean peak plasma total genistein levels in three men receiving 8mg/kg (~560 mg) was ~ 20 μM, with a pseudo $t_{1/2}$ of ~9.2 h, and the major route of elimination through the urine (56). Thus, safe, biologically active does of genistein may prevent or treat breast cancer by not only inhibiting Akt and its downstream effector, NF-κB, but by increasing the efficacy of chemotherapy such as genistein. Moreover, monitoring NF-κB activity in peripheral blood mononuclear cells (PBMNC) may serve as an surrogate marker of genistein activity in clinical trials.

2.5 Rationale of the study

2.5.1 Clinical Rationale

Systemic chemotherapy can palliate symptoms as well as prolong survival of patients with metastatic breast cancer (4, 5). Introduction of novel agents in this setting is important in order to further improve the clinical outcome of patients. Gemcitabine at a dose of 800-1200 mg/m^2 has significant activity comparable to the taxanes as second line agent in breast cancer (14, 17, 57). The toxicity of gemcitabine is mainly myelosuppression. Dose limiting myelosuppression occurs in about 10% of patients treated with gemcitabine (16). However, in the Phase II trials in metastatic breast cancer no treatment related mortality was observed (16). Genistein has garnered increasing scientific interest as a breast chemoprevention agent. Genistein may inhibit several molecular targets that are relevant to human breast cancer, and may modulate sensitivity to gemcitabine. These include Akt and NF-κB, which are overexpressed and/or activated in the majority of breast cancer cell lines and primary tumors (39, 58). Overexpression of the NF-κB in cancer cell lines results in resistance to gemcitabine (45). Inhibition of NF-κB can sensitize cells to the effects of chemotherapeutic agents (44), including gemcitabine (46). Genistein is an inhibitor of the NF-κB pathway as demonstrated in healthy volunteers (54) as well as in cancer cell lines (48, 59). Genistein at doses proposed in this study has no side effects (Hussein et al., manuscript submitted) (55). Thus, there is increasing evidence that genistein may have chemoprevention, or chemotherapeutic applications, especially in combination with other cytotoxic chemotherapies such as gemcitabine. Further clinical studies with genistein are clearly indicated. We hypothesize that combining genistein and gemcitabine in patients with metastatic breast cancer could improve the therapeutic effectiveness of gemcitabine through decreasing chemo-resistance via modulation of the Akt and NF-κB pathways without added toxicity.

2.5.2 Translational Rationale

The two general translational aims are to: 1. Determine if higher plasma levels of genistein are associated with clinical responses to the combined treatment; and 2. Explore effects of genistein alone on in vivo tumor biology. Although there have been numerous recent reports of the in vitro effects of genistein on human cancer cell lines (reviewed in Section 2.5), there are no studies that have corroborated or even explored the in vivo effects in human tumor specimens. Therefore, in patients who

have easily accessible tumor and are willing to undergo biopsies before and after seven days of genistein, we will perform studies to determine if genistein inhibits cell cycle, induces apoptosis, and inhibits Akt and NF-κB activation as would be predicted from preclinical studies. Further, we will explore the effects on other potential biomarkers through cDNA microarray analysis when sufficient cDNA can be prepared from paired specimens. This will provide novel insights and a better understanding on the mechanisms of genistein effects on malignant breast cancer cells.

3.0 DRUG INFORMATION

3.1 Gemcitabine (Gemzar)

Chemistry: Gemcitabine HCl is 2-deoxy-2, 2-difluorocytidine monohydrochloride (beta isomer).

Clinical Pharmacology: Gemcitabine pharmacokinetics are linear and are described by a 2-compartment model. Population pharmacokinetic analyses of combined single-and multiple-dose studies showed that the volume of distribution of gemcitabine was significantly influenced by duration of infusion and gender. Clearance was affected by age and gender. Differences in either clearance of volume of distribution based on patient characteristics or the duration of infusion; result in changes in half-life and plasma concentrations.
Gemcitabine half-life for short infusions ranged from 32-94 minutes, and the value for long infusions varied from 245-638 minutes, depending on age and gender, reflecting a greatly increased volume of distribution with longer infusions. The lower clearance in women and the elderly result in higher concentrations of gemcitabine for any given dose. The volume of distribution was increased with infusion length. Volume of distribution of gemcitabine was 50 L/m2 following infusions lasting <70 minutes, indicating that gemcitabine, after short infusions, is not extensively distributed into tissues. For long infusions, the volume of distribution rose to 370 L/m2, reflecting slow equilibration of gemcitabine within the tissue compartment. The maximum plasma concentrations of dFdU (inactive metabolite) were achieved up to 30 minutes after discontinuation of infusions, and the metabolite is excreted in urine without undergoing further biotransformation. The metabolite did not accumulate with weekly dosing, but its elimination is dependent on renal excretion, and could accumulate with decreased renal function. The effects of significant renal or hepatic insufficiency on the disposition of gemcitabine have not been assessed.

Human Toxicity: *Hematologic:* Myelosuppression is the dose-limiting toxicity with gemcitabine, but <1% of patients discontinued therapy for anemia, leukopenia, or thrombocytopenia. Red blood cell transfusions were required by 19% of patients. The incidence of sepsis was less than 1%. Petechiae or mild blood loss (hemorrhage), from any cause, were reported in 16% of patients; less than 1% of patients required platelet transfusions. Patients should be monitored for myelosuppression during gemcitabine therapy and dosage modified or suspended according to the degree of hematologic therapy (see Section 8.0). *Gastrointestinal:* Nausea and vomiting were commonly reported (69%) but were usually mild to moderate. Severe nausea and vomiting (WHO grade 3/4) occurred in <15% of patients. Diarrhea was reported by 19% of patients, and stomatitis by 11% of patients. *Hepatic*: gemcitabine was associated with transient elevations of serum transaminases in approximately two-thirds of patients, but there was no evidence of increasing hepatic toxicity with either longer duration of exposure to gemcitabine or with greater total cumulative dose. *Renal*: Mild proteinuria and hematuria were commonly reported. Clinical findings consistent with the hemolytic-uremic syndrome (HUS) were reported in 6/24 patients (0.25%) receiving gemcitabine in clinical trials. Four patients developed HUS on gemcitabine therapy, two immediately post-therapy. Renal failure may not be reversible, even with discontinuation of therapy, and dialysis may be required. *Fever*: The overall incidence of fever was 41%. This is in contrast to the incidence of infection (16%) and indicates that gemcitabine may cause fever in the absence of clinical infection. Fever was frequently associated with other flu-like symptoms and was usually mild and clinically manageable. *Rash*: Rash was reported in 30% of patients. The rash was typically a macular or finely granular maculopapular pruritic eruption of mild-to-moderate severity, involving the trunk and extremities. Pruritus was reported for 13% of patients. *Pulmonary:* Dyspnea

was reported in 23% of patients, severe dyspnea in 3%. Dyspnea may be due to underlying disease, such as lung cancer (40% of study population) or pulmonary manifestations of other malignancies. Dyspnea was occasionally accompanied by bronchospasm (<2% of patients). Rare reports of parenchymal lung toxicity consistent with drug-induced pneumonitis have been associated with the use of gemcitabine. *Edema:* Edema (13%), peripheral edema (20%), and generalized edema (<1%) were reported. Less than 1% of patients discontinued due to edema. *Flu-like Symptoms:* "Flu syndrome" was reported for 19% of patients. Individual symptoms of fever, asthenia, anorexia, headache, cough, chills, and myalgia, were commonly reported. Fever and asthenia were also reported frequently as isolated symptoms. Insomnia, rhinitis, sweating, and malaise were reported infrequently. Less than 1% of patients discontinued due to flu-like symptoms. *Infection:* Infections were reported for 16% of patients. Sepsis was rarely reported (<1%). *Alopecia:* Hair loss, usually minimal, was reported by 15% of patients. *Neurotoxicity*: There was a 10% incidence of mild parasthesias and a <1% rate of severe parasthesias. *Extravasation:* Injection-site-related events were reported for 4% of patients. There were no reports of injection-site necrosis. Gemcitabine is not a vesicant. *Allergic*: Bronchospasm was reported for less than 2% of patients. Anaphylactoid reaction has been reported rarely. Gemcitabine should not be administered to patients with a known hypersensitivity to this drug. *Cardiovascular*: Two percent of patients discontinued therapy with gemcitabine due to cardiovascular events such as myocardial infarction, cerebrovascular accident, arrhythmia, and hypertension. Many of these patients had a prior history of cardiovascular disease.

Pharmaceutical Data: Gemcitabine is supplied as a lyophilized powder in sterile vials containing 200 mg or 1 g of gemcitabine as the hydrochloride salt (expressed as the free base), mannitol, and sodium acetate. Drug vials will be reconstituted with normal saline added to the vial to make a solution ideally containing 10 mg/mL or less. The concentration for 200 mg and 1 g vials should be nor greater than 40 mg/mL. An appropriate amount of drug will be prepared with normal saline and administered as a continuous infusion for 30 minutes. Once the drug has been reconstituted, it should be stored at room temperature and used within 24 hours.

Storage and Stability: Store at controlled room temperature (20-250C), should be handled and disposed of in a manner consistent with other anti-cancer drugs.

Route of Administration: Intravenous infusion over 30 minutes.

Supplier: Commercially available from Eli Lilly and Company, Indianapolis, Indiana, 46285.

3.2 Genistein (Soy Isoflavones)

Novasoy ® tablets containing 50 mg soy isoflavones will be obtained at no charge from Archer Daniels Midland (ADM) Company (Decatur, IL). Novasoy tablet is a well-defined soy concentrate tablet manufactured to contain 50 mg soy isoflavones per tablet. The contents have been analyzed by HPLC and contain soy isoflavones genistein, daidzein and glycitein at a ratio of 1.1:1:0.1, thus each 50 mg tablet provides roughly 25 mg of genistein. The isoflavones are present in both conjugated (40%) and unconjugated (60%) forms. In our previous studies we have measured serum genistein and daidzein levels and found them to significant increase after one week and remain stable for the remaining period upon continuous dosing without change in serum concentrations. The half-life of genistein after a single oral dose is approximately 3-4 hours. We have shown in our previous studies that Novasoy taken 1 tablet (50 mg) daily is sufficient to have a biological effect with significant reduction in oxidative stress markers. Soy isoflavones are generally considered safe and are available in nutrition stores and pharmacies without prescription. No adverse health effects have been attributed to use of soy isoflavone supplements.

Chemistry: Genistein is supplied as a major component of an isoflavone extract of soy and is classified as a nutritional product. Genistein's chemical structure is 4,5,7-trihydroxyisoflavone.

Administration: Patients will be instructed to take their capsules twice daily with breakfast and dinner. They may also take the study capsules at any time during the day if they forget to take them with the designated meals. Patients will return their remaining study capsules. A capsule count will be made and the number of remaining capsules will be placed in patient's study folder by the study coordinator. Capsule containers (partial and empty) need to be returned for accountability.

Human toxicity: Genistein did not have any side effects in previous studies; therefore, we do not expect toxicity in this study.

Storage: At room temperature.

Supplier: Archer Daniels Midland, Decatur, IL

4.0 ELIGIBILITY CRITERIA

4.1 Inclusion Criteria

a) Patients must have histological or cytological diagnosis of breast cancer.

b) Patients must have clinical and/or radiological evidence of metastatic disease (stage IV).

c) Patients must have measurable disease by RECIST criteria. Prior radiation permitted as long as at least one measurable disease site is outside the radiation field

d) Patients may have had any prior cytotoxic chemotherapy treatments for metastatic disease excluding gemcitabine. Any prior adjuvant therapy is permitted (except for gemcitabine). Patients must have failed within 24 months of completion of adjuvant (or neoadjuvant) taxane-based therapy or after a taxane therapy for metastatic disease.

e) Patients with prior hormone therapy must have documented disease progression on the hormone therapy.

f) Patients must not have received any prior gemcitabine.

g) Patients must have performance status of 0-2 on SWOG scale (see Appendix III).

h) Patients must have adequate bone marrow function: absolute neutrophil count $\geq$1,500/cmm, platelet count $\geq$100,000/cmm, and hemoglobin $\geq$ 10g/l.

i) Adequate liver function: bilirubin $\leq$ 3.0 mg/dL; transaminases (AST/ALT) $\leq$ 2.5 times upper limit of institutional normal.

j) Adequate renal function: creatinine $\leq$ 1.5 mg/dL.

k) Patients must be informed of the investigational nature of this study and must give written informed consent prior to the receiving of treatment per this protocol.

l) Women with child bearing potential must practice effective birth control while receiving treatment.

m) Prior chemotherapy, biologic, investigational , or major surgical therapy must be completed at least 3 weeks prior to initiation of protocol therapy.

n) Prior radiation therapy is allowed, however, patients must have recovered from the acute effects of the treatment, and have completed radiation at least 4 weeks prior to starting protocol therapy.

o) Patients may have hormone receptor positive or negative breast cancer. Any prior hormonal therapy, either adjuvant or for metastatic disease is permitted, as long as patients are off all hormone therapy for at least 2 weeks prior to starting protocol therapy.

p) All soy supplements (including all soy based pills, liquids, concentrates) must be discontinued for at least 1 week. Dietary soy that may be normally part of a meal (e.g., tofu) may be ingested once per week. A daily multivitamin pill is permitted.

q) Patients must discontinue all other nutritional supplements, herbal agents, high doses of antioxidants (e.g., vitamin C, D, or E) including any that may interact with, antagonize, or alter or imitate the potential effects of either gemcitabine or genistein.

4.2 Exclusion Criteria

a) History of active central nervous system (CNS) metastases. Patients with previously treated CNS metastases are permitted if $\geq$ 3 months prior to enrollment, clinically stable without steroids or antiseizure medications.

b) Serious concomitant systemic disorders incompatible with the study (at the discretion of the investigator).

c) History of other malignancy, except for cervical carcinoma, or basal or squamous skin cancers. For these cancers, patient must have been treated with a curative intent be in complete in remission.

d) Unresolved bacterial infection requiring treatment with antibiotics.

e) Pregnant or lactating women may not participate in the study.

j) Patients infected with HIV-1 virus because of the undetermined effect of this chemotherapy regimen in these patients, and the potential for interaction with anti-HIV medications.

5.0 TREATMENT PLAN

5.1 Dosage Selection and Administration Procedures

TABLE 2. The Starting Dose Levels

	Cycle and Day	Gemcitabine (I.V.)	Novasoy (P.O.)[1]
Dose		1000 mg/m^2	100 mg

Schedule	Cycle 1, Days -7 to -1	----------	BID x 7 days[2]
	Cycles 1 to N, Days 1-21	Days 1 and 8 Q 21 days	BID[2] Days 1-21 Q21 days

[1]Genistein (Novasoy) should be taken with breakfast and dinner.
[2]Patients should be told not to take genistein A.M. dose on cycle 1, days –7 and –1, and on cycle 2, day 1, until plasma for genistein level is collected in clinic.

The body surface area will be calculated according to actual height and weight at the beginning of treatment and this will be used to calculate the dose of gemcitabine. Doses can be recalculated if weight declines by >10%. Treatment may be administered on an outpatient basis.

Gemcitabine 1,000 mg/m^2 intravenously administered over 30 minutes on days 1 and 8 of each 21-day cycle. Please refer to Table 2 for the initial dose level.

5.2 Duration of Therapy

Patients with metastatic cancer will continue until disease progression and/or undue or intolerable toxicity.

5.3 Concomitant Therapy

No other chemotherapy, immunotherapy, hormonal therapy (excluding anticoagulants, appetite stimulants and replacement steroids), nutritional or herbal supplements (except for a multi-vitamin) radiation therapy, or experimental medications will be permitted while the patients are on study. To prevent nausea and vomiting, the prophylactic use of anti-emetics including dexamethasone, is allowed. Any disease progression requiring other forms of specific anti-tumor therapy will be a cause for early discontinuation from this study. Bisphosphonates are permitted. Patients should receive full supportive care as necessary. Patients may receive hematopoietic growth factors as clinically indicated.

6.0 TREATMENT MODIFICATIONS FOR TOXICITY, AND CRITERIA FOR DISCONTINUATION FROM STUDY

*** Dose reductions and treatment delays only apply to gemcitabine**

6.1 Dose delay and modification on day 1 of each cycle based on toxicities *on day of treatment*

TABLE 3. Day 1 dose delay or modification due to hematologic toxicity on day of treatment

ANC	Platelet	Delay, until ANC≥1500 and Platelet ≥ 100,000	Percent dose reduction of gemcitabine	Percent dose reduction of genistein
≥ 1,500	≥100,000	None	0	0
<1,500	<100,000	≤ 1 week	0	0
		> 1 to 2 weeks	25	0
		> 2 weeks	Off treatment	

TABLE 4. Day 1 dose delay or modification due to *non*-hematologic toxicity on day of treatment (excludes alopecia, nausea, or vomiting, based on NCI Common Tox. Criteria 3.0)

Grade	Delay, until non-hematologic toxicity = 0-1	Percent dose reduction of gemcitabine	Percent dose reduction of genistein
0-1	None	0	0
2	0 to 1 week	0	0
	> 1 to 3 weeks	25	0
	> 3 weeks	**Off treatment**	
3-4	0 to 1 week	0	0
	> 1 to 3 weeks	25	0
	> 3 weeks	**Off treatment**	

6.2 Dose modification on day 1 of each cycle based on toxicity of the *previous* cycle

TABLE 5. Dose modification on day 1 of each cycle based on previous cycle's hematologic toxicity

Worst toxicity during previous cycle		Percent reduction in gemcitabine dose from the previous cycle	Percent reduction of the genistein dose from previous cycle
ANC	**or Platelets**		
≥0.250	≥40	0	0
<0.250	<40	25	0

Note: Dose modifications should be repeated in subsequent cycles if hematologic toxicity recurs.

TABLE 6. Dose modification on day 1 of each cycle based on previous cycle's *non*-hematological toxicity during previous cycle (excluding pulmonary toxicity, alopecia, nausea and vomiting)

Worst toxicity during previous cycle	Percent reduction in gemcitabine dose from the previous cycle	Percent reduction of the genistein dose from previous cycle
0	0	0
1-2	0	0
3-4	25	0

TABLE 7. Dose modification on day 1 of each cycle based on previous cycle's *non*-hematological *pulmonary* toxicity during previous

Pulmonary toxicity	Percent reduction in gemcitabine dose from the previous cycle	Percent reduction of the genistein dose from previous cycle
0	0	0
1	25	0
2-4	**Off treatment**	**Off treatment**

If pneumonitis grade 2 or higher develops in a given cycle and is related to gemcitabine, gemcitabine should be promptly discontinued and the patient should be removed from protocol treatment. Treatment with corticosteroids should be given according to established guidelines.

6.3 Dose modifications on *day 8* of each cycle based on hematologic toxicities

TABLE 8. Dose modifications on day 8 of each cycle

ANC	Platelet (X 10^9)	Percent dose reduction of gemcitabine	Percent dose reduction of genistein
≥ 1.0	≥75	0	0
0.75-0.999	50-74	25	0
0.50-0.749	40-49	50	0
<0.50	<40	100	100

6.4 Discontinuation from the Study Treatment

A patient will be discontinued from the study under the following circumstances:

6.4.1 There is evidence of progressive disease.

6.4.2 The attending physician thinks the best interests of the patient require a change of therapy.

6.4.3 The patient requests discontinuation.

6.4.4 The drug exhibits unacceptable toxicity.

6.5.5 Patient becomes pregnant or fails to use adequate birth control (for those patients who are potentially able to conceive).

7.0 CRITERIA FOR RESPONSE EVALUATION AND TOXICITY REPORTING

7.1 Definition of clinical response:

7.1.1 Measurable lesions: Lesions that can be measured in at least one dimension as ≥ 20 mm with conventional techniques or as ≥ 10 mm with spiral CT scan.

7.1.2 Evaluable disease: Includes unidimensionally measurable lesions, masses with poorly defined margins, palpable nodal disease (measured by two observers), lesions with diameter less than 0.5 cm.

7.1.3 Non-measurable lesions: All other lesions including small lesions (longest diameter < 20 mm

with conventional techniques) and other non-measurable lesions including: pleural effusions, ascites, lesions on bone scans and disease documented by indirect evidence (e.g. biochemical abnormalities). Non-evaluable disease does not influence objective response assessment except for the determination of CR when all disease must be absent and in the determination of progression (if new non-evaluable disease develops).

7.1.4 Target lesions: All measurable lesions up to a maximum of 10 lesions. Target lesions are selected for their size and suitability for accurate repetitive measurements. The sum of the longest diameter of all target lesions will be calculated and reported as the baseline sum longitudinal diameter (LD). This will be used as a reference to further quantify objective response.

7.1.5 Non-target lesions: All other lesions are identified as non-target lesions and should be followed as present or absent.

7.1.6 Objective response criteria:

Complete response (CR): Complete disappearance of all measurable and evaluable disease for at least 3 weeks without the appearance of any new lesions. Persistent effusions after therapy should be cytologically negative of malignant cells.

Partial Response (PR): Greater than or equal to 30% reduction in the sum longest diameters of target lesions taking as reference the baseline sum of the longest diameters without the appearance of any new lesions.

Progressive disease (PD): Greater than 20% increase in the sum of longest diameter of target lesions taking as reference the smallest sum of the longest diameter recorded since the treatment started OR appearance of new lesions.

Stable disease (SD): Neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD taking as reference the smallest sum of the longest diameters of target lesions since treatment started for a minimum interval of 6 weeks.

Best objective response: This will be determined from serial objective response assessments in a given patient using the response criteria mentioned above. For patients whose disease sites are assessed every three to six weeks, two consecutive objective CRs are required to assign the best response of CR. Similarly, two successive evaluations documenting PR or SD are required before assigning the patient to either.

Best overall response: This is the best response from the start of the treatment until disease progression/recurrence. In general, the patient's best response assignment will depend on the achievement of both measurement and confirmation criteria.

7.1.7 Evaluable Patients: **A participant who completes 2 cycles of therapy is evaluable for response. A patient who receives any amount of drug is evaluable for toxicity.**

TABLE 9. Overall response definitions

Target lesions	Non-Target lesions	New lesions	Overall response
CR	CR	No	CR
CR	Non-CR/Non-PD	No	PR
PR	Non-PD	No	PR
SD	Non-PD	No	SD

PD	Any	Yes or No	PD
Any	PD	Yes or No	PD
Any	Any	Yes or No	PD

7.1.7 Duration of response: Time from point at which response (PR or CR) is first noted, until first clinical or radiologic evaluation that shows progressive disease.

7.1.8 Time to progression: Time from date of registration to the date of documented progressive disease or death.

7.1.9 Overall survival: Time from date of registration to date of death or last follow up.

7.2 Toxicity definitions for evaluation of this protocol

Toxicity reported for patients in this trial will be defined by NCI CTC 3.0 criteria.

8.0 SCHEDULED EVALUATIONS ON STUDY

8.1 Within 4 weeks before study initiation all patients must have:

- Bone scan.
- Chest x-ray.
- Tumor imaging to document measurable disease (Chest and abdominal CT scans, or MRI scans).

8.2 Within two weeks before study initiation all patients must have:

- History and physical exam, including height and weight.
- Performance status.
- CBC with differential white cell and platelet counts
- Serum chemistries: electrolytes, BUN, creatinine, LDH, alk. phosphatase, total bilirubin, AST(GOT), ALT(GPT), magnesium, calcium, phosphate and albumin.
- Amylase and lipase.
- Tumor biopsy from easily accessible tumor, before first genistein dose (Optional – if patient has easily accessible tumor and patient agrees to procedure).

8.3 On treatment evaluations:

8.3.1 On cycle 1, day –7

- 5 ml plasma for genistein level, **before A.M. genistein dose**
- Tumor biopsy before A.M. dose, or earlier (Optional)

8.3.2 On cycle 1, day –1, on **day 7 of genistein alone** (before cycle 1, day 1)

- 5 ml plasma for genistein level, before A.M. genistein dose, and 4 h after A.M. dose

- Tumor biopsy from same site as before (Optional)

8.3.3 On cycle 2 only, day 1

- 5 ml plasma for genistein level, **before A.M. genistein dose, and 4 h after A.M. dose.**

8.3.3 On cycles 2 through cycle N, day 1 (before genistein and gemcitabine administration):

- History and physical examination.
- Performance status.
- Toxicity evaluation using NCI-CTC criteria.
- CBC with differential white cell and platelet counts.
- Electrolytes, SGOT (AST), SGPT (ALT), alkaline phosphatase, bilirubin, creatinine, calcium, phosphate.
- Amylase and lipase.

8.3.4 On day 8 and 15 of every cycle:

- CBC with differential white cell and platelet counts. Therefore all patients will have weekly CBC and differential throughout their treatment period.

8.3.5 After every 2 cycles:

- Repeat all imaging tests to evaluate objective tumor response

9.0 CORRELATIVE STUDIES: INSTRUCTIONS

For schedule please see Appendix, Study Calendar.

9.1 Blood samples for plasma genistein and other isoflavone levels:

- Plasma will be collected:
  - **before first dose of genistein on cycle 1, day –7** (or earlier)
  - **cycle 1, day –1 (on day 7 of genistein alone) before A.M. dose, then 4 h after A.M. dose**
  - **cycle 2, day 1 before A.M. dose, then 4 h after A.M. dose**
- Blood will be collected into (1) 10 ml or (2) 5 ml **citrated** tubes. Within 2 hours of collection tubes should be spun at 1000g for 10 m and plasma stored at -70° C in (2) cryovials, each with ~2 ml aliquots.
- **Cryovials should be labeled with**:
  - Institution name
  - patient number (assigned at registration)
  - plasma
  - date and time collected
  - cycle number
  - either as “cycle 1, day-7” or “cycle 1, day-1” or “cycle 2, day 1”
  - either as “pre-AM dose” or “4-hour post AM dose”
- Samples should be sent on dry ice by next AM express mail to:

  Translational Research Lab (Dr. Sarkar’s lab)
  HWCRC, room 703
  Karmanos Cancer Institute
  Lab Phone: (313) 576-8314 or (313) 576-8315

- Contact Dr. F. Sarkar's lab at (313) 576-8314 or(313) 576-8315, for questions or sample pick up.

9.2 Tumor tissue samples to assess effects of **genistein alone** on Akt and NF-κB activation and on potential cDNA biomarkers:

- Patients with tissue easily accessible for biopsy (i.e. skin metastasis) should be requested to volunteer to biopsy for tumor tissue. This is a recommended but optional study component.

- Tumor tissue collection should be arranged at least **2 days in advance of treatment initiation** by calling Dr. Sarkar's lab at (313) 576-8315, or (313) 576-8314.

- Tumor tissue will be obtained, before first genistein on **cycle 1**, day –7 (or earlier), and on **cycle 1** day–1 (day 7 of **genistein alone**).
- At least 0.1 g of tumor should be collected (equivalent to a 5 mm cube of tumor, or 3 core (not FNA) cutting needle biopsies should be taken at each time point.

- **Tumor will be immersed immediately into sterile plastic containers with at least 4 volumes of RNAlater solution (Ambion, 800-888-8804).**

- **The container should be labeled with**:

    - Institution name
    - patient number (assigned at registration)
    - metastatic site biopsied
    - date and time
    - "pre-genistein" or "post 7 days of genistein."

- After immersion in **RNAlater**, the tumor samples must be refrigerated or iced until brought to the Translational Lab (Room 703 HWCRC, (313) 576-8314, or (313) 576-8315) **before 5 pm the day of collection**. From outside institutions, they should be stored in ice or 4C and delivered the same day, or express shipped for delivery the morning after the biopsy in a container containing 4C cold packs).

- Upon arrival to the lab:

    - For core biopsies, one will be sent to pathology for routine H and E staining. The other two cores will be transferred to one labeled 2 ml cryovial with 4 volumes RNAzol and stored at –70C.
    - For incision or excision biopsies, they will be divided in 3 parallel sections, and the middle 1/3 of the sample will be sent to pathology for routine H and E staining, while the two flanking 1/3 pieces will be transferred to one labeled 2 ml cryovial with 4 volumes RNAzol and stored at –70C

- Shipping address:

    Karmanos Cancer Institute
    Translational Research Lab
    4100 John R
    HWCRC, room 703
    Detroit, MI 48201

    Lab Phone: (313) 576-8314 or (313) 576-8315

- Histologic analysis of tumor biopsies taken before and on day 7 of genistein alone:
  - Routine H and E staining
  - TUNEL staining for determination of percent apoptotic tumor cells (Calbiochem)
  - IHC will be performed using established staining methods for:
    - Ki67 to estimate percent cells in G1-S phase using specific antibody (Dako, #Ki-S5).
    - Expression of activated Akt using anti-phosphoserine473-Akt antibody (Cell Signaling, #9277).
    - Expression of activated NF-κB using an antibody specific for the activated form of NF-κB p65 that is free of IκB (Chemicon, #12H11).
    - Interpretation of IHC for activated Akt or NF-κB:

      For p-Ser473-Akt and activated NF-κB, IHC each stain will be interpreted based a composite score of two parameters: *percent tumor cell staining* (0 = 0 - 9%, 1 = 10 - 49%, 2 = ≥ 50%), and relative *staining intensity* (0 = absent - minimal, 1 = moderate, 2 = strong staining). Appropriate controls will include tumor xenografts that are known to overexpress either marker**.** A tumor will be positive for expression only it scores 1 or 2 for both percent and intensity parameters; otherwise expression will be considered negative. In addition, the sum score of the parameters for each biomarker will be recorded (i.e., 0 – 4).

- CDNA microarray analysis of tumor biopsies taken before and on day 7 of genistein alone
  - Paired analysis of each patient's pre- and post- gensistien effects will be done if the H and E staining demonstrates ≥50 % tumor cells.
  - The –70C frozen tumor specimens will be crushed on dry ice, then processed in RNAzol to collect total RNA. If the quantity and quality of the pair of tumor RNAs is confirmed (each specimen yielding ≥ 10 ug of non-degraded RNA gel analysis), pairs of matched RNAs will be subjected to microarray analysis.
  - Microarray analysis will be performed using established methods ((60) and manuscript in preparation).

10.0 REGISTRATION PROCEDURE

All patients entering into this study will be registered with the Clinical Trials Office (CTO) at the Karmanos Cancer Institute, Detroit, Michigan, within 7 days *prior* to starting treatment (313) 576-8994. Each patient will be assigned an alphanumeric code, indicating the *overall* number of the patient on study, and the institution acronym (e.g., "05-KCI" would indicate the fifth patient registered, and he/she was from KCI.

11.0 REPORTING OF ADVERSE EVENTS

11. 1 General principles:

- Reporting of adverse events will include those that occur within 60 days of last treatment (FDA requirement is 30 days).

- All adverse events that warrant reporting should use the **Wayne State University (WSU) adverse event (AE) form**, available on the Human Investigations Committee (HIC) web site: www.hic.wayne.edu).

- Note that certain events require reporting within 3 calendar days of *awareness* of adverse event, while less urgent events are to be reported within 10 days of *awareness*. These deadlines may be met by verified fax, with the original sent by mail. In addition, for deaths and immediately life threatening events, the Principle Investigator (PI) and WSU Clinical Trials Office (CTO) should be contacted by phone immediately so that compliance with contacting other agencies within 24 h can be facilitated.

- The **WSU CTO** staff will coordinate the reporting process between the PI and the WSU human Investigations Committee (HIC, a.k.a. IRB) as well as other applicable reporting agencies (FDA, CTEP, NIH, OBA, and industry). Copies of all related correspondence and reporting documents will be maintained in the regulatory file. All deaths, immediately life threatening, and reportable serious adverse events will be reported and documented on Form FDA 3500 A (MedWatch Form) and forwarded directly to Eli Lilly. This includes serious, related, expected and serious, related, unexpected events. Reports should be sent to Eli Lilly, US Pharmacovigilance Department by fax at 908-243-6800, within 24 hours of receipt by the investigator. Fax transmission should include the Grant-In-Aid Study Number, Study Title, and name of Principal Investigator.

- **Reporting of AEs: Contact information:**

  - Study PI of the lead institution, WSU/KCI:
    Amy M. Weise, D.O.
    Phone: (313) 576-8952 E-mail: weise@karmanos.org
    Pager: (313) 745-5111 #0585
    Fax: (313) 576-8767

    Address:
    Karmanos Cancer Institute
    4100 John R
    4-HWCRC
    Detroit, MI 48201

  - Data Manager, Clinical Trials Office (CTO) at WSU/KCI:
    Matthew Gretkierewicz
    Phone: (313) 576-8994 E-mail: gretkiem@karmanos.org
    Pager: (313) 745-5111 #98026
    Fax: (313) 576-8368

    Address:
    Clinical Trials Office
    Harper Professional Bldg., Room 711
    Karmanos Cancer Institute
    4160 John R
    Detroit, MI 48201

11.2 Events to be reported by phone within 24 h, and within 3 calendar days, by WSU AE form.

- **Deaths**

  Death from any cause must be reported by phone to the Karmanos Cancer Institute Clinical Trials Office within 24 hours of the death (while receiving this treatment or up to 60 days from the last dose of treatment). Any death that occurs after 60 days of the last treatment that is felt to be a drug-related toxicity should also be reported. An WSU AE form must be faxed/sent to the WSU Clinical Trials Office within 3 calendar days. In addition, the PI and CTO will be responsible for reporting deaths to the HIC of WSU/KCI (phone 313-577-1628, fax 313-993-

7122), with formal written notification faxed within 3 days of the event. Formal notification includes the FDA and/or sponsor AE reporting forms attached to the completed WSU AE reporting form. Deaths must be reported to the FDA within 3 days.

- **Immediately life threatening events**

  These events must be reported by phone to the Karmanos Cancer Institute Clinical Trials Office within 24 hours of the event (while receiving this treatment or up to 60 days from the last dose of treatment). An WSU AE form must be sent to the WSU Clinical Trials Office within 3 days. In addition, the PI and CTO will be responsible for reporting of immediately life threatening events to the HIC of WSU/KCI (phone 313-577-1628, fax 313-993-7122), with formal written notification within 3 days of the event. Formal notification includes the FDA and/or sponsor AE reporting forms attached to the completed WSU AE reporting form. Deaths must also be reported to the FDA within 3 days.

11.3 Events to be reported within 3 calendar days, by WSU AE form.

- **Serious Adverse Events (SAEs) that are grade 3 and 4, and:**
  - **Unexpected, and/or**
  - **Expected, but definitely or probably related to protocol therapy (i.e., relationship to treatment cannot be ruled out)**

  These SAEs should reported to the PI and CTO of WSU within 3 days of awareness.

  Serious adverse events that are expected, but a relationship to the study treatment is ruled out do not have to be reported.

11.4 Non-serious adverse events that are to be reported within 10 days by WSU AE form.

- **Non-serious adverse events (generally grade 1 – 2) that are:**
  - **Unexpected, and/or**
  - **Expected, but more intense, longer in duration, or permanent, *and* definitely, or probably related to protocol therapy (i.e., relationship to treatment cannot be ruled out)**

  Non-serious adverse events that are expected, but a relationship to the study treatment is ruled out, do not have to be reported.

11.5 If the adverse event requires modification of the study protocol and the informed consent document, then they should be provided to the local IRB with the report of the adverse event. Consent revisions for sponsored research studies must receive sponsor approval **prior** to submission to the local IRB.

11.6 Any serious clinical adverse event or laboratory value occurring during the course of the study even if unrelated to the therapy should be reported to the Karmanos Cancer Institute Clinical Trials Office. All adverse events will be noted in the case report forms.

12.0 DATA AND SAFETY MONITORING

12.1 Scheduled meetings or conference calls will occur **every month**. These meetings will include the protocol principal investigators and any data managers involved with the conduct of the protocol.

12.2 During these meetings the investigators will discuss:

1. Safety of protocol participants (AE reporting and regulatory compliance)
2. Monitoring for excessive toxicity after the first 9, 11, 17 and 20 patients enrolled and treated, as described in Section 13.2;
3. Validity and integrity of the data;
4. Enrollment rate relative to expectations and the characteristics of participants;
5. Retention of participants and adherence to the protocol (potential or real protocol violations);
6. Completeness of collected data.

12.3 Data and Safety Monitoring Reports of these regular meetings will be kept on file in the Karmanos Cancer Institute Clinical Trials Office. The data manager assigned to the trial will be responsible for completing the report. These reports will be signed by the PI or one of the Co-PI's.

13.0 STATISTICAL CONSIDERATIONS

13.1 **Primary Objective:** To estimate the objective response rate of patients with metastatic breast cancer treated with the combination of gemcitabine and genistein.

13.1.1 Assumptions/Hypothesis

The primary statistical endpoint is the objective (complete plus partial) response rate. We would consider this regimen not promising if the true response rate was 0.20 in these pretreated patients, since the average response rate of the 5 phase II studies in Table 1 that have an average of 1 prior chemotherapy treatment, is 0.23. The regimen would be promising for further study if the true response rate were at least 0.40. Only response evaluable (RE) patients will contribute to this assessment. In designing this Phase II trial, we have set significance level = 0.05, and power = 0.80.

13.1.2 Estimation of response rate:

Based upon the Simon minimax design (61), stage 1 will accrue 18 response RE patients. If 4 or fewer responses are observed, we will recommend the trial for early termination and conclude that the regimen has a true response rate of 0.20 or less. If there are $\geq$ 5 responders, we will proceed to stage 2 and enroll and additional 15 RE patients. If at least 11 responders are observed among the 33 RE patients, we will conclude that the regimen has a true response rate of 0.40 or more. Otherwise, we will conclude that the true response rate is 0.20 or less.

13.1.3 Sample size, accrual rate, and follow-up:

In this two-stage Simon minimax design 18 RE patients will be accrued in stage 1, and 15 RE patients will be accrued in stage 2 (if necessary), for a total of 33 RE patients. Three extra patients should insure 33 RE patients. Therefore, 36 total patients are anticipated for accrual at a combined rate equivalent to about 36 patients over 18 months (1.5 years) (i.e., about 2/month). We anticipate accrual from two other institutions so that each institution would contribute 12 patients over 18 months, or 8 patients per 12 months (or 0.5 – 1 per month). Thus, we expect to enroll the 18 patients needed for Stage 1 in about 9 months, and (if necessary) the additional 15 – 18 patients for Stage 2 in about 9 more months. Additional time (a few months beyond 18 months) may be required to assess response in the last few patients, and because some patients will not become response evaluable until after registration and treatment is started. Patients will be followed for 60 days after the development of disease progression.

13.2 Monitoring for excessive toxicity

Using exact upper 1-sided 80% confidence limits (computed in StatXact 4 via the Casella methodology (62)), we have determined a monitoring plan for excessive toxicity for the first 20 patients entered. From prior studies of gemcitabine alone, we expect about a 10% rate of grade 4 toxicity (of any type). We wish to have 80% confidence that the true grade 4 toxicity rate of this new regimen is less than 30%. That will be assured if, among the first 9 patients there is at most 1 patient with a grade 4 toxicity, and if among the first 11 patients at most 2 Grade 4 toxicities, and if among the first 17 patients at most 3 grade 4 toxicities, and if among the first 20 patients at most 4 grade 4 toxicities. If at any of these accrual points, these respective numbers of grade 4 toxicity are exceeded, then the potential incidence of grade 4 toxicity would be $\geq$ 30% and enrollment will be terminated.

13.3 Secondary endpoints:

Since all the secondary endpoints are exploratory and intended for hypothesis generation, the results will primarily descriptive, and no formal hypothesis testing is presented.

13.3.1 Overall survival: Patients will be followed from the time the last patient comes off study treatment for one year to monitor survival

13.3.2 Duration of response: For patients with an objective response, this will be measured from the time when response is first documented, to the last restaging that continues to confirm the response.

13.3.3 Time to disease progression: This will be measured from the time that treatment is initiated until the time restaging indicates progressive disease.

13.3.4 Explore if there is an association between plasma genistein levels and responses.

- Exploratory associations: The response evaluable patients will be grouped by whether the cycle 1 day –1, or cycle 2 day 1 trough (if elevated) or 4 h peak fall above ("high genistein") or below the median values ("low genistein"). The number of responses will be determined for either high or low level genistein groups. The possible association of genistein levels with responses will be tested by the Fisher exact test.

13.3.5 Explore the in vivo effects of genistein on human breast cancer tumor biomarkers (Ki67, TUNEL, p-Akt and activated NF-κB immunohistochemistry (IHC), and cDNA expression by microarray analysis.

- Exploratory associations: Genistein treatment for 7 days will be associated with: 1. Decreased tumor cell cycling by Ki67 staining; 2. Increased apoptosis as detected by TUNEL staining; 3. Decreased expression of activated Akt or NF-κB by IHC; and 4. Modulation in expression of other potential cDNA biomarkers.
- Methods:
  - The mean and S.D. of the Ki67 or TUNEL percentages all pre-genistein versus post-genistein tumor biopsies will be calculated, along with 95% confidence intervals.
  - The mean and S.D. of the sum expression of p-Akt or NF-κB of all pre-genistein versus post-genistein tumor biopsies will be calculated, along with 95% confidence intervals.
  - For each tumor after normalization for control housekeeping gene expression, genes that have $\geq$ 3 fold induction or repression by genistein will be identified. Those genes which are found to be modulated consistently in all of the paired specimens (e.g., occurring in 5 out of 5 patients in whom paired tumor biopsies

are collected and technically successful cDNA array analysis is done) will be preliminarily considered as a significant results. These results are to be used in association with ongoing in vitro cell culture and animal models, as well as for hypothesis generation for future research.

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15.0 APPENDICES

Appendix I: Study Schema

Metastatic Breast Cancer
any prior chemotherapies for metastatic disease
Except Gemcitabine

↓

Pretreatment baseline studies

↓

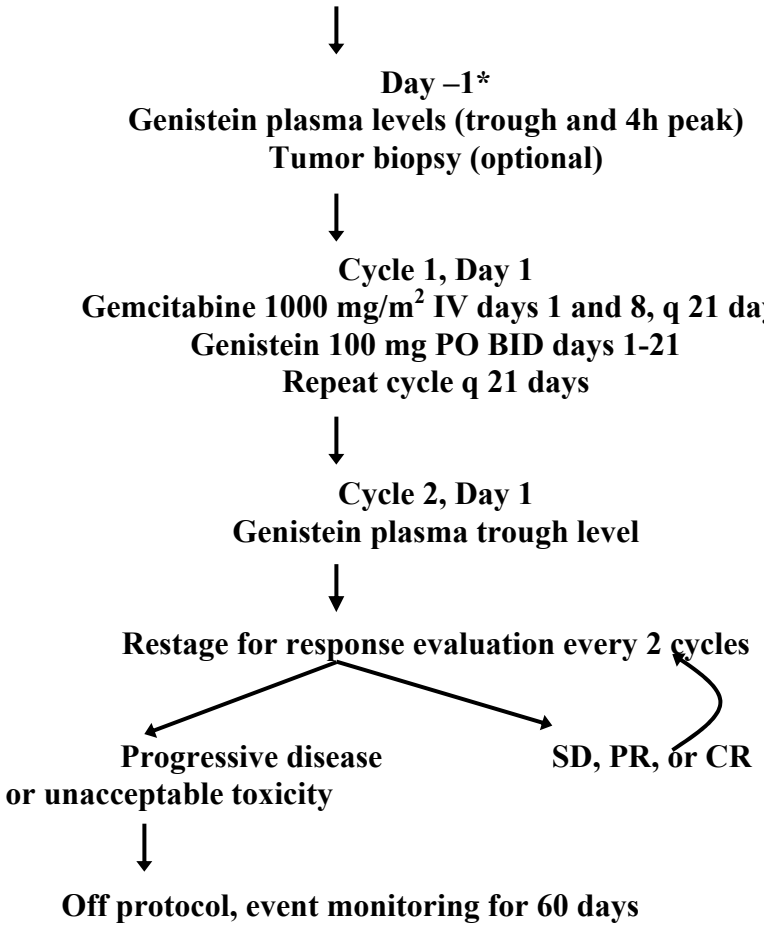
Registration

↓

Baseline genistein plasma levels
Baseline Tumor biopsy (optional)

↓

Genistein 100 mg PO BID x 7 days (Day –7 to –1*)



(*Day-1 = day <u>before</u> cycle 1 day 1 of Gemcitabine and Genistein)

Appendix II: Study Calendar

	Pre-study	Cycle 1 Day-7 (Day 1 of genistein alone)	Cycle 1 Day-1[1] (Day 7 of genistein alone)	Cycle 1, Day 1 (gemcitabine and genistein)	Cycle N, Day 1	Cycle N, Day 8 and 15	After every 2 cycles
History and Physical	X[2]			X	X		
Performance status	X[2]			X	X		
Height and Weight	X[2]			X	X		
CBC, differential and platelets	X[2]			X	X	X (weekly)	
Serum Chemistry[4]	X[2]			X	X		
Amylase and Lipase	X[2]			X	X		
CXR	X[3]						
Bone Scan	X[3]						
Tumor site(s) imaging[5]	X[3]						X
Toxicity assessment				X	X		
Genistein plasma level before a.m. dose[6]		X	X		X[7]		
Genistein peak plasma level 4 h after a.m. dose[6]			X		X[7]		
Tumor biopsy[8]		X[9]	X[9]				

1. Day –1 = day before Cycle 1, Day1 of gemcitabine and genistein
2. Within two weeks of registration.
3. Within four weeks of registration.
4. Serum chemistry: electrolytes, BUN, creatinine, LDH, alk. phosphatase, total bilirubin, AST(SGOT), ALT(SGPT), magnesium, calcium, phosphate and albumin.
5. Radiologic studies to document measurable disease; i.e., CT and/or MRI scans.
6. Collection of 5 ml of plasma and store at –70C, then shipped on dry ice to Translational Lab, 703 HWCRC, KCI, 4100 John R, Detroit, MI 48201; (313) 576-8314, or (313) 576-8315.
7. Cycle 2 only.
8. Optional. Pre-arrange tissue collection ≥ 1 day prior, by calling (313) 576-8314, or (313) 576-8315. Tissue must be immediately placed in RNAlater solution, then kept at 4C or on ice until transported same day, or next-A.M. express shipped at 4°C to Translational Lab, 703 HWCRC, KCI, 4100 John R, Detroit, MI 48201; (313) 576-8314, or (313) 576-8315.
9. Biopsies are prior to first dose of genistein alone (on cycle 1, day-7, or earlier), and then on day 7 of genistein alone (on day-1).

Appendix III: Performance Status

SWOG Scale

Grade	Scale
0	Fully active; able to carry on all pre-disease activities without restriction. (Karnofsky 90-100)
1	Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, e.g., light housework, office work. (Karnofsky 70-80)
2	Ambulatory and capable of all self-care but unable to carry out any work activities. Up and about more than 50% of waking hours. (Karnofsky 50-60)
3	Capable of only limited self-care; confined to bed or chair. Up and about less than 50% of waking hours (Karnofsky 30-40)
4	Completely disabled. Cannot carry on any self-care. Totally confined to bed or chair. (Karnofsky 10-20)
5	Dead

APPENDIX IV: NCI-CTEP COMMON TOXICITY CRITERIA (CTC version 3.0)

(see Internet Website http://www.ctep.info.nih.gov)