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TITLE: A PILOT AND PHASE II STUDY OF ENTINOSTAT AND ANASTROZOLE/TAMOXIFEN IN WOMEN WITH TRIPLE NEGATIVE BREAST CANCER TO EVALUATE BIOMARKERS AND SURROGATES FOR RESPONSE

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Contract #	
NCI Supplied Agent:	Entinostat or SNDX-275 (IND 61198; NSC 706995)
Commercial Agents:	Anastrozole (AstraZeneca)

Protocol History:

Version Date	Description of Action
10/29/09	Original Submission to NCI
1/8/10	Follow Response
10/28/10	Amendment clarifying the shipping of the anastrozole, updating the research team, and defining the primary end points.
1/19/11	Version date 1/19/11. Face Sheet: Added Dr. Henry of U Mich as Investigator/participating site. Removed Dr. Kamboj from NorthShore, Dr. Kraft and MUSC as a participating site. Added California Cancer Consortium before listing of CCC sites. Adjusted investigator table as needed. Section 10.3 Study Days: deleted Methylation study profile on week 3, day 15.
11/9/11	Version date 11/9/11. In response to RRA for Entinostat risk profile update. Section 7.1 updated with Entinostat CAEPR updated to Version 2.3, October 18, 2011.
11/20/11 - Disapproved	Version date 11/20/11. Face sheet: Title updated, added Northwestern, Indiana, and St. Joseph's Medical PI and contact info, removed Loyola and Medical College of Wisconsin, updated Phase II Regulatory Manager and Responsible Data Manager. Schema updated with addition of metastatic triple negative breast cancer and premenopausal women. Section 1 Objectives updated with references to tamoxifen. Section 2.4 updated with tamoxifen clinical experience. Section 2.5 rationale for using tamoxifen was added. Section 3.1 Inclusion Criteria updated with the number of unstained slides required and inclusion criteria for first and second cohort. Section 5.1 Agent Administration updated in response to addition of new cohorts. Section 5.2.1 was updated to evaluate entinostat and anastrozole and entinostat and tamoxifen separately to determine the dose for phase II. Section 5.4 updated to specify duration of therapy for each cohort. Section 6.1 amended due to addition of tamoxifen. Sections 7.2 and 8 were updated with

	tamoxifen information. Section 9 updated with instructions on obtaining a core specimen from the surgical sample after treatment for the first cohort and instructions on obtaining core biopsy specimens for the second cohort and timing clarifications were made to blood sample collections. Section 10 study calendar for first cohort updated with pregnancy test and tamoxifen information and study calendar for second cohort added and visit schedule was updated with these changes. Section 12 Statistical Considerations updated with changes for the new cohorts. References updated. Appendix C updated with tamoxifen.
1/3/12	Version date 1/3/12. Face sheet: Title updated, added Northwestern, Indiana, and St. Joseph's Medical PI and contact info, removed Loyola and Medical College of Wisconsin, updated Phase II Regulatory Manager and Responsible Data Manager. Schema updated with addition of metastatic triple negative breast cancer and premenopausal women. Section 1 Objectives updated with references to tamoxifen. Section 2.4 updated with tamoxifen clinical experience. Section 2.5 rationale for using tamoxifen was added. Section 3.1 Inclusion Criteria updated with the number of unstained slides required and inclusion criteria for first and second cohort. Section 4 removed old U of C registrar. Section 5.1 Agent Administration updated in response to addition of new cohorts. Section 5.2.1 was updated to evaluate entinostat and anastrozole and entinostat and tamoxifen separately to determine the dose for phase II. Section 5.4 updated to specify duration of therapy for each cohort. Section 6.1 amended due to addition of tamoxifen. Section 7.5.1 updated to as paper AdEERS forms are no longer accepted. Sections 7.2 and 8 were updated with tamoxifen information. Section 7.5.2 updated Cancer Clinical Trials Office contact information. Section 9 updated with instructions on obtaining a core specimen from the surgical sample after treatment for the first cohort and instructions on obtaining core biopsy specimens for the second cohort and timing clarifications were made to blood sample collections. Section 9.1.2 updated with the requirement of ≥3 slides. Section 10 study calendar for first cohort updated with pregnancy test and tamoxifen information and study calendar for second cohort added and visit schedule was updated with these changes. Section 12 Statistical Considerations updated with changes for the new cohorts. References updated. Appendix C updated with tamoxifen.
8/15/12	Amendment submitted to clarify inclusion criteria, logistics regarding samples and shipping updated and non-participating sites removed from the face page of the protocol.
4/15/14	Change in the sponsorship of the trial, the coordinating center, registration process, agent ordering, and AE/SAE reporting.
9/9/14	PI change to Dr. Katherine Tkaczuk

SCHEMA

A PILOT AND PHASE II STUDY OF ENTINOSTAT AND ANASTROZOLE/TAMOXIFEN IN WOMEN WITH TRIPLE NEGATIVE BREAST CANCER TO EVALUATE BIOMARKERS AND SURROGATES FOR RESPONSE

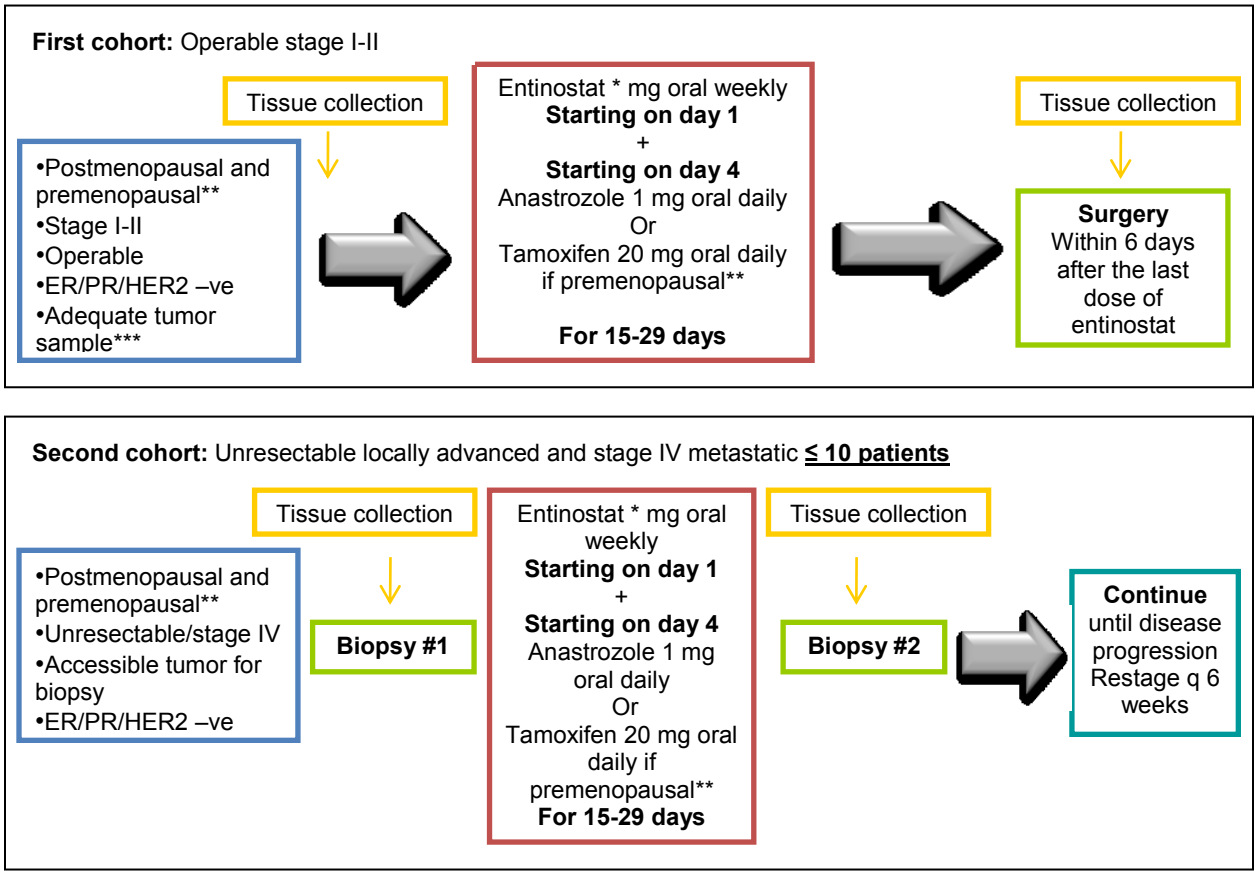
Patient population:

First Cohort: Operable stage I-II triple negative breast cancer (ER, PR, and HER2 negative) in pre- and postmenopausal women

Second Cohort: Unresectable locally advanced or stage IV metastatic triple negative breast cancer in pre- and postmenopausal women with ≤ 2 previous systemic chemotherapy and accessible tumors for biopsies, excluding bone only metastasis

Sample size: 12-41 patients

Schema:



* As per recommended dose for phase II
* *For premenopausal patients, tamoxifen 20 mg oral daily will be used instead of anastrozole.
***≥ 3 unstained slides of 5 micron thickness

REGIMEN DESCRIPTION					
Agents	**Premedications/Precautions**	**Dose**	**Route**	**Schedule**	**Length of Treatment**
Entinostat	Taken with a meal after the first two or three bites	*** mg	oral	weekly starting on day 1	15-29 days (Preferably 22 days)
Anastrozole	-	1 mg once daily	oral	everyday starting on day 4	
Tamoxifen	-	20 mg once daily	oral	everyday starting on day 4	
***Doses as appropriate for assigned dose level.					

Dose Modification Schema for Postmenopausal Women		
Dose Level	**Dose**	
	Entinostat (mg)	**Anastrozole (mg) daily**
-1	3 mg weekly (on day 1, 8, 15, 22, and 29)	1
1	5 mg weekly (on day 1, 8, 15, 22, and 29)	1

Dose Modification Schema for Premenopausal Women		
Dose Level	**Dose**	
	Entinostat (mg)	**Tamoxifen (mg) daily**
-1	3 mg weekly (on day 1, 8, 15, 22, and 29)	20
1	5 mg weekly (on day 1, 8, 15, 22, and 29)	20

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1. OBJECTIVES

1.1. Primary Objectives

Pilot Phase:

1. To evaluate the safety and tolerability of entinostat in combination with anastrozole or tamoxifen.
2. To determine the optimal dose of entinostat in combination with anastrozole or tamoxifen for phase II.

Phase II:

1. To determine baseline and percentage change in proliferative index (Ki67) before and after treatment with entinostat and anastrozole/tamoxifen in triple negative breast cancer (TNBC).
2. To determine the estrogen receptor (ER) expression after treatment with entinostat and anastrozole/tamoxifen in TNBC.

1.2. Secondary Objectives

1. To evaluate baseline and change in the expression levels of progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), epidermal growth factor receptor (EGFR), cytokeratin 5/6 (CK5/6), and aromatase before and after treatment with entinostat and anastrozole/tamoxifen.
2. To assess baseline and change in tumor tissue histone H3 and H4 acetylation before and after treatment with entinostat and anastrozole/tamoxifen.
3. To assess the clinical and pathological response to preoperative combination of entinostat and anastrozole/tamoxifen in TNBC.

1.3. Exploratory Objectives

1. To correlate the levels of histone H3 and H4 acetylation in tumors with the changes in Ki67 and ER.
2. To evaluate baseline and change in gene methylation silencing and expression of candidate genes in tissues and in circulating DNA, including estrogen receptor (ER)-alpha, ER-beta, RAR-beta, cyclin D2, Twist, RASSF1A, and HIN-1.
3. To correlate entinostat trough concentrations with histone H3 and H4 acetylation in tumors as well as the change in Ki67 and ER.
4. To evaluate baseline and change in the global gene expression profile before and after treatment with entinostat and anastrozole/tamoxifen.

2. BACKGROUND

In the era of postgenomic cancer biology, it is becoming increasingly evident that epigenetic modulation of gene expression plays a crucial role in tumorigenesis and tumor progression. Multiple epigenetic treatments have been validated to be successful strategies in cancer therapy. While the mortality rate in breast cancer has decreased in the past few decades, some subsets of breast cancer still have poor outlooks. With the advent of targeted therapy, particularly endocrine and anti-human epidermal growth factor receptor 2 (HER2) therapies, the outcome of breast cancer has changed dramatically. Nevertheless, approximately one fourth of breast cancers unfortunately lack expression of all three constructive cell surface receptors, namely estrogen receptor (ER), progesterone receptor (PR), and HER2, hence the term “triple negative” breast cancer (TNBC). Due to an absence of ER and/or PR, these tumors conventionally do not respond to endocrine therapy like tamoxifen and aromatase inhibitors, which have minimal toxicities and are generally well tolerated. For that reason, the treatment of these tumors depends solely on chemotherapy[1]. In our preliminary data, we found that entinostat, a histone deacetylase (HDAC) inhibitor, can induce re-expression of estrogen receptor and aromatase in TNBC cell lines and render them to be sensitive to aromatase inhibitor. With this promising data, we plan to evaluate the efficacy of this novel strategy in the neoadjuvant setting to target TNBC.

2.1 Epigenetics

Epigenetics is a heritable process which refers to changes in gene expression caused by mechanisms other than alterations in the DNA sequence[2]. Epigenetic regulation plays a crucial role in normal development and allows for the transcriptional control of gene expression without changing the DNA sequence. Key machineries of epigenetic regulation include methylation of the promoter regions of the genes and posttranslational modification of histone, the main protein in the chromatin structure which serves as spools for DNA to wind[3]. In cancer cells, abnormal patterns of DNA methylation have been observed. The abnormal patterns include both loss of methylation in the CpG-depleted regions where most CpG dinucleotides should be methylated and gains in methylation of CpG islands in gene promoter regions resulting in abnormal silencing of critical genes such as the tumor-suppressor genes[4].

2.2 Histone Modification and Epigenetics

Chromatin is a complex structure that is comprised of DNA, RNA, and histones. Nucleosome is the fundamental repeating unit of chromatin which allows proper packaging of large genome in the nucleus. Each nucleosome composes of approximately 147 base pairs of DNA that wrapped around a histone octamer[5]. Posttranslational modifications of histones, mainly acetylation and methylation, determine the accessibility of the nucleosomal DNA to transcription factors and thereby the gene transcriptional activity. HDACs and histone acetyltransferases (HATs) are the key enzymes that are involved in posttranslational modifications of histones by selectively deacetylating or acetylating the ε-amino groups of lysine located near the amino termini of core histone proteins. Acetylation of the chromatin by HATs is associated with open chromatin and transcriptional activation, whereas deacetylation by HDACs correlates with close chromatin and gene silencing. The presence of the hypo-acetylated and hypermethylated histones H3 and H4 alone in the absence of the promoter hypermethylation can also silence certain genes with tumor-suppressor–like properties, such as *p21WAF1*[6]. Eighteen human HDACs have been identified, which are generally subdivided into class I (HDACs 1, 2, 3, 8), class II (HDACs 4, 5, 6, 7, 9, 10), class III (sirtuins 1-7) and class IV (HDAC 11) based on sequence and functional homology. There is evidence that HDACs are associated with a wide range of tumors including neuroblastomas, melanomas, lymphomas, breast, prostate, lung, ovarian, bladder, and colon cancers.

2.3 Triple Negative Breast Cancer

Approximately 15-20% of all breast cancers do not express functional cell surface receptors like ER, PR, and HER2. Triple-negative tumors are typically associated with a higher histologic grade, increased mitotic count, pushing margins of invasion, and a stromal lymphocytic response. Patients with TNBC often have poorer prognosis compared to other breast cancer subtypes. It is commonly misconceived that TNBC is more sensitive to chemotherapy. Although higher pathologic complete response rate (pCR) was observed in TNBC (25–45%) compared to luminal breast cancers (6–7%), patients with TNBC had worse four-year distant disease free and overall survival[7]. It has been demonstrated in multiple studies that the loss of ER protein expression is a result of epigenetic changes in the tumors. Hypermethylation of the CpG islands within the ERα promoter has been observed in a significant fraction of breast cancers[8]. Moreover, HDAC1 has been reported to interact with and suppresses transcription activity of ERα. The interaction of HDAC1 with ERα is mediated by the activation function-2 (AF-2**)** domain and DNA-binding domain of ERα. Treatment of ER-negative breast cancer cells with TSA, an HDAC inhibitor, induced re-expression of ERα. These findings strongly suggest that HDAC1 affects breast cancer progression by promoting cellular proliferation in association with a reduction in both ERα protein expression and transcriptional activity. Therefore, HDAC1 may be a potential target for therapeutic intervention in the treatment of a subset of ER-negative breast cancers[9].

2.4 Entinostat Clinical Experience

Entinostat is a histone deacetylase (HDAC) inhibitor that selectively inhibits HDACs 1, 2, and 3. By promoting hyperacetylation of nucleosomal histones, entinostat promotes transcriptional activation of certain set of genes which leads to inhibition of cell proliferation, induction of terminal differentiation, and apoptosis. Multiple studies both *in vitro* and *in vivo* demonstrated that entinostat can inhibit growth of multiple tumors including breast, lung, prostate pancreatic, renal cell, and glioblastoma[10,11]. Due to the selectivity of entinostat towards HDACs 1, 2, and 3, entinostat has a distinct safety and efficacy profiles compared to pan-HDAC inhibitors like vorinostat. Early clinical studies showed that entinostat has a remarkably long half-life which allows weekly or biweekly treatment dosing[12]. In phase I pharmacokinetic study, entinostat has a half-life ranging from 40 hours to 120 hours. The level of entinostat was still detectable at 168 hours after the dose of 6 mg/m^2 to 12 mg/m^2. Pharmacodynamic studies confirm an inhibition of HDAC by entinostat in phase I study participants at all dose levels (ranging between 2 and 12 mg/m^2 and from once daily to every 2 weeks) tested to date by demonstrating increase in the histone hyperacetylation. Anti-cancer activity of entinostat has been observed in wide range of advanced solid tumors and hematologic malignancies across all range of tested doses. For single agent, the MTD for solid malignancies is 4 mg/m² weekly 3 weeks on 1 week off, or

10 mg/m² every other week. In hematologic malignancies, the MTD is 6 mg/m² weekly 3 weeks on 1 week off. However, further analysis based on the body surface area and drug clearance showed that the fixed dosing is considered to be as accurate as dosing based on body surface area[13]. Entinostat is currently in phase 2 clinical development in multiple solid tumors and hematologic malignancies.

In breast cancer, Syndax currently has an ongoing study of entinostat at the dose of 5 mg oral weekly in combination with exemestane, a steroidal aromatase inhibitor, 25 mg oral daily in the setting of endocrine resistant metastatic breast cancer. From personal communication with Syndax, this study demonstrates that this combination is safe with minimal toxicity. Based on these clinical experiences with entinostat, a dose of 5 mg given weekly has been selected as the regimen for this study in combination with anastrozole. Additional information on the chemistry, pharmacology, toxicology, preclinical findings, and clinical experience to date may be found in the Investigator's Brochure.

Although there is no prior clinical trial of entinostat in combination with tamoxifen, we do not expect to see significant increase in toxicity when compare between entinostat and tamoxifen vs. entinostat and aromatase inhibitor, particularly because these endocrine therapy agents are generally well tolerated drugs. In contrast to entinostat which specifically inhibits class I HDACs, vorinostat is a pan-histone deacetylase inhibitor that inhibits both class I and class II HDACs[14]. Due to the specificity of entinostat, the side effect profile is better with entinostat compared to vorinostat. Nevertheless, a prior phase II study of vorinostat in combination with tamoxifen in hormone receptor-positive metastatic breast cancer demonstrated that this combination is safe and tolerable[15]. In this trial, vorinostat was given at the dose of 400 mg daily for 21 out of 28 days and tamoxifen was given at the daily dose of 20 mg continuously. Most of the toxicities are grade 1 and 2. Grade 3 toxicities range from 2-16%. Most common toxicities include fatigue (16%) and neutropenia (16%) which are common toxicities of HDAC inhibitors rather than tamoxifen. There are only 7% of patients who developed thrombosis related to tamoxifen. Taken this together, tamoxifen will be given at the dose of 20 mg daily in combination with entinostat 5 mg weekly.

Since this trial was opened for accrual in 2009 under the National Cancer Institute's Cancer Therapy Evaluation Program (NCI CTEP) IND 61198, there were a total of 7 women enrolled to date. However, due to funding and slow accrual considerations, NCI CTEP discontinued support for the trial earlier in 2013. Among these 7 patients, 4 patients were treated with entinostat in combination with anastrozole and 3 patients were treated with entinostat and tamoxifen. There were 5 patients in the first cohort (operable stage I-III) and 2 patients in the second cohort (unresectable locally advanced and stage IV). With regards to response, there was one patient in the first cohort who achieved pathological complete response (pCR) after the treatment with entinostat and anastrozole prior to surgery. There was one more patient in the first cohort who had an increase in the expression of ER from 0% to 1%. She was treated with entinostat and tamoxifen. However, there was no reduction in Ki67 observed in this particular patient. Therefore, she did not meet our pre-specified criteria for response in this trial.

With regards to toxicity, there was no dose limiting toxicity observed at the starting dose levels for both tamoxifen and anastrozole. Furthermore, there was no surgical delay due to toxicity from this preoperative treatment. The adverse events are as followed:

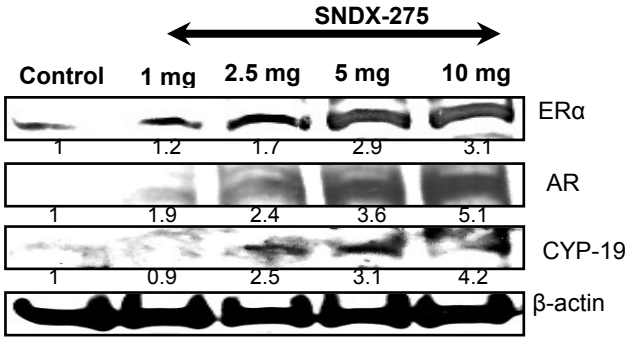
Adverse Events	***Grade***	***Number of patients***
Gastroesophageal reflux disease	1-2	4
Nausea	1-2	3
Fatigue	1-2	3
Diarrhea	1-2	2
Headache	2	1
Hot flashes	1	1
Myalgias	3	2
White blood cell decreased	1-2	2
Hypophosphatemia	2	1
Neutrophil count decreased	3*	1

*Entinostat was held and neutrophil count recovered to ≥ 1,000/mm^3 within 3 days.

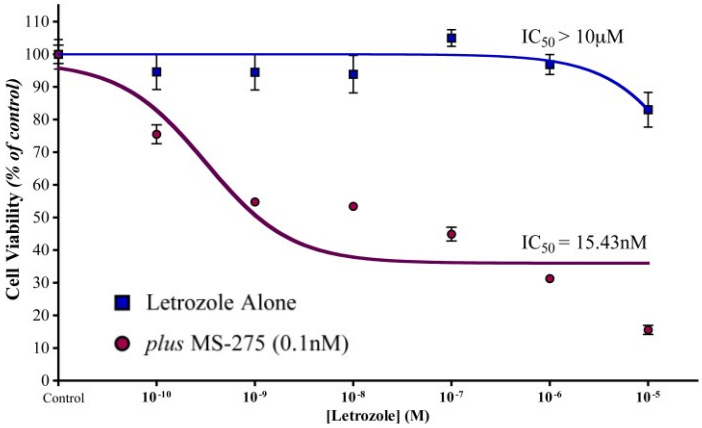
2.5 Rationale

In collaboration with Dr. Angela Brodie and Dr. Gauri Sabnis at the University of Maryland Greenebaum Cancer Center (UMGCC), we have found that entinostat can induce re-expression of ERα, androgen receptor, and

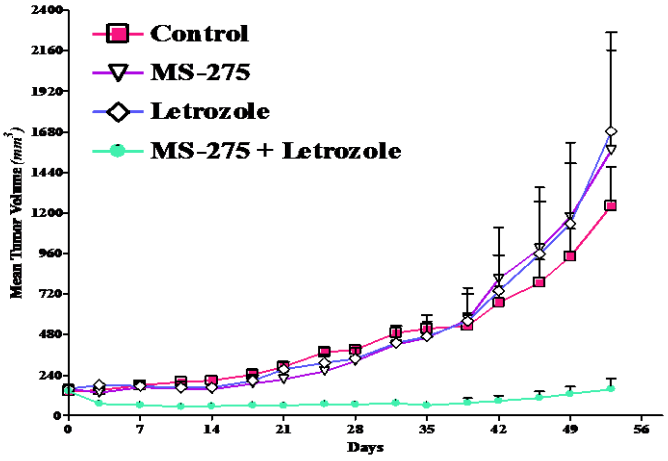
aromatase enzyme (CYP-19) in both *in vitro*[16] and TNBC xenografts[17], MDA-MB-231. This effect is dose dependent as shown below.



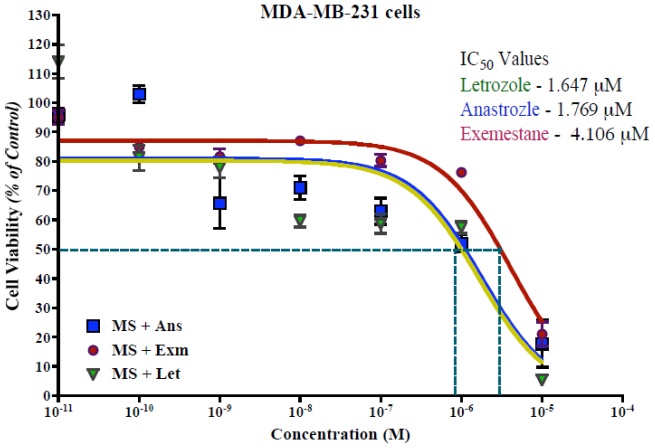
As a single agent, entinostat is quite active in MDA-MB-231 with the half maximal inhibitory concentration (IC_{50}) of 84.2 nM. The combination of entinostat and letrozole demonstrated a marked synergy as shown in the figure below.



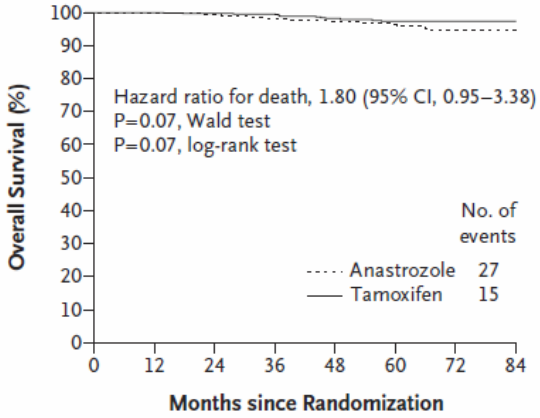
By re-expressing aromatase and ERα, we **hypothesized** that entinostat can sensitize these TNBC cells to endocrine therapy. To test this hypothesis, we treated mice bearing MDA-MB-231 tumors with vehicle control, entinostat, letrozole, and the combination of letrozole and entinostat. In the combination group, entinostat was started 3 days prior to letrozole. Intriguingly, we found that the combination of letrozole and entinostat results in a significant and durable reduction in the tumor volume of MDA-MB-231 xenografts[18] as illustrated in the figure below. In this xenograft model, starting entinostat 3 days prior to letrozole in order to induce expression of ER and aromatase is more effective than starting both of the treatment at the same time (data not shown).



In vitro study of anastrozole in combination with entinostat also demonstrated a comparable result with letrozole and entinostat in MDA-MB-231 as shown in the figure below. Therefore, we propose to conduct a pilot and phase II study of entinostat in combination with anastrozole in postmenopausal women with operable TNBC.

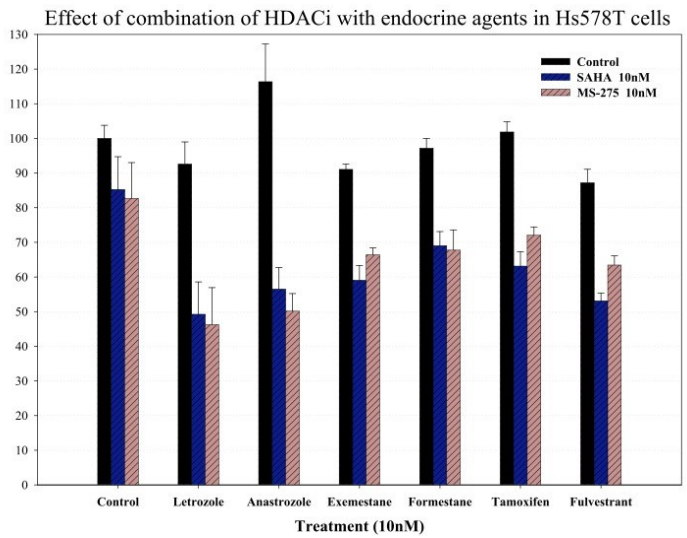


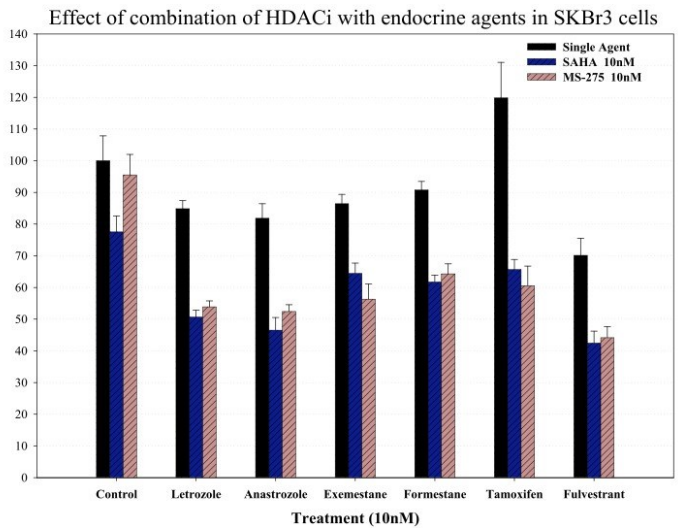
In premenopausal women, tamoxifen will be used instead of anastrozole. This is based on the fact that anastrozole with ovarian suppression may not adequately suppress estrogen production within 2-5 weeks after injection. It is commonly known that ovarian suppression with GnRH agonist initially produce surge of estrogen production, particularly within the first 2 weeks after injection[19]. This estrogenic surge can last up to 6 weeks which could have detrimental effect on tumor growth and our biologic endpoints, particularly the Ki67 expression. Furthermore, the data from The Austrian Breast and Colorectal Cancer Study Group trial 12 (ABCSG12 trial) demonstrated a trend toward poor overall survival in premenopausal women receiving anastrozole in combination with goserelin compared to tamoxifen (figure below)[20]. This effect is more pronounced in overweight patients with BMI ≥ 25 kg/m^2 which may be related to higher level of aromatase from subcutaneous tissue. Subset analysis of overweight patients demonstrated shorter disease free survival (DFS) and overall survival (OS) in patients taking anastrozole compared to tamoxifen (HR 0.625 95%CI 0.387-1.009 p = 0.052, and HR 0.336 95%CI 0.149-0.76, p = 0.006, respectively)[21]. This effect is believed to be secondary to an inadequate suppression of estrogen production with aromatase inhibitor and GnRH agonist in premenopausal women.



Gnant et al NEJM 2009;679

In our preclinical studies, it appears that tamoxifen also has similar synergistic effect with entinostat compared to other aromatase inhibitors in estrogen receptor-negative breast cancer cell lines *in vitro* (figures below).



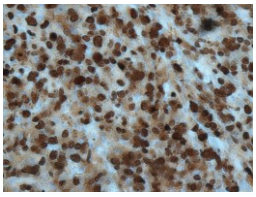


2.6 Correlative Studies Background

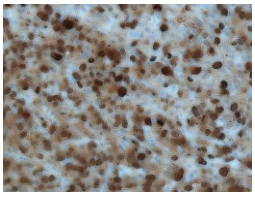
Ki67: Ki67 protein is a cellular marker for proliferation. Ki67 labeling has been reported to be a good prognostic marker and correlate well with the S phase fraction[22] and mitotic index[22,23]. A change in the expression of Ki67 after short-term exposure to the investigational agent is frequently used to determine the efficacy, particularly in the neoadjuvant setting. For neoadjuvant chemotherapy, two previous studies[24-26] showed a significant correlation between the reduction in Ki67 of more than 25% and a longer disease-free survival. However, subsequent studies[26-28] on the reproducibility of Ki67 measurements in the core needle biopsy suggested that a change in Ki67 score of at least 32-50% between two determinations is required to consider statistically significant and attributable to treatment effect for an individual patient. For neoadjuvant endocrine therapy, Dowsett et al. [29] reported that a reduction in Ki67 could be observed in 2 weeks after neoadjuvant endocrine therapy and was largely maintained after 12 weeks of treatment in the majority of patients in the Immediate Preoperative “Arimidex” (anastrozole), Tamoxifen, or Arimidex Combined with Tamoxifen (IMPACT) trial. In this study, postmenopausal women with previously untreated ER-positive breast cancer were randomized to receive a daily treatment of anastrozole, tamoxifen, or the combination of anastrozole and tamoxifen for 12 weeks before surgery. Biomarkers were measured in the core-cut biopsied specimens taken before and after 2 weeks of treatment as well as surgical specimens after 12 weeks of treatment. The geometric means of reduction in Ki67 at 2 and 12 weeks for anastrozole were 76% and 82% respectively. In their subsequent publication[30], Ki67 expression after 2 weeks of treatment depending on the tertiles of tumor Ki67 expression was more strongly associated with recurrence free survival (log-rank P = .008) than tumor Ki67 expression at baseline (log-rank P = .07). However, there was no correlation made between the percentage change in the Ki67 expression and recurrence free survival.

In our same preclinical study, there was a 63% reduction in Ki67 with the combination of entinostat and letrozole. There seems to be no significant change in Ki67 expression with single agent letrozole or entinostat. The figure below illustrates the Ki67 staining by immunohistochemistry in MDA-MB-231 (a TNBC cell line) mouse tumor xenografts treated with vehicle, letrozole, entinostat, or the combination of letrozole and entinostat. The percentage of Ki67-positive cells is quantified by the ChromaVision ACIS system for immunohistochemistry image analysis which will also be used to analyze the specimens in this clinical trial. Therefore, we will use the cutoff point of 50% reduction in Ki67 to evaluate our primary endpoint.

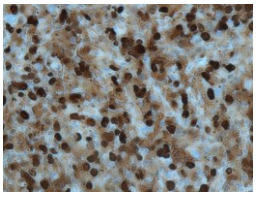
Ki67 Expression



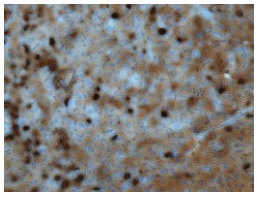
Control = 39.7%



Letrozole = 33.1%



SNDX-275 = 35.3%



Combination = 14.7%

EGFR and Cytokeratin 5/6: Recent data have identified five distinct subtypes of breast cancer based on gene expression profiling: luminal A, luminal B, HER2 overexpressing, normal breast-like, and basal-like[31]. These different subtypes of breast cancer are associated with different clinical course. Basal-like subtype comprises approximately 20% of the breast cancer and has a poor prognosis[32,33]. Basal-like breast cancer is not entirely synonymous with TNBC since ER expression can be seen in 5%to 45% of basal-like cancers and up to 14% of basal-like cancers also express HER2[34]. In contrast, TNBC are not necessarily basal-like cancers since a significant proportion of normal breast–like cancer subtype as defined by expression arrays would also lack hormone receptors and HER2. While the normal breast–like subtype is still poorly characterized, they are reported to have a better prognosis compared to basal-like cancers[32,35]. Nielsen *et al.*[36] have reported that a panel of four antibodies (ER, HER1, HER2, and cytokeratin 5/6) can precisely identify basal-like tumors using standard immunohistochemical staining. Moreover, overexpression of EGFR was found to be associated with poor survival independent of tumor size and nodal status.

Gene Expression Profile: Chromatin remodeling induced by different classes of HDAC inhibitors can result in significant changes in multiple gene expression. The recent development of high-throughput technologies provides a prevailing tool for a global analysis of gene expression. In the past, global gene expression profiling in the formalin-fixed, paraffin-embedded (FFPE) tissue has been technically challenging since these technologies typically require substantial quantities of fresh or frozen tissue. Novel method like the cDNA-mediated Annealing, Selection, Extension, and Ligation (DASL) assay from Illumina has been developed to allow more comprehensive gene expression profiling of degraded RNA extracted from paraffin blocks using bead array format. The DASL assay has been shown to be highly reproducible and can be performed with as little as 50 ng of total RNA isolated from FFPE tissue that had been stored over 10 years[37].

Methylation Profile: In breast cancer, multiple genes are methylated and therefore silenced. Gene methylation is likely related to cancer progression. HDAC inhibitors may also alleviate gene repression that is mediated through promoter hypermethylation. It is presumed that HDAC inhibitors can increase expression of the genes that are not methylated but may not induce the expression of hypermethylated genes. Some of the genes that are often methylated in breast cancer include growth promoting hormone receptors such as ER-alpha, an important predictive factor of response to endocrine manipulations. Other genes that are often hypermethylated in breast cancer include cyclin D, Twist, RASSF1A, APC and HIN-1. Dr. Sukumar and other Johns Hopkins investigators have evaluated hypermethylation of a panel of seven genes using methylation-specific polymerase chain reaction (MSP) in a variety of breast tissues[38-40]. In invasive breast carcinomas, up to 100% of specimens contained at least one hypermethylated gene, 80% contained two, and 60% contained three or more methylated genes[39]. In 44 ductal carcinoma in situ (DCIS) specimens, 95% had at least one methylated gene. In contrast, the percentage of women with benign breast disease having at least one methylated gene was only 15%. Only one of 8 reduction mammoplasty specimens contained hypermethylated genes. More recently, Dr. Sukumar and colleagues have developed a novel method, quantitative multiplex methylation specific PCR (QM-MSP) to globally assess promoter hypermethylation for many genes simultaneously in small samples. QM-MSP is highly sensitive (1 in 104-105 copies of DNA) and linear over 5 orders of magnitude[39]. Therefore, it is also feasible to use the DNA sample obtained from the peripheral blood to perform this assay.

3. PATIENT SELECTION

3.1 Inclusion Criteria

3.1.1 Female greater than or equal to 18 years.
3.1.2 Eastern Cooperative Oncology Group (ECOG) performance status ≤2 (see Appendix A).
3.1.3 Histologically confirmed adenocarcinoma of the breast.
3.1.4 Evidence of hormone insensitivity (ER and PR negative) of primary tumor tissue. ER negative is define as ER 0 or < 1% staining by immunohistochemistry. PR negativity is defined as PR ≤ 10% staining by immunohistochemistry.
3.1.5 HER2 negative in the primary tumor tissue as defined by:

- Immunohistochemistry (IHC) Grade 0 as defined by no staining observed or membrane staining that is incomplete and is faint/barely perceptible and within ≤ 10% of the invasive tumor cell
- IHC 1+ as defined by incomplete membrane staining that is faint/barely perceptible and within > 10% of the invasive tumor cell
- IHC Grade 2+ staining intensity by means of IHC analysis with no gene amplification below.
- No gene amplification on ISH based on
    - Single-probe average HER2 copy number < 4.0 signals/cell
    - Dual-probe HER2/CEP17 ratio < 2.0 with an average HER2 copy number < 4.0 signals/cell

3.1.6 Ability to understand and the willingness to sign a written informed consent document.
3.1.7 Patients must not have received any prior chemotherapy, radiation therapy, or endocrine therapy for their current breast cancer. Patients who received tamoxifen or raloxifene or another agent for prevention of breast cancer may be included as long as the patient has discontinued the treatment at least one month prior to baseline study biopsy.
3.1.8 Women of childbearing potential must have negative (serum or urine) pregnancy test within 7 days prior to registration.
3.1.9 Patients must have adequate tumor tissue sample prior to the enrolment available for correlative studies as defined below:

- Core needle biopsy or incisional biopsy samples that can provide ≥ 3 unstained sections of 5 micron thickness. Fine needle aspiration (FNA) sample alone is not sufficient except in the second cohort.
- Additional core needle biopsy needs to be performed in the patients who agree to participate in this study and do not have adequate tumor tissue sample.

3.1.10 Patients must have adequate organ and marrow function as defined below:

• Hemoglobin	≥ 9.0 g/dL
• Leukocytes	≥2,500/mcL
• Absolute neutrophil count	≥1,100/mcL
• Platelets	≥100,000/mcL
• Total bilirubin	within normal institutional limits
• AST(SGOT)/ALT(SGPT)	≤2.5 x institutional upper limit of normal
• Creatinine	within normal institutional limits or creatinine clearance ≥ 60 mL/min/1.73 m^2 for patients with creatinine levels above institutional normal

Additional Inclusion Criteria for the First cohort:

3.1.11 Unresected operable breast cancer that meets the following clinical stages (see Appendix B):

- T1b, T1c, or T2
- N0 or N1
- M0 (No distant metastasis)

Additional Inclusion Criteria for the Second cohort:

3.1.12 Unresectable, inoperable, recurrent local-regional breast cancer or
3.1.13 Metastatic (stage IV) breast cancer
3.1.14 Patients must have measurable or evaluable disease (i.e. ascites or pleural/pericardial effusion). Patients with bone metastatic only will be excluded.
3.1.15 Patients must not have rapidly progressive disease, extensive visceral involvement, or any high risk characteristics that are not appropriate for this treatment as per investigator's discretion.
3.1.16 Patients must receive at least one prior line of chemotherapy but not more 2 prior chemotherapy regimens for stage IV breast cancer. Prior chemotherapy in the adjuvant and /or neoadjuvant setting is permitted. However, patients must have finished chemotherapy at least 2 weeks prior to enrollment.
3.1.17 Patients must have an accessible tumor lesion from which a fine needle aspirate or preferably a core biopsy specimen can be obtained. Patients with FNA only samples are allowed in this cohort. Ascites or pleural/pericardial effusion alone is not sufficient.

3.1.18 Patients must be willing to provide consents for 2 research biopsies. However, the pretreatment biopsy can be omitted in patients who have recent biopsy but have not been started on breast cancer treatment within 12 weeks prior to the registration and there is adequate tumor tissue sample as per section 3.1.9.

3.2 Exclusion Criteria

3.2.1 Patients may not be receiving any other investigational agents.
3.2.2 Prior exposure to other HDAC inhibitors. However, prior valproic acid exposure is allowed providing ≥ 30 days wash-out period.
3.2.3 History of allergic reactions or hypersensitivity to compounds of similar chemical or biologic composition to entinostat, benzamide, anastrozole, or tamoxifen.
3.2.4 Any medical condition which in the opinion of the investigator puts the patient at risk of potentially serious complications while on this therapy.
Examples: HIV, unstable angina, uncontrolled heart failure or hypertension, uncontrolled hyperlipidemia, uncontrolled diabetes mellitus, uncontrolled systemic infection.
3.2.5 Previous or current systemic malignancy within the past 3 years other than breast cancer or adequately treated cervical carcinoma in situ or basal/squamous carcinoma of the skin.

3.3 Inclusion of Minorities

Women of all races and ethnic groups are eligible for this trial.

Accrual Targets							
Ethnic Category	**Sex/Gender**						
	Females			**Males**		**Total**	
Hispanic or Latino	10		+	0	=	10	
Not Hispanic or Latino	35		+	0	=	35	
Ethnic Category: Total of all subjects	45	(A1)	+	0 (B1)	=	45	(C1)
Racial Category							
American Indian or Alaskan Native	5		+	0	=	5	
Asian	5		+	0	=	5	
Black or African American	5		+	0	=	5	
Native Hawaiian or other Pacific Islander	5		+	0	=	5	
White	25		+	0	=	25	
Racial Category: Total of all subjects	45	(A2)	+	0	=	45	
	(A1 = A2)			(B1 = B2)		(C1 = C2)	

Accrual Rate: 2-3 pts/month **Total Expected Accrual:** 12 Min 45 Max

4. REGISTRATION PROCEDURES

4.1 General Guidelines

Eligible patients will be entered on study at the University of Maryland Greenebaum Cancer Center by the Study Coordinator. Following registration, patients should begin protocol treatment within 3 business days. All of the patients' documents must be kept confidential in a secured area.

To register a patient, the following documents should be completed by a research team member and sent to the Study Coordinator, Nancy Tait or Jane Lewis, via fax, (410) 328-1741, or e-mail, ntait@umm.edu or jlewis@umm.edu

- Copy of required laboratory tests
- Signed patient consent form
- HIPAA authorization form
- Other appropriate forms (e.g., Eligibility Screening Worksheet, Documentation of Pathology and Diagnosis, Registration Form)

To complete the registration process, the Coordinator will

- Assign the patient a study number
- Register the patient on the study

4.2 Pretrial Screening

Patients will be screened at the participating centers before entry to establish eligibility. The following assessments must be obtained within 3 weeks of trial entry:

- Medical history including all significant conditions.
- Concomitant therapy before entry documented to establish eligibility.
- ECOG performance status
- Physical examination including vital signs, height and weight
- Clinical tumor assessment by a breast examination including clinical tumor size, character, mobility, and location of the breast mass.
- Laboratory studies: CBC with diff; chemistries including sodium, potassium, chloride, bicarbonate, BUN, creatinine, glucose, total bilirubin, calcium, total protein, albumin, AST (SGOT), ALT (SGPT) , alkaline phosphatase, LDH, uric acid, phosphorus, magnesium; and coagulation including PT with INR and PTT
- 12-lead electrocardiogram (ECG) at baseline

Following baseline breast imaging can be obtained within 6 weeks of trial entry:

- Baseline mammogram, breast ultrasound, or breast magnetic resonance imaging (MRI) as indicated

5. TREATMENT PLAN

5.1 Agent Administration

Treatment will be administered on an outpatient basis. Reported adverse events and potential risks for anastrozole and entinostat are described in Section 7. Appropriate dose modifications for entinostat and anastrozole are described in Section 6. No investigational or commercial agents or therapies other than entinostat, anastrozole, and tamoxifen may be administered with the intent to treat the patient's malignancy.

In the first cohort:

- Treatment will be continued for a minimum of 15 days and a maximum of 29 days. If clinically acceptable, treatment should be preferably continued for 22 days prior to the definitive surgery.
- Anastrozole will be administered continuously starting on day 4 at a fixed dose of 1 mg oral once daily.
- In patients who are not postmenopause as defined by any of the following criteria, tamoxifen will be administered continuously instead of anastrozole starting on day 4 at a fixed dose of 20 mg oral once daily.

Postmenopausal as defined by any of the following criteria:

- Age ≥ 60
- Age < 60 with amenorrhea > 12 months and an intact uterus and an FSH and estradiol level within the postmenopausal range by institutional laboratory parameters.
- Age < 60 with previous hysterectomy must have FSH and estradiol level within the postmenopausal range by institutional laboratory parameters.
- Any age having undergone bilateral oophorectomy

- Entinostat will be administered at the assigned dose once a week (on day 1, 8, 15, 22, and 29) until the definitive surgery. Entinostat should be taken with a meal after the first two or three bites.
    - The initial starting dose for entinostat is 5 mg oral weekly. Dose de-escalation to 3 mg/week is permitted in the pilot phase if dose limiting toxicity is observed as defined in section 5.2.1.
    - Dose level for entinostat in phase II part of this study will be determined after the pilot phase is completed.

In the second cohort:

1. Pretreatment biopsy (biopsy#1) will be performed within 3 weeks after registration. However, the pretreatment biopsy can be omitted in patients who have recent biopsy but have not been started on breast cancer treatment within 12 weeks prior to the registration and there is adequate tumor tissue sample as per section 3.1.9.

- Treatment will be started after pretreatment biopsy and will be continued until disease progression. The post-treatment biopsy (biopsy#2) will be performed after a minimum of 15 days and a maximum of 29 days of treatment, but preferably close to 29 days. The post-treatment biopsy must take place within 6 days (preferably within 3 days) after the last dose of entinostat. After the post-treatment biopsy, treatment will be continued until disease progression regardless of the result of post-treatment biopsy. Restaging scans will be performed every 6 weeks while on treatment. In the event that a patient cannot undergo the post-treatment biopsy for any reason, they will continue to be treated per study protocol. If a patient discontinues the study prior to 15 days, an attempt should be made to collect the biopsy when the decision is made to discontinue and prior to initiation of additional treatment, if possible.
- Anastrozole will be administered continuously starting on day 4 at a fixed dose of 1 mg oral once daily until disease progression.
- In patients who are not postmenopause as defined by any of the previously described criteria, tamoxifen will be administered continuously instead of anastrozole starting on day 4 at a fixed dose of 20 mg oral once daily until disease progression.
- Entinostat will be administered at the assigned dose once a week until disease progression. Entinostat should be taken with a meal after the first two or three bites.
    - The initial starting dose for entinostat is 5 mg oral weekly. Dose de-escalation to 3 mg/week is permitted if dose limiting toxicity is observed as defined in section 5.2.1.
    - Dose level for entinostat in phase II part of this study will be determined after the pilot phase is completed.

The dose schemas are as shown below for both postmenopausal and premenopausal women:

Dose Modification Schema for Postmenopausal Women		
Dose Level	Dose	
	Entinostat (mg)	Anastrozole (mg) daily
-1	3 mg weekly (on day 1, 8, 15, 22, and 29)	1
1	5 mg weekly (on day 1, 8, 15, 22, and 29)	1

or

Dose Modification Schema for Premenopausal Women		
Dose Level	Dose	
	Entinostat (mg)	Tamoxifen (mg) daily
-1	3 mg weekly (on day 1, 8, 15, 22, and 29)	20
1	5 mg weekly (on day 1, 8, 15, 22, and 29)	20

5.1.1 Entinostat

Entinostat is supplied as light brown (1 mg) or intense yellow (5 mg) coated tablets. Patients will self-administer entinostat as per assigned dose level shown in section 6.1. Tablets should be taken with a meal after the first two or three bites. If the patient's dose requires more than one tablet, the tablets should be taken one at a time. Unused tablets or empty bottles will be returned to the clinic for accountability purposes. Dose reductions for toxicity are summarized in section 6.2. The definitive surgery or post-treatment biopsy (biopsy#2) must take place within 6 days (preferably within 3 days) after the last dose of entinostat.

Entinostat may cause fatigue or malaise; advise patient to exercise caution while driving a vehicle or operating machinery. Delay administration of H_2-antagonists, antacids, or proton pump inhibitors, or other drugs that lower acidity for at least 2 hours after dosing entinostat.

5.1.2 Anastrozole

Anastrozole will be taken by mouth once daily on a continuous basis starting on day 4 at the fixed dose of 1 mg per day. Anastrozole should be continued until the day of the surgery in the first cohort and until disease progression in the second cohort.

5.1.3 Tamoxifen

In patients who are not postmenopause as defined in section 5.1 (premenopausal women), tamoxifen will be administered instead of anastrozole. Tamoxifen will be taken by mouth once daily on a continuous basis starting on day 4 at the fixed dose of 20 mg per day. Tamoxifen should be continued until the day of the surgery in the first cohort and until disease progression in the second cohort.

5.2 Study Plan

5.2.1 Pilot Phase

In the initial pilot phase, we will separately evaluate the safety of the combination of entinostat and anastrozole as well as entinostat and tamoxifen to find the optimal dose for the subsequent phase II part.

The combination of entinostat and anastrozole will be administered in postmenopausal patients as defined in section 5.1. Even though there is no previous clinical trial of entinostat in combination with anastrozole, we do not expect significant toxicity of this combination. An ongoing study of entinostat at the dose of 5 mg oral weekly with exemestane, a steroidal aromatase inhibitor, 25 mg oral daily in postmenopausal women with metastatic estrogen receptor-positive breast cancer progressing on a non-steroidal aromatase inhibitor demonstrates that so far this combination is safe with minimal toxicity (from personal communication with Syndax). Therefore, the first cohort of 3

postmenopausal patients will be treated with entinostat at the dose of 5 mg oral weekly with anastrozole 1 mg oral daily (dose level 1 as shown in the table below). If we do not observe any dose limiting toxicity (DLT), up to 3 additional patients will be treated with dose level 1. If there is ≥ 1 patient experiencing DLT at dose level 1, the dose will be de-escalated to level -1. Up to 6 additional patients can be enrolled in the dose level -1.

The dose modification schemas are as follow for both postmenopausal women:

Dose Modification Schema for Postmenopausal Women		
Dose Level	Dose	
	Entinostat (mg)	Anastrozole (mg) daily
-1	3 mg weekly (on day 1, 8, 15, 22, and 29)	1
1	5 mg weekly (on day 1, 8, 15, 22, and 29)	1

The combination of entinostat and tamoxifen will be administered in premenopausal patients as defined in section 5.1. There is no previous study of tamoxifen in combination with entinostat. Based on previous phase II study of vorinostat and tamoxifen, we also do not expect significant toxicity of this combination. The first cohort of 3 premenopausal patients will be treated with entinostat at the dose of 5 mg oral weekly with tamoxifen 20 mg oral daily (dose level 1 as shown in the table below). If we do not observe any DLT, up to 3 additional patients will be treated with dose level 1. If there is ≥ 1 patient experiencing DLT at dose level 1, the dose will be de-escalated to level -1. Up to 6 additional patients can be enrolled in the dose level -1.

The dose modification schemas are as follow for both premenopausal women:

Dose Modification Schema for Premenopausal Women		
Dose Level	Dose	
	Entinostat (mg)	Tamoxifen (mg) daily
-1	3 mg weekly (on day 1, 8, 15, 22, and 29)	20
1	5 mg weekly (on day 1, 8, 15, 22, and 29)	20

Therefore, the total number of patients for the pilot phase of the study will be 6-12 for each treatment groups (entinostat and anastrozole as well as entinostat and tamoxifen) or the total of 12-24 patients.

The phase II part will continue with the dose level in which there is ≤ 1 out of 6 patients experiencing a DLT. However, if there are ≥ 2 out of 6 patients experiencing DLT at dose level -1, the study will be terminated due to toxicity.

The patients in the pilot phase will be included in the overall evaluation of response.

Toxicity will be graded according to the active version of National Cancer Institute (NCI)-Common Terminology Criteria for Adverse Events (CTCAE) as per section 7.3. Patients in both cohorts (the first cohort-operable stage I-II and the second cohort-unresectable locally advance/metastatic) in the pilot phase will be used to evaluate for DLT. In the second cohort, only adverse events (AEs) that occur during the first 29 day will be considered for DLT. DLT is defined as one or more of the following treatment-related:

1. Hematologic AEs
    a. Absolute neutrophil count (ANC) < 500/mm^3 regardless of duration
    b. Failure of ANC to recover ≥ 1,000/mm^3 within 14 days
    c. Grade ≥ 4 thrombocytopenia regardless of duration or grade 3 thrombocytopenia with significant bleeding.
    d. Failure of platelet to recover ≥ 50,000/mm^3 within 14 days
    e. Hemoglobin < 6.5 g/dL despite transfusion lasting > 7 days
2. Grade ≥ 3 nonhematologic AEs (except for fatigue, diarrhea, and nausea/vomiting if manageable).
3. Grade ≥ 3 diarrhea lasting > 2 days despite being treated with optimal medical therapy, or associated with fever or severe dehydration.
4. Grade ≥ 3 fatigue lasting > 7 consecutive days.

5.2.2 Phase II period

After the pilot phase has been completed, the phase II period will be started with the dose level in which there is ≤ 1 out of 6 patients experiencing any DLT.

5.3 General Concomitant Medication

All concomitant treatments and therapies, or medication administered during the 28 days preceding the screening study visit must be reported. The generic name of the drug (or trade name for combination drugs) must be specified along with the route of administration, dosage, frequency of dosage, and duration of treatment. Any medication which is considered necessary for the patient's welfare, and which is not expected to interfere with evaluation of the study drug, may be given at the discretion of the investigator. Patients must notify the investigator of all concomitant medications taken through completion of the study.

Throughout the study, investigators may prescribe any concomitant medications or treatments deemed necessary to provide adequate supportive care, such as potassium and phosphorus supplements and anti-emetics, with the exception of those listed below.

The following concomitant medications are prohibited:

- **Valproic acid, Zolinza™ (vorinostat), or any other HDAC inhibitor**
- **DNA methyltransferase inhibitors**
- **Other anti-cancer agents including Lupron or other approved luteinizing-hormone releasing hormone (LHRH) agonists such as goserelin or leuprolide**
- **Bisphosphonates initiated within 4 weeks prior to study start**
- **Other investigational agents**

Delay administration of H_2-antagonists, antacids, or proton pump inhibitors, or other drugs that lower acidity for at least 2 hours after dosing entinostat.

5.4 Duration of Therapy

- In the first cohort, treatment will be administered for a minimum of 15 days and up to 29 days prior to the definitive surgery. However if clinically acceptable, the preferred treatment duration is 22 days. The treatment should be continued until the time of the surgery (up to 29 days) or until one of the criteria in section 5.8 applies.
    - Anastrozole or tamoxifen treatment should be continued until the time of the surgery for a maximum of 29 days or until one of the criteria in section 5.8 applies.
    - Entinostat should be administered within 6 days prior to the definitive surgery. A minimum of 3 doses (day 1, 8, and 15) are required to complete the study.
- In the second cohort, treatment including entinostat and anastrozole/tamoxifen will be continued until disease progression. Restaging scans will be performed every 6 weeks while on treatment.

5.5 Surgical Considerations for the First Cohort (Operable Stage I-II)

5.5.1 Marker Placement Prior to Initiation of Therapy

A titanium marker or "clip" should be placed at the tumor site(s) prior to starting treatment in women who may be candidates for breast conservation surgery. This will identify the tumor location(s) for the surgical procedure in the event that there is a complete response to the preoperative treatment.

5.5.2 Breast Surgery

Following preoperative treatment, women will undergo breast conserving surgery or a mastectomy at the discretion of the treating surgeon. It is recommended that the definitive surgery take place within 6 days (preferably within 3 days) after the last dose of entinostat. Anastrozole should be continued until the day of the surgery.

5.5.3 Axillary Staging

Pre-treatment

- Fine needle aspiration (FNA) or core biopsy of an axillary lymph node is permitted for any patient.
- A pre-neoadjuvant therapy sentinel lymph node biopsy (SLNB) for patients with clinically *negative* axillary lymph nodes is permitted.

Post-treatment

- Post-neoadjuvant therapy axillary staging is required for all patients and will be performed according to the widely accepted American Society of Clinical Oncology (ASCO) guideline[41].

5.6 Postoperative Treatments

5.6.1 Chemotherapy

It is recommended that postoperative chemotherapy be administered. The adjuvant chemotherapy regimen is at the discretion of the treating physicians.

5.6.2 Radiation Therapy

Radiation therapy will be administered at the conclusion of all protocol treatment, when appropriate.

5.7 Duration of Follow Up

Patients will be followed for 30 days after the last dose of study medication or until death, whichever occurs first. In patients who receive other primary treatment after entinostat and anastrozole administration and prior to surgery, we will collect a summary of the treatment given to correlate, if needed, with tissue samples from the surgical procedure – no additional data will be collected after that time. Patients continuing to experience adverse events attributable to the study drug will be followed as needed until resolution or stabilization of the adverse events. Patients who either are found to be ineligible or refuse to start treatment after consenting will not be followed and their information will not be collected.

5.8 Criteria for Removal from Study

Patients will be removed from study when any of the criteria listed below applies. The reason for study removal and the date the patient was removed must be documented in the case report form.

- Evidence of rapid disease progression during treatment at the discretion of the treating investigator.
- Non-compliance with the study protocol; including, but not limited to not attending the majority of scheduled visits. The Protocol Chair will determine when non-compliance should lead to removal from study.

Note: The patients will still be included in the overall evaluation of response (intent-to-treat analysis).

- Unacceptable major toxicity or the dosing delay and/or delay in the surgery ≥ 2 weeks.

Note: The patients will still be included in the overall evaluation of response (intent-to-treat analysis).

- Intercurrent illness or condition that would, in the judgment of the treating investigator, affect assessment of clinical status to a significant degree or require discontinuation of study treatment.
- At subject's own request.

Note: The reason for discontinuation from the study must be documented. The patients will be included in the overall evaluation of response (intent-to-treat analysis) if any protocol therapy was administered prior to withdrawal.

- Study is closed for any reason (e.g. new information shows that the patient's welfare would be at risk if she continued study treatment).
- Subject withdraws consent for follow-up.
- Subject is lost to follow-up.
- Study is terminated for any reason.

6. DOSING DELAYS/DOSE MODIFICATIONS

6.1 Recommended Dose Adjustments

Although entinostat and anastrozole/tamoxifen have distinct toxicity profiles, they do share some adverse events such as fatigue and nausea. There is the theoretical possibility that one agent may potentiate the other and hence drug causality will not always be clear. In the event of uncertainty, dose reductions and/or delays will follow the most conservative approach, i.e., withholding both drugs until resolution of the event.

6.1.1 Non-Hematologic Toxicities of Entinostat

The following rules will be followed for the management of adverse events that are definitely or probably due to entinostat alone. Grades are according to the active version of NCI CTCAE as per section 7.3.

Toxicity	Hold dose	Treatment	Recovery	Dose reduction
Grade ≥ 3	Until recovery to grade 1 or baseline	Symptomatic remedies, start prophylaxis	If recovered by next scheduled dose	Resume at one dose level below the current dose
			If not recovered by next scheduled dose	Skip this dosing; resume at one dose level below the current dose after recovery
Recurrent Grade ≥ 3 despite dose reduction	Until recovery to grade 1 or baseline	Symptomatic remedies, start prophylaxis	If recovered by next scheduled dose	Resume at one dose level below the current dose
			If not recovered by next scheduled dose	Skip this dosing; resume at one dose level below the current dose every other week after recovery
Grade ≤ 2	Until recovery to grade 1 or baseline	Symptomatic remedies, start prophylaxis	If recovered by next scheduled dose	Resume at the current dose level
			If not recovered by next scheduled dose	Skip this dosing; resume at the current dose after recovery

The dose modification schemas are as follow for both postmenopausal and premenopausal women:

Dose Modification Schema for Postmenopausal Women		
Dose Level	**Dose**	
	Entinostat (mg)	**Anastrozole (mg) daily**
-1	3 mg weekly (on day 1, 8, 15, 22, and 29)	1
1	5 mg weekly (on day 1, 8, 15, 22, and 29)	1

or

Dose Modification Schema for Premenopausal Women		
Dose Level	**Dose**	
	Entinostat (mg)	**Tamoxifen (mg) daily**
-1	3 mg weekly (on day 1, 8, 15, 22, and 29)	20
1	5 mg weekly (on day 1, 8, 15, 22, and 29)	20

6.1.2 Hematologic Toxicities of Entinostat

Myelosuppression is a common side effect of higher doses of entinostat. The guidelines in the table below will be followed for hematologic toxicities.

ANC at planned dose		***PLT at planned dose***	***Cycle Delivery***
≥ 1.0 x 10^9/L	and	≥ 80 x 10^9/L	Resume at the current dose level
< 1.0 x 10^9/L	or	< 80 x 10^9/L	Delay until recovery to grade 1 or

	baseline

If the treatment is delayed for more than 2 weeks, the patient will be discontinued from treatment and followed until event resolution, and treated off-protocol at the investigator's discretion.

6.1.3 Toxicities likely from both drugs (diarrhea, anorexia, fatigue, and nausea)

In the event that one of the above toxicities occurs, patients should be treated symptomatically and prophylactic treatment should be considered for subsequent administration of entinostat. In case grade 3 or 4 toxicity is observed both drugs should be held until recovery to grade 1 or baseline.

If dosing is to be delayed by more than 2 weeks the patient will go off study.

6.1.4 Other Adverse Events

For any other NCI-CTCAE Grade 3 or 4 adverse events or any clinically significant, lower-grade adverse event, treatment with entinostat and anastrozole/tamoxifen should be interrupted for a maximum of 14 days until the patient recovers completely or the adverse event reverts to NCI-CTCAE Grade 1 or to baseline grade. If recurrence of adverse event after drug holiday / interruptions is observed a dose reduction to dose level -1 is recommended. Dose reduction should only be implemented when all supportive care measures have been exhausted without an improvement of patient status. In all cases where the subject is withdrawn due to unusual or unusually severe adverse event considered related to entinostat, the investigator must report the withdrawal as an SAE.

6.2 Supportive Care Guidelines

6.2.1 Dehydration/dysgeusia

In other studies, the use of popsicles and Gatorade has been found to be useful by some investigators in maintaining adequate hydration in the setting of dysgeusia.

6.2.2 Diarrhea

Diarrhea should be treated promptly with appropriate supportive care, including loperamide. Instruct patients to begin taking loperamide at the first signs of: 1) poorly formed or loose stool; 2) occurrence of more bowel movements than usual in one day; or, 3) unusually high volume of stool. Loperamide should be taken in the following manner: 4 mg at first onset of diarrhea, then 2 mg after each unformed stool. Daily dose should not exceed 16 mg/day. Advise patients to drink plenty of clear fluids to help prevent dehydration caused by diarrhea. Avoid loperamide if there is the presence of blood or mucus in the stool or if diarrhea is accompanied by fever. If grade 3 or 4 diarrhea recurs despite treatment, discontinue further treatment with entinostat.

6.2.3 Additional Information

When possible, symptoms should be managed symptomatically. In case of toxicity, appropriate medical treatment should be used (including anti-emetics for nausea/vomiting, anti-diarrheals for diarrhea, as above, etc.).

7. ADVERSE EVENT REPORTING AND DATA SAFETY AND MONITORING PLAN

7.1 Adverse Events (AEs)

An Adverse Event is any untoward occurrence reflecting a change from baseline state after signing informed consent and up to 30 days from last administration of the study medication. A Serious Adverse Event (SAE) is any AE that is 1) Fatal, 2) Life Threatening, 3) Requires or prolongs hospital stay, 4) Results in a persistent or significant disability or incapacity. 5) Results in a congenital anomaly or birth defect or 6) Is considered by the Investigator as an important medical event.

7.1.1 The descriptions and grading scales found in the revised NCI Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 (Available through http://ctep.cancer.gov) will be utilized for AE reporting. AEs of any grade will be documented in the case report form.

7.1.2 AEs can be “Expected” if described in the Informed Consent or “Unexpected”. The attribution of the AE is Definite if the AE is clearly related to study treatment; Probable if it is likely related to the study treatment; Possible if it is equally likely related to study treatment or to some other circumstance, and Unlikely if most probably some other event was responsible for the adverse event, and Unrelated if the Adverse Event is entirely explained by circumstances not caused by the study medication.

7.1.3 Data Safety Monitoring. The UMGCC Data Safety Monitoring / Quality Assurance Committee will review the study. The specific functions of the DSMB /QAC are contained in the UMGCC Clinical Investigators Handbook, available through http://www.umgcc.org/research/clinical_research.htm which is modeled closely after NCI policies and procedures for example, for adverse event reporting. (See http://www.ctep.nci.nih.gov.)

7.2 Expedited reporting of severe adverse events

The University of Maryland IRB has specific guidelines for expedited adverse event reporting. These policies can be found in the IRB’s policies and procedures manual which can be accessed using the following URL: http://www.hrpo.umaryland.edu/default.asp. The UM policy requires that any harm experienced by a subject or other individual, which in the opinion of the investigator is unexpected and probably related, be expeditiously reported to the IRB within 5 business days.

For those events that require a change to the informed consent, the data entry staff who input these data will notify the appropriate research coordinator and regulatory coordinator of this change.

7.3 Expedited reporting by investigator to Syndax

Serious adverse events (SAE) are defined above. The investigator must inform Syndax in writing using a SAE form or MEDWATCH 3500A form of any SAE within 24 hours of being aware of the event. The date of awareness should be noted on the report. The written report must be completed and supplied to Syndax by facsimile within 24 hours/1 business day at the latest on the following working day. The initial report must be as complete as possible, including details of the current illness and (serious) adverse event, and an assessment of the causal relationship between the event and the investigational product(s). Information not available at the time of the initial report (e.g., an end date for the adverse event or laboratory values received after the report) must be documented on a follow-up report. A final report to document resolution of the SAE is required. If this is a multicenter trial, please participating study sites must report SAEs to Syndax as described and within 24 hours of awareness. Participating sites should also report SAEs to the primary study site.

All SAEs will be reported to Syndax Drug Safety as soon as possible, but no later than fifteen calendar days from first knowledge of the event. A copy will be sent to the FDA (if applicable) by the investigative site:

Syndax Pharmaceuticals, Inc.
Drug Safety/ Regulatory
Attn: Miranda Rees
460 Totten Pond Road, Suite 650
Waltham, MA 02451
mrees@syndax.com
Main: 781-419-1400
Direct: 781-419-1417

CC: jhasapidis@syndax.com

8. PHARMACEUTICAL INFORMATION

8.1 Anastrozole

8.1.1 Description

Anastrozole or Arimidex® is a non-steroidal aromatase inhibitor. It is chemically described as 1,3-Benzenediacetonitrile, a, a, a’, a’-tetramethyl 5-(1H-1,2,3-triazol-1-ylmethyl) and the molecular formula $C_{17}H_{19}N_5$, with a molecular weight of 293.4.

8.1.2 Formulation

The tablets are white, biconvex, film-coated containing 1 mg of anastrozole. The tablets are impressed on one side with a logo consisting of a letter “A” (upper case) with an arrowhead attached to the foot of the extended right leg of the “A” and on the reverse with the tablet strength marking “Adx1”.

8.1.3 Availability and Storage

The commercial version of anastrozole will be supplied by AstraZeneca Pharmaceuticals.

These tablets are supplied in bottles of 30 tablets of 1 mg anastrozole tablet (NDC 0310-0201-30). Anastrozole should be stored at controlled room temperature, 20-25^{oC} (68-77^{oF}).

Additional information regarding the formulation as well as storage and handling instructions can be found in the approved package insert.

8.1.4 Drug Accountability

Agent Inventory Records – The investigator, or a responsible party designated by the investigator, must maintain a careful record of the inventory and disposition of anastrozole.

8.1.5 Agent Ordering

Anastrozole may be requested by the Principal Investigator (or their authorized designees). Anastrozole will be supplied by AstraZeneca Pharmaceuticals.

Agent may be requested by contacting Emmanuel Semmes, Investigational Drugs Clinical Specialist Pharmaceutical Services (telephone number 773-834-8749) (fax number 773-834-7461). The contact person at AstraZeneca Pharmaceuticals is Ms. Karen Bartels, ISS Manager (telephone number 302-886-1507).

8.2 Entinostat

8.2.1 Description

Entinostat is a synthetic small molecule bearing the chemical name N-[[4-[[(2-aminophenyl)amino]carbonyl]phenyl]methyl]-, 3- pyridinylmethyl ester and the molecular formula $C_{21}H_{20}N_4O_3$, with a molecular weight of 376.41. SNDX-275 or MS-275 or Entinostat is classified as an anti-neoplastic agent, specifically functioning as an inhibitor of histone deacetylases by promoting hyperacetylation of nucleosomal histones, allowing transcriptional activation of a distinct set of genes that leads to the inhibition of cell proliferation, induction of terminal differentiation, and/or apoptosis.

8.2.2 Formulation

Entinostat is orally bioavailable and is supplied as light brown or yellow coated tablets containing 1 mg and 5 mg of active ingredient, respectively. Each tablet contains mannitol, carboxymethylstarch sodium, hydroxypropyl cellulose, potassium bicarbonate, and magnesium stearate as inactive ingredients. The tablet coat contains hydroxypropyl-methyl cellulose, talc, titanium dioxide, and ferric oxide pigment as a coloring agent.

8.2.3 Availability and Storage

Entinostat is an investigational agent supplied by Syndax Pharmaceuticals, Inc.

Entinostat is supplied as 1 mg (light brown, in bottles of 40), or 5 mg (intense yellow, in bottles of 40) film-coated tablets (round-biconvex). Each tablet also contains mannitol, carboxymethyl starch sodium, hydroxypropyl cellulose, potassium bicarbonate, and magnesium stearate, hydroxypropyl methylcellulose, talc, titanium dioxide, iron oxide pigment (yellow) and iron oxide pigment (red).

Entinostat tablets may be shipped and stored at room temperature (15-30ºC), and should be protected from light. Avoid temperature extremes. The pharmacist will dispense the investigational material to the patient at appropriate intervals throughout the study in childproof containers. Shelf life stability studies of the intact bottles are on-going.

8.2.4 Drug Accountability

Agent Inventory Records – The investigator, or a responsible party designated by the investigator, must maintain a careful record of the inventory and disposition of entinostat.

8.2.5 Agent Ordering

Agent may be requested by contacting Jeannette Hasapidis , VP of Clinical Operations (office phone 781-419-1404, mobile phone 781-724-6909, and email jhasapidis@syndax.com).

8.3 Tamoxifen

8.3.1 Description

Tamoxifen or Nolvadex® is a nonsteroidal antiestrogen. It is the trans-isomer of a triphenylethylene derivative. The chemical name is (Z)2-[4-(1,2-diphenyl-1-butenyl) phenoxy]-N, N-dimethylethanamine 2-hydroxy-1,2,3-propanetricarboxylate (1:1) with a molecular weight of 563.62.

8.3.2 Formulation

The tablets are round, biconvex, uncoated, white tablet identified with NOLVADEX 604 debossed on one side and a cameo debossed on the other side. Each tablet contains 20 mg equivalent of tamoxifen.

8.3.3 Availability and Storage

Tamoxifen will be prescribed by the treating physician and covered by the patients insurance.

These tablets should be stored at controlled room temperature 20-25°C (68-77°F). Dispense in a well closed, light-resistant container.

Additional information regarding the formulation as well as storage and handling instructions can be found in the approved package insert.

9. CORRELATIVE STUDIES

Both blood samples and tissue samples should be collected from both pilot and phase II subjects.

9.1 Tumor Tissue Samples

The submission of pathology materials from **BOTH** the original diagnostic procedure and the definitive surgery or biopsies are required for **ALL** patients.

9.1.1 Collections

In the first cohort:

Tumor tissue samples from the following time points will be obtained:

1. Prior to starting treatment or tissue sample at the original diagnostic procedure. (T0)
2. At the time of definitive surgery (i.e., mastectomy or lumpectomy). A separate tumor biopsy is not needed on the day of surgery; tissue will be taken from the surgical specimen. (T1)

Note: If a subject receives additional preoperative treatment after discontinuing study treatment and prior to surgery, every attempt should be made to collect a tissue sample for the study prior to initiation of the new treatment; in these cases, tissue will still be collected at the time of definitive surgery, as above.

Every effort should be made to provide a core specimen from the surgical sample after treatment. The site pathologist should remove a core samples during the dissection of the surgical specimen (using a 5-mm skin punch biopsy device). The samples should be processed preferably within 1 hour, but may be processed up to 2 hours of removal from the patients. This core specimen should be fixed in formalin and processed separately as per the American Society of Clinical Oncology/College of American Pathologists Guideline Recommendations published in Journal of Clinical Oncology 2010[42].

In the second cohort:

Approximately **six** core biopsy specimens will be obtained with each biopsy. Alternative to core needle biopsy, fine needle aspirate (FNA) is allowed in this cohort of patients. Cytology cell blocks should be made from FNA specimens using routine institutional cytology protocol. Biopsy should be obtained from soft tissue metastasis such as breast, liver, skin, lung, or intra-abdominal implants. Bone biopsy, paracentesis (ascites), and thoracocentesis (pleural effusion) specimens are not adequate.

Tumor tissue samples from the following time points will be obtained:

1. Pretreatment biopsy (biopsy#1) will be performed within 3 weeks after registration. However, the pretreatment biopsy can be omitted in patients who have recent biopsy but have not been started on breast cancer treatment within 12 weeks prior to the registration and there is adequate tumor tissue sample as per section 3.1.9. (T0-Second cohort)
2. Post-treatment biopsy (biopsy#2) will be performed after a minimum of 15 days and a maximum of 29 days of treatment, but preferably close to 29 days. The post-treatment biopsy must take place within 6 days (preferably within 3 days) after the last dose of entinostat. If a patient discontinues the study prior to 15 days, an attempt should be made to collect the biopsy when the decision is made to discontinue and prior to initiation of additional treatment, if possible.

9.1.2 Paraffin Embedded Tissue Submission

All samples must be submitted **within ONE month** after the patient's definitive surgery or biopsy. All materials must be labeled with the institutional surgical pathology number, the study number, the patient's study number, the cohort number (first or second), the treatment group (tamoxifen or anastrozole), and the designated specimen type (T0 or T1). The samples should not contain patients' identifying information.

Please be sure to use a method of shipping that is secure and traceable. Extreme heat precautions should be taken when necessary. Please use papers to pad the specimen shipments. Do not use bubble wrap. Shipping should be arranged only on Monday-Thursday by overnight service to assure receipt. **Do not ship specimens on Friday or Saturday**. Please notify all of the contact persons listed below via email and/or telephone **PRIOR** to shipping.

A copy of the tissue tracking form should be included in the shipping package. Please note that if multiple samples are sent, only one copy of the form needs to be included.

The specimens should be shipped as directed below:

Specimens	***Purpose***	***Shipping Address and Contact Information***
≥3 unstained slides*	ER, PR, HER2, EGFR, CK5/6, Aromatase, histone H3, H4 acetylation, and Ki67 expression by immunohistochemistry **NOTE**: A copy of the pathology report from the treating and/or referring institution and a copy of the operative report should be included	Kimberly C. Tuttle, HT (ASCP) Pathology Biorepository and Research Core University of Maryland Medical Center 22 S. Greene Street, Rm NBW58 Baltimore, MD 21201 Phone: (410) 328-5524 Fax: (410) 328-5508 Email: ktuttle@umm.edu

	in this package.	
Tumor/lymph node block or ≥3 unstained slides∞	Gene expression profiling and Methylation profiling	Saraswati Sukumar, Ph.D. Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins 1650 Orleans Street, Rm 143 CRB Baltimore MD 21231-1000 Phone: (410) 614-2479 Fax: (410) 614-4073 Email: sukumsa@jhmi.edu

*Five-micron (5 µM) thickness mounted on charged slides (do not oven bake slides) from both original diagnostic tumor blocks **AND** residual primary tumor or a positive lymph node blocks should be submitted as described above.

∞The original diagnostic tumor block and at least one representative block of residual primary tumor or a positive lymph node containing the maximum amount of tumor are required. Although it is preferred that tissue blocks are submitted, for institutions that do not allow submission of the tissue blocks, please submit ≥3 unstained sections of 5 µM thickness mounted on charged slides.

9.1.3 Methods

9.1.3.1 Immunohistochemistry

Immunohistochemistry (IHC) will be used to determine the pre-treatment and post-treatment expression of ER, PR, HER2, and Ki67. The level of expression of these proteins will be quantified by ChromaVision ACIS Assisted Quantitative Image Analysis. These studies will be performed by immunohistochemistry technicians in the pathology department at the University of Maryland which is certified by both Clinical Laboratory Improvement Amendments (CLIA) and College of American Pathologists (CAP). The specimens will be de-identified with a unique patient's study number and stored in the tumor bank facility at University of Maryland. Hematoxylin and eosin stained sections will be examined by a pathologist to confirm the tissue source. Five-micron sections of formalin-fixed, paraffin-embedded tissue will be tested for the presence of immunohistochemically detectable ER, PR, HER2, and Ki67 expression. After 60 minutes of heat induced epitope retrieval in citrate buffer, strepavidin biotin detection systems using Ventana reagents and instruments will be used. We will use ER, PR, HER2, Ki67, EGFR, and CK5/6 antibodies that have been validated and are currently in clinical use at University of Maryland pathology department as listed below.

Immunostaining	***Antibody***
ER	SP1 rabbit monoclonal Ventana prediluted anti-ER antibody
PR	1E2 rabbit monoclonal Ventana prediluted anti-PR antibody
HER-2	4B5 rabbit monoclonal Ventana prediluted anti-HER-2/neu antibody
Ki67	30-9 rabbit monoclonal Ventana prediluted anti-Ki67 antibody
EGFR	3C6 mouse monoclonal Ventana prediluted anti-EGFR antibody
CK5/6	D5&16B4 mouse monoclonal Ventana prediluted anti-CK5/6 antibody
Aromatase	Mouse monoclonal antibody (Novartis #677)[43]

Scoring systems:

- ER and PR: Will be scored as the percentage of positive staining tumor nuclei on the examined slide. Five percent immunoreactivity is considered a positive result. This is based on comparison of our results to that of clinically validated ER and PR assays, determined by several large clinical studies.
- HER-2/neu: Will be scored as followed - score 0: no membrane staining is observed. 1+: weak, incomplete staining in any proportion of the invasive tumor cells. 2+: complete membrane staining in less than or equal to 30% of the invasive tumor cells. 3+: strong complete membrane staining in more than 30% of the invasive tumor cells.

- Ki-67: Will be assessed by counting 500 to 1000 cells, and will be reported as percent positive cells based on nuclear expression using ChromaVision ACIS system to quantify.
- EGFR: Will be assessed by counting 500 to 1000 cells, and will be reported as percent positive cells based on membrane expression using ChromaVision ACIS system to quantify.
- CK5/6: Will be assessed by counting 500 to 1000 cells, and will be reported as percent positive cells based on cytoplasmic expression using ChromaVision ACIS system to quantify.
- Aromatase: Will be assessed by counting 500 to 1000 cells, and will be reported as percent positive cells based on cytoplasmic expression using ChromaVision ACIS system to quantify.

Various other cancer specimens (expression in > 30% of the tumor cells) will be used as positive controls. Buffer will replace primary antibody for negative controls. All of the immunohistochemical staining will be quantitatively analyzed by ChromaVision ACIS system for immunohistochemistry image analysis. Quantitation of both staining intensity as well as assessment of numbers of positive stained cells will be determined by the agreement of a consensus of pathologists and researchers. These studies will be carried out under the direction of Dr. Olga Ioffe in the Department of Pathology at University of Maryland.

9.1.3.2 Gene Expression Profiling

Additional biopsy for fresh tissue sample is not required in this study. We will use the cDNA-mediated Annealing, Selection, Extension, and Ligation (DASL) assay from Illumina which has been developed to allow more comprehensive gene expression profiling of degraded RNA extracted from paraffin blocks using bead array format as previously described[37]. Illumina's Bead Array Technology is based on 3-micron silica beads that self assemble in microwells on either of two substrates: fiber optic bundles or planar silica slides. When randomly assembled on one of these two substrates, the beads have a uniform spacing of ~5.7 microns. Each bead is covered with hundreds of thousands of copies of a specific oligonucleotide that act as the capture sequences in one of Illumina's assays. BeadArray technology is utilized in Illumina's iScan System for a broad range of DNA and RNA analysis applications.

All promising findings of gene expression changes will also be validated by the quantitative RT-PCR methods. Change in expression of genes as a result of treatment will be determined by IHC or quantitative RT-PCR. Although IHC can be easily accomplished on a single slide, it may not be sensitive to small changes in expression and expression measurements are semi-quantitative. Quantitative (real-time) RT-PCR can provide better quantification of expression of gene product, and recent studies have shown that this approach can be applied to RNA isolated from paraffin-embedded tissues, when small sequences are amplified. The overall goal of these studies is to identify candidate markers for response and molecular profiles that might be relevant to an understanding of drug mechanisms. Although statistical methods will provide some indication of significance of particular molecular changes, validation will ultimately require additional testing in independent samples.

9.1.3.3 Methylation of Relevant Genes

Both gene expression profiling and Methylation of relevant genes will be performed under the direction of Dr. Saraswati Sukumar, the co-director of breast cancer program at the Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins.

Methylation of relevant genes (ERalpha, APC-1, RARbeta, cyclin D2, Twist, RASSF1A, and HIN-1) will be evaluated by Quantitative Multiplex Methylation-Specific Polymerase Chain Reaction (QM-MSP) as previously described[39,44]. This analysis provides a quantitative assessment of methylation in each gene as well as units of cumulative methylation of the gene panel. The prediction is that methylation in specific genes will reduce as a result of treatment and will result in re-expression of the genes. Re-expression of the silenced genes will be tested by Q-R-PCR in RNA extracted from the same samples.

9.1.3.4 Tumor Histone H3 and H4 Acetylation

Analysis for tumor histone H3 and H4 acetylation will be performed at University of Maryland Pathology Department under direction of Dr. Olga Ioffe. The histone acetylation will be assessed in formalin-fixed, paraffin-embedded section by immunohistochemical staining using the method that was previously described in a phase II trial of vorinostat in recurrent glioblastoma multiforme[45]. However, we will use ChromaVision ACIS system for immunohistochemistry image analysis to quantify the expression of tumor histone H3 and H4 acetylation. The polyclonal antibodies that will be used are listed below:

- Acetylated histone H3, lysine 9 (1:25 dilution; Cell Signaling technology catalog# 9671L)
- Acetylated histone H4, lysine 8 (1:100 dilution; Cell Signaling technology catalog# 2594S)

9.2 Blood Samples

9.2.1 Collections

Blood samples from the following time points will be obtained in all groups of patients including both tamoxifen/anastrozole and the first/second cohort. Blood samples should be collected in the following venipuncture tubes:

1. For pharmacokinetic study (PK): One 6 ml, sodium heparin coated venipuncture tubes (BD vacutainer® green top tube).
2. For Methylation profile (MP): Two 8.5 ml, Red/Gray tiger top serum separator tubes (SST)

For patients in the pilot phase and stage 1 of the phase II portion (the initial 12 patients):

Day of Rx	***Label***	***Time Points***	***Purposes***
1	B0	Prior to starting treatment	PK and MP
1	B1	30 minutes after the first dose of entinostat	PK
15	B2	Day 15 (±1 day) after the first treatment BEFORE entinostat	PK
15	B3	Day 15 (±1 day) after the first treatment 30 minutes AFTER entinostat	PK
Surgery/Biopsy	B4	At the time of definitive surgery (i.e., mastectomy or lumpectomy) or the post-treatment biopsy*	MP

For patients in the stage 2 of the phase II portion (20 patients):

Day of Rx	***Label***	***Time Points***	***Purposes***
1	B5	Prior to starting treatment	PK and MP
15	B6	Day 15 (±1 day) after the first treatment BEFORE entinostat	PK
Surgery/Biopsy	B7	At the time of definitive surgery (i.e., mastectomy or lumpectomy) or the post-treatment biopsy*	MP

*Blood sample at the time of definitive surgery or post-treatment biopsy can be omitted if definitive surgery or post-treatment biopsy occurs within 3 days after day 15 blood collection.

9.2.2 Blood Sample Processing and Storage

All samples should be labeled with the study number, patient's study number, the date and time of collection, the cohort number (first or second), the treatment group (tamoxifen or anastrozole), and designated specimen type in section 8.2.1 (i.e. B0/B1/B2/B3). The samples should not contain patients' identifying information.

9.2.2.1 Pharmacokinetic Samples

Blood will be collected for entinostat pharmacokinetics in a sodium heparin vacutainer. Samples will be obtained at baseline on Day 1 and pre-dose Day 15. In the initial dose finding portion of the study, patients will also have 2 additional samples obtained at 0.5 hr (historical time to C_{max}) on Day 1 and Day 15. Each sample will require 5 mL of patient blood. Samples will be placed **on ice immediately** and centrifuged **within 30 minutes** of collection at 1,000 x g for 10 minutes in a refrigerated centrifuge. Plasma will be stored at –20°C until analysis. Entinostat concentrations will be determined using a validated LC/MS/MS method that will be developed by the Analytical Pharmacology Core Laboratory at the Sidney Kimmel Comprehensive Cancer Center (SKCCC) at Johns Hopkins [46].

9.2.2.2 Methylation Profile

The samples should be processed within **two hours** of drawing the blood.

- Draw blood into two 8.5 ml SST red/grey vacutainer tubes (BD vacutainer® catalog# 367988) and invert gently 5 times to initiate clotting.
- Allow blood to clot for a minimum of 30 minutes, but not longer than 2 hours.
- Centrifuge 15-20 minutes at 3,000 rpm (1,100-2,000 x g) at room temperature or 4°C.

- Aliquot the serum (top layer) into 6 cryovials (~1 ml each aliquots). There should be approximately 3 ml of serum per vacutainer tube. All of the serum should be stored.
- Serum will be stored at -70ºC or colder until batch shipment or analysis.

9.2.3 Blood Sample Submission

Instructions for the collection and shipping of samples are included below. All materials must be labeled with the study number, the patient's study number, patient initials, date of collection, the cohort number (first or second), the treatment group (tamoxifen or anastrozole), and the designated specimen type (i.e. B0, B1, or B2). The samples should not contain patients' identifying information. Please be sure to use a method of shipping that is secure and traceable. Extreme heat precautions should be taken when necessary. Shipping should be arranged only on Monday-Wednesday by overnight service to assure receipt. **Do not ship specimens on Thursday, Friday, Saturday, or Sunday** (specimens drawn on Friday should be sent the following Monday). Please notify the contact person listed below for each type of specimens via email and/or telephone **PRIOR** to shipping.

A copy of the tissue tracking form and/or the blood and serum tracking form should be included in the shipping package. Please note that if multiple samples are sent, only one copy of the form needs to be included.

9.2.3.1 Pharmacokinetic Samples

All specimens should be properly labeled as described above. NOTE: In the event that baseline specimens are available but the patient is not successfully registered to the protocol, do not submit the patient's specimens. Blood samples for pharmacokinetic study should be processed and stored until batch shipment at the end of the study. Samples should be shipped on dry ice and overnighte shipmentsshould occur on **Monday through Wednesday** except when the following day is a holiday. A fax or call should be place to the Analytical Pharmacology Core Laboratory prior to shipment providing the shipment tracking information.

Address shipments and any question regarding specimen processing to:

Analytical Pharmacology Core Laboratory
Attn: Entinostat/Anastrozole Study Samples
1650 Orleans Street CRB1, Rm 184
Baltimore, MD 21231-1000
Phone: 410-955-1129
Phone with voicemail: 410-502-7192
Fax: 410-502-0895

9.2.3.2 Methylation Profile

All specimens should be properly labeled as described above. Blood samples for Methylation profile should be processed and stored until batch shipment at the end of the study. Samples should be shipped in dry ice overnight on **Monday through Wednesday** except when the following day is a holiday. Please contact Dr. Saraswati Sukumar prior to the shipment and the shipment tracking information should be provided.

Address shipments and any question regarding specimen processing to:
Saraswati Sukumar, Ph.D.
Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins
1650 Orleans Street, Rm 143 CRB
Baltimore MD 21231-1000
Phone: (410) 614-2479
Fax: (410) 614-4073
Email: sukumsa@jhmi.edu

9.2.4 Methods

9.2.4.1 Pharmacokinetic Study

Pharmacokinetic analysis will be performed by Michelle A. Rudek, Pharm.D, Ph.D. at the Analytical Pharmacology Core Laboratory at SKCCC at Johns Hopkins. Patients in the pilot phase as well as stage 1 of the phase II

component of the study will have plasma samples collected four times to assess entinostat pharmacokinetics. Steady state trough concentrations ($C_{ss,min}$) will be the pretreatment concentration on Day 15. These pharmacokinetic parameters will be used to explore the relationship between entinostat exposure with pharmacodynamic (PD) endpoints such toxicity and histone acetylation in the tumors. Levels of entinostat will be determined using a validated LC/MS/MS method by Dr. Michelle Rudek at the Analytical Pharmacology Core Laboratory at Johns Hopkins[46]. The pharmacokinetic schedule has been summarized below.

For patients in the pilot phase and stage 1 of the phase II portion (the initial 12 patients):

1. Day 1 Pretreatment B0
2. Day 1 0.5 hour after entinostat (Cmax) B1
3. Day 15 Pretreatment (Css,min) B2
4. Day 15 0.5 hour after entinostat (Cmax) B3

For patients in the stage 2 of the phase II portion (20 patients):

1. Day 1 Pretreatment B5
2. Day 15 Pretreatment (Css,min) B6

9.2.4.2 Methylation Profiling

DNA Methylation studies will be performed at the Breast Cancer Research Laboratories at Johns Hopkins under the direction of Dr. Sara Sukumar. We expect that over 70% of patients will have circulating DNA at baseline. QM-MSP studies will be done on DNA extracted from serum obtained from each subject. Primers and probes for QM-MSP analysis have been designed to specifically amplify hypermethylated promoter sequences of Cyclin D2, RAR-beta, APC-1, Twist, RASSF1A, HIN-1, and ER-alpha. PCR will be performed in separate wells for each primer/probe set. Fluorogenic PCR amplification will be carried out using a 7,900 Sequence detector (Perkin-Elmer Applied Biosystems) as previously described.

10. STUDY CALENDAR AND VISIT SCHEDULE

Baseline evaluations are to be conducted within 3 weeks prior to start of protocol therapy.

For the first cohort:

Procedure	Pre-study	Week 1		Week 2		Week 3		Week 4		Week 5		Surgery [a]	Off treatment
		D1	D4-7	D8	D9-14	D15	D16-21	D22	D23-28	D29	D30-32		
		Minimum treatment					Optional						
Treatment:													
Entinostat		A		A		A		A		A			
Anastrozole/Tamoxifen			B	B	B	B	B	B	B	B	B		
Clinical assessment:													
Informed consent	X												
Demographics	X												
Medical history	X												
Current medications	X	X		X		X		X		X		X	X
Physical exam	X	X		X		X		X		X		X	X
Vital signs	X	X		X		X		X		X		X	X
Height	X												
Weight	X	X		X		X		X		X		X	X
Performance status	X	X		X		X		X		X		X	X
Adverse event evaluation		X		X		X		X		X		X	X
Tumor measurement [b]	X	X		X		X		X		X		X	
Laboratory test:													
CBC w/diff, plts	X	X		X		X		X		X		X	X
Chemistries [c]	X	X		X		X		X		X		X	X
PT/INR, PTT	X											X	
Electrocardiogram	X												
Pregnancy test [g]	X												
Correlative studies (Research procedures):													
Pharmacokinetic study													
• Pilot phase, stage 1		X[d]				X[d]							
• Stage 2		X[e]				X[e]							
Methylation profile		X[f]										X[f]	
Tumor sample	X											X	

A: Entinostat: as per appropriate dose level PO weekly on day 1, 8, 15, 22 and 29 ± 2 days. Preferably 22 days.
B: Anastrozole for postmenopausal women: 1 mg PO daily starts on day 4 and continue until the day of definitive surgery (lumpectomy or mastectomy).
Tamoxifen for premenopausal women: 20 mg po daily starts on day 4 and continue until the day of definitive surgery (lumpectomy or mastectomy).

a: Within 6 days (preferably within 3 days) after the last dose of entinostat.
b: Tumor measurement: clinical tumor size, character, mobility, and location of the breast mass.
c: Chemistries: sodium, potassium, chloride, bicarbonate, BUN, creatinine, glucose, total bilirubin, calcium, total protein, albumin, AST (SGOT), ALT (SGPT) , alkaline phosphatase, LDH, uric acid, phosphorus, and magnesium
d: Day 1: Prior to starting treatment
30 minutes after the first dose of entinostat
Day 15 (±1 day): BEFORE entinostat
30 minutes AFTER entinostat
e: Day 1: Prior to starting treatment
Day 15 (±1 day): BEFORE entinostat
f: Day 1: Prior to starting treatment
The day of definitive surgery (lumpectomy or mastectomy)
g: Serum or urine pregnancy test should be performed within 7 days prior to registration in women of childbearing potential

NOTE: Visit Windows ± 2 days

For the second cohort:

Procedure	Pre-study	Week 1		Week 2		Week 3		Week 4		Week 5		Continue until disease progression [h]	Off treatment
		D1	**D4**-7	D8	D9-14	D15	D16-21	D22	D23-28	D29	D30-32		
Treatment:													
Entinostat		A		A		A		A		A			
Anastrozole/Tamoxifen			B	B	B	B	B	B	B	B	B		
Clinical assessment:													
Informed consent	X												
Demographics	X												
Medical history	X												
Current medications	X	X		X		X		X		X			X
Physical exam	X	X		X		X		X		X			X
Vital signs	X	X		X		X		X		X			X
Height	X												
Weight	X	X		X		X		X		X			X
Performance status	X	X		X		X		X		X			X
Adverse event evaluation		X		X		X		X		X			X
Tumor measurement for palpable or visible tumors [b]	X	X		X		X		X		X			
CBC w/diff, plts	X	X		X		X		X		X			X
Chemistries [c]	X	X		X		X		X		X			X
PT/INR, PTT	X												
Electrocardiogram	X												
Pregnancy test [g]	X												
Correlative studies (Research procedures):													
Pharmacokinetic study													
• Pilot phase, stage 1		X[d]				X[d]							
• Stage 2		X[e]				X[e]							
Methylation profile		X[f]							X[f]				
Biopsies	X								X[a]				

A: Entinostat: as per appropriate dose level PO weekly until disease progression.
B: Anastrozole: 1 mg PO daily starts on day 4 and continue until disease progression.
Tamoxifen for premenopausal women: 20 mg po daily starts on day 4 and continue until disease progression.

a: The post-treatment biopsy (biopsy#2) will be performed after a minimum of 15 days and a maximum of 29 days of treatment, but preferably close to 29 days. The post-treatment biopsy must take place within 6 days (preferably within 3 days) after the last dose of entinostat.
b: Tumor measurement for palpable or visible tumor: clinical tumor size, character, mobility, and location of the breast mass. Restaging scans will be performed every 6 weeks while on treatment.
c: Chemistries: sodium, potassium, chloride, bicarbonate, BUN, creatinine, glucose, total bilirubin, calcium, total protein, albumin, AST (SGOT), ALT (SGPT) , alkaline phosphatase, LDH, uric acid, phosphorus, and magnesium
d: Day 1: Prior to starting treatment
30 minutes after the first dose of entinostat
Day 15 (±1 day): BEFORE entinostat
30 minutes AFTER entinostat
e: Day 1: Prior to starting treatment
Day 15 (±1 day): BEFORE entinostat
f: Day 1: Prior to starting treatment
The day of post-treatment biopsy
g: Serum or urine pregnancy test should be performed within 7 days prior to registration in women of childbearing potential

h: After the post-treatment biopsy, treatment will be continued until disease progression. Restaging scans will be performed every 6 weeks while on treatment. Follow up visits after the post-treatment biopsy will be every 4 weeks or at physician's discretion.

NOTE: Visit Windows ± 2 days

VISIT SCHEDULE

The study consists of a maximum 21-day (3 weeks) screening phase prior to registration, start of study drug administration within 3 business days of registration. The follow-up phase will last 30 days after the last dose of study medication or until death, whichever occurs first.

10.1 Screening

Each potential patient will be screened before the start of the study to determine her eligibility for participation. These tests are to be conducted within 3 weeks prior to study registration. The following procedures will be performed:

- Informed consent (signed and dated by patient)
- Medical history and demographics
- Inclusion/Exclusion Criteria
- Concomitant medications and treatments will be recorded from 1 month prior to the start of study treatment.
- Physical examination including examination of major body systems, height, body weight, ECOG performance status, and vital signs (temperature, blood pressure, heart rate, respiratory rate)
- Clinical tumor assessment by a breast examination including clinical tumor size, character, mobility, and location of the breast mass. The patient will be examined by one of the treating investigators or his/her designee at each specified time point and after the final dose of treatment.
- Laboratory studies: CBC with diff; chemistries including sodium, potassium, chloride, bicarbonate (HCO_3^- or CO_2), BUN, creatinine, glucose, total bilirubin, calcium, total protein, albumin, AST (SGOT), ALT (SGPT) , alkaline phosphatase, LDH, uric acid, phosphorus, and magnesium
- Coagulation study: PT with INR and PTT
- 12-lead electrocardiogram (ECG)
- Collection of tumor sample: Parrafin blocks and/or unstained slides

10.2 Registration

Registration will take place once the consented patient has completed the necessary screening procedures and is deemed eligible for study entry by the investigator or designee. Each registered patient will be assigned a unique identification number. Treatment should begin within 3 business days after registration. Please see registration process in more detail in section 4.2.

10.3 Study Days

The period called "Study Days" begins when the patient receives the initial dose of entinostat (Week 1 Day 1). Assessments are scheduled once a week with a visit window of ± 2 day until the time of definitive surgery (lumpectomy or mastectomy) or the post-treatment biopsy. However, this may be repeated more often, as clinically indicated. A maximum 2-week delay for resolution of study drug-related toxicities is allowed. A patient should be discontinued from the study if a greater than 2-week delay occurs.

Week 1 Day 1

On Week 1 Day 1 only, the required assessment may be omitted if an acceptable screening assessment was performed within 3 days prior to Week 1 Day 1. Week 1 Day 1 is dictated by the day the patient receives the first dose of entinostat.

- Assessment of Concomitant Treatments including medication use

- Physical examination including examination of major body systems, height, body weight, ECOG performance status, and vital signs (temperature, blood pressure, heart rate, respiratory rate)
- Clinical tumor assessment by a breast examination including clinical tumor size, character, mobility, and location of the breast mass.
- Laboratory studies: CBC with diff; chemistries including sodium, potassium, chloride, bicarbonate, BUN, creatinine, glucose, total bilirubin, calcium, total protein, albumin, AST (SGOT), ALT (SGPT) , alkaline phosphatase, LDH, uric acid, phosphorus, and magnesium
- Study Drug Administration, and Drug Accountability
- Methylation profile study
- Pharmacokinetic study:
    - Pilot phase and stage 1 patients
        - Prior to the first dose of entinostat
        - 30 minutes after the first dose of entinostat
    - Stage 2 patients
        - Prior to the first dose of entinostat
- Administration of entinostat as per appropriate dose level orally

Week 3 Day 15

Day 15 after the first dose of entinostat.

- Assessment of Concomitant Treatments including medication use
- Physical examination including examination of major body systems, height, body weight, ECOG performance status, and vital signs (temperature, blood pressure, heart rate, respiratory rate)
- Clinical tumor assessment by a breast examination including clinical tumor size, character, mobility, and location of the breast mass.
- Laboratory studies: CBC with diff; chemistries including sodium, potassium, chloride, bicarbonate, BUN, creatinine, glucose, total bilirubin, calcium, total protein, albumin, AST (SGOT), ALT (SGPT) , alkaline phosphatase, LDH, uric acid, phosphorus, and magnesium
- Study Drug Administration, and Drug Accountability
- Pharmacokinetic study:
    - Pilot phase and stage 1 patients
        - Prior to the day 15 dose of entinostat
        - 30 minutes after the day 15 dose of entinostat
    - Stage 2 patients
        - Prior to the day 15 dose of entinostat
- Administration of entinostat as per appropriate dose level orally

Day 8, 22, and 29 after the first dose of entinostat

- Assessment of Concomitant Treatments including medication use
- Physical examination including examination of major body systems, height, body weight, ECOG performance status, and vital signs (temperature, blood pressure, heart rate, respiratory rate)
- Clinical tumor assessment by a breast examination including clinical tumor size, character, mobility, and location of the breast mass.
- Laboratory studies: CBC with diff; chemistries including sodium, potassium, chloride, bicarbonate, BUN, creatinine, glucose, total bilirubin, calcium, total protein, albumin, AST (SGOT), ALT (SGPT) , alkaline phosphatase, LDH, uric acid, phosphorus, and magnesium
- Study Drug Administration, and Drug Accountability
- Administration of entinostat as per appropriate dose level orally

For the first cohort:

Surgery day

The definitive surgery (i.e. lumpectomy or mastectomy) should be performed within 6 days (preferably within 3 days) after the last dose of entinostat.

- Assessment of Concomitant Treatments including medication use
- Physical examination including examination of major body systems, height, body weight, ECOG performance status, and vital signs (temperature, blood pressure, heart rate, respiratory rate)

- Clinical tumor assessment by a breast examination including clinical tumor size, character, mobility, and location of the breast mass.
- Laboratory studies: CBC with diff; chemistries including sodium, potassium, chloride, bicarbonate, BUN, creatinine, glucose, total bilirubin, calcium, total protein, albumin, AST (SGOT), ALT (SGPT) , alkaline phosphatase, LDH, uric acid, phosphorus, and magnesium
- Coagulation study: PT with INR and PTT
- Collection of tumor sample: Parrafin blocks and/or unstained slides
- Methylation profile study

For the second cohort:

The post-treatment biopsy (biopsy#2) day

The post-treatment biopsy (biopsy#2) will be performed after a minimum of 15 days and a maximum of 29 days of treatment, but preferably close to 29 days. The post-treatment biopsy must take place within 6 days (preferably within 3 days) after the last dose of entinostat.

- Assessment of Concomitant Treatments including medication use
- Physical examination including examination of major body systems, height, body weight, ECOG performance status, and vital signs (temperature, blood pressure, heart rate, respiratory rate)
- Clinical tumor assessment by a breast examination including clinical tumor size, character, mobility, and location of the breast mass.
- Laboratory studies: CBC with diff; chemistries including sodium, potassium, chloride, bicarbonate, BUN, creatinine, glucose, total bilirubin, calcium, total protein, albumin, AST (SGOT), ALT (SGPT) , alkaline phosphatase, LDH, uric acid, phosphorus, and magnesium
- Coagulation study: PT with INR and PTT
- Collection of tumor sample: Parrafin blocks and/or unstained slides
- Methylation profile study

After the post-treatment biopsy, treatment will be continued until disease progression.

- Restaging scans will be performed every 6 weeks while on treatment.
- Follow up visits after the post-treatment biopsy will be every 4 weeks or at physician's discretion.

10.4 End of Study (Treatment) / Withdrawal

All patients must continue to be observed for 30 days after the last dose of study medication or until death; whichever occurs first. At the end of the study treatment, the following procedures should be performed within 4 weeks.

- Assessment of Concomitant Treatments including medication use
- Physical examination including examination of major body systems, height, body weight, ECOG performance status, and vital signs (temperature, blood pressure, heart rate, respiratory rate)
- Laboratory studies: CBC with diff; chemistries including sodium, potassium, chloride, bicarbonate, BUN, creatinine, glucose, total bilirubin, calcium, total protein, albumin, AST (SGOT), ALT (SGPT) , alkaline phosphatase, LDH, uric acid, phosphorus, and magnesium

11. MEASUREMENT OF EFFECT

For the purposes of this study, patients should be reevaluated weekly until the definitive surgery.

11.1 Pathologic Response Determination

Pathological response in the breast, and when appropriate, in the axilla will be assessed.

11.1.1 Primary Endpoint Definitions for Ki67 and ER Expression

For the primary endpoints, pathological response for Ki67 and ER expression will be assessed in the primary breast tumor. As per the updated guideline from the American Society of Clinical Oncology (ASCO) and Colleges of

American Pathologists (CAP) published in June 2010, the recommended ER positivity is at least 1% positive tumor nuclei in the tumor samples.

- Ki67: ≥ 50% reduction in Ki67 after treatment with entinostat and anastrozole.
- ER: ≥ 1% staining of ER is considered a positive result.

11.1.2 Pathologic Response

- Pathologic Complete Response (pCR): No viable invasive cancer in pathologic specimen, as determined by histological examination. We will report whether in situ disease only was present in the specimen versus no disease. We will also report for each woman whether she had a pCR in the breast only, in the lymph nodes only, or in both.
- Nodal Disease: We will report the number of women who had positive lymph node prior to starting study drugs, or at the time of surgery: 0, 1-3, 4-10, or >10 lymph nodes.

11.2 Clinical Response Determination

Clinical response in the breast will be evaluated, and when appropriate, in the axilla, infraclavicular, supraclavicular regions, and skin. At the time of study enrollment, patients will have a breast examination. Physical examination findings will be documented including clinical tumor size, character, mobility, and location of the breast mass. In addition, T4 features must be described. The patient will be examined by one of the treating investigators or his/her designee at each specified time point and at the time of the surgery.

11.2.1 Endpoint Definitions for Clinical Response (UICC criteria)

- Complete response (cCR) in the breast on physical exam will be defined as the absence of any palpable abnormality: i.e., no skin or breast thickening, mass, or associated skin or nipple changes. CR will be recorded separately for the breast and axilla.
- Partial response (cPR) in the breast will be defined as a 50% or greater decrease in the product of biperpendicular diameters as measured with a ruler, compared with the prechemotherapy measurement.
- Stable disease (cSD) in the breast will be a defined as palpable disease which does not fit the definition of PR or PD.
- Progressive disease (cPD) will be defined as an increase in the product of biperpendicular diameters of 25% or greater compared to the original measurement.
- Inevaluable disease (cID) is defined as breast cancer that is not palpable in two dimensions.

12. STATISTICAL CONSIDERATIONS

12.1 Study Design/Endpoints

This is a Pilot and Phase II, single-arm, open-labeled, multi-center study of entinostat in combination with anastrozole/tamoxifen in women with triple negative breast cancer. The main objective of this study is to assess the safety and efficacy of the combination of entinostat and anastrozole/tamoxifen. The primary endpoints, that we will use to evaluate the efficacy of the entinostat and anastrozole/tamoxifen, are the reduction in proliferative index (Ki67) and upregulation of ER after treatment with entinostat and anastrozole/tamoxifen therapy. We define patient's response as at least 50% reduction in the Ki67 index AND greater than 1% ER-positivity OR pathologic complete response in the post-treatment surgical specimens. For the evaluation of these biological endpoints, the analysis will include ALL of the patients in both cohorts (the first cohort-operable stage I-II and the second cohort-unresectable locally advance/metastatic) as well as both anastrozole and tamoxifen groups.

In the pilot phase, the classic cohort-of-3-study design will be used. The primary endpoint of this part of the study is to determine the recommended dose for the phase II part as well as to evaluate the safety of both combinations. The dose levels are defined below for both pre- and postmenopausal groups. For safety, each combination (entinostat and anastrozole as well as entinostat and tamoxifen) will be evaluated separately. For both groups (anastrozole and tamoxifen), the first cohort of 3 patients will be treated starting at dose level 1 (d_1). If ≤ 1 patient experiences DLT, an additional 3 patients will be treated at this dose level (d_1). If ≥ 2 patients in the initial cohort of 6 patients suffer DLT, then up to 6 additional patients will be enrolled at the dose level -1. The phase II part will continue with the highest dose level with < 2 out of 6 patients experiencing a DLT. Therefore, we will need 6-12 patients for each combination

in this part of the study. The 6 patients from the pilot phase of each combination that are treated with the recommended dose level for the phase II will be included in the primary endpoint analysis.

The dose modification schemas are as follow for both postmenopausal and premenopausal women:

Dose Modification Schema for Postmenopausal Women		
Dose Level	Dose	
	Entinostat (mg)	Anastrozole (mg) daily
-1	3 mg weekly (on day 1, 8, 15, 22, and 29)	1
1	5 mg weekly (on day 1, 8, 15, 22, and 29)	1

or

Dose Modification Schema for Premenopausal Women		
Dose Level	Dose	
	Entinostat (mg)	Tamoxifen (mg) daily
-1	3 mg weekly (on day 1, 8, 15, 22, and 29)	20
1	5 mg weekly (on day 1, 8, 15, 22, and 29)	20

In the phase II part, the Simon's two-stage design will be used for the study to ensure that the number of patients exposed to this new treatment is minimized. The analysis for this part of the study will include ALL of the patients in both cohorts (the first cohort-operable stage I-II and the second cohort-unresectable locally advance/metastatic) as well as both anastrozole and tamoxifen groups. The total sample size for the phase II part of the study is 32 with 12 and 20 patients in the first and second stage respectively. The 6 patients in the pilot phase of each combination that are treated with the recommended dose level for phase II will be included in the first stage of the phase II part of the study. The study will be terminated, if there is no response among the first 12 patients. If ≥ 1 response in these 12 patients is observed, then 20 more patients will be accrued. The combination will be considered promising, if ≥ 4 out of 32 patients have a response. This design yields 87% power. The probability of early stopping and declaring that this combination has no sufficient activity is 0.54, if the true success rate is 5% and 0.07 if the true response rate is 20%. If the true proportion of patients with Ki67 reduction combined with ER up-regulation is 0.20, the probability of concluding that the drug has sufficient activity is 0.87 and 0.07 if the true proportion is 0.05. To allow about 10% inevaluability, the phase II trial will accrue 35 eligible patients. Statistical analysis will be done using S-plus (TIBCO, version 8.0). All descriptive statistics will be reported. The multivariate analysis of variance or its non-parametric alternative will be applied to compare biomarkers' expression levels. The 90% confidence intervals will be constructed for the observed proportions. All statistical tests will be two-sided, and done at the 0.05 level of significance.

12.2 Sample Size/Accrual Rate

For the pilot phase, the study will require 12-24 patients to complete, allowing for 3-6 patients per dose level and 2 predefined dose levels for 2 combination treatments (entinostat and anastrozole as well as entinostat and tamoxifen). In the phase II period, the study will require 12-35 patients to complete. However, we will include the 12 patients in the pilot phase that are treated with the recommended dose level of each combination for phase II in the primary endpoint analysis. Therefore, the total sample size for this study is **12-41 patients**. At an anticipated accrual rate of 15-20 patients/year, we anticipate that the phase II part will need to accrue patients for anywhere from 7 to 33 months.

NOTE: The maximum total sample size will most likely remain 41 patients. The only situation that this trial will require additional 6 patients is that there are ≥ 2 patients in the first cohort of 6 patients suffer DLT in dose level 1 in both combination treatments. As of November 2011, there were 3 patients enrolled in the pilot phase of this study. There is no DLT observed in this first 3 patients treated with entinostat and anastrozole. Furthermore, a phase II study of

entinostat and AIs including anastrozole was recently completed which demonstrated that this combination is safe and tolerable with the majority of AEs being only grade 1 or 2[47]. Taken this together, it is highly unlikely that there will be ≥ 2 patients in the first cohort of 6 patients suffer DLT in dose level 1 in entinostat and anastrozole group.

12.3 Analysis of Primary Endpoints

Pilot Phase

To address the safety of both regimens, a maximum width 90% confidence interval for any grade 3 or higher toxicity will be approximately 30%. For 35 patients in this study, if the true unknown probability of a rare toxicity is 10%, the probability of observing 1 or more toxicities is 97%, and if the true toxicity rate is 5% then the probability of observing one or more rare toxicities is 83%.

In the first cohort, because the study participants are awaiting a curative surgery, greater than 10% delay in surgery (more than 2 weeks) is unacceptable. Thus, if ≥ 2 delays are observed in the first ten participants, the dose will be de-escalated to level -1. If ≥ 2 additional delays are observed after dose modification, consideration will be made to further change in dose or schedule of study medication.

Phase II

Regarding the ER and Ki67 analysis, we will also include the evaluable patients from the pilot phase for this analysis. For the evaluation of these biological endpoints, the analysis will include ALL of the patients in both cohorts (the first cohort-operable stage I-II and the second cohort-unresectable locally advance/metastatic) as well as both anastrozole and tamoxifen groups. When the sample size is 41, a single-group repeated measures analysis of variance with a 0.05 significance level will have adequate above 80% power to detect a difference in average expression levels of ER and Ki67 between the baseline and after treatment values when an effect size is 0.1. With effect size of 0.125 and higher, power increases to 90%.

The 95% confidence intervals will be constructed for the observed proportions. Exploratory data analysis and appropriate graphs will be used to decide whether data transformation (e.g. log or square-root) is necessary to assure an approximate normality. All descriptive statistics will be reported for ER and Ki67 expression. We will use general linear model approach and/or its non parametric alternative, the Wilcoxon test, to assess whether there is any evidence of changes due to treatment. All statistical tests will be two-sided done at the 0.05 level of significance. Statistical analysis will be done using S-plus (TIBCO version 8.0).

12.4 Analysis of Secondary Endpoints

Baseline and after treatment PR, HER2, EGFR, CK5/6, aromastase, tissue histone H3 and H4 acetylation will be treated as continuous variables. We may also use the multivariate analysis of variance to compare correlated biomarkers' expression. Correlation between biomarkers will be estimated and tested. The repeated measures model approach will be also used. Categorical outcome data (e.g., number of proteins expressed) will be recorded, proportions will be estimated and compared using the Fisher's exact test.

Clinical and pathological response rate with the corresponding 90% or 95% CI will be estimated for all eligible patients. The possible association between response rate and dose level will also be assessed.

12.5 Analysis of Exploratory Endpoints

12.5.1 To correlate the levels of histone H3 and H4 acetylation with the changes in Ki67 and ER, the appropriate to the data coefficient of correlation will be calculated. With 35 eligible subjects on the study we will have above 80% power to test the null hypothesis of some positive association against the research hypothesis of strong positive correlation, for instance, rho of 0.30 versus rho of at least 0.70.

12.5.2 To evaluate gene methylation profile, the outcomes of interest are gene methylation silencing and expression of candidate genes in tissue and in circulating DNA (ER-alpha, ER-beta, RAR-beta, cyclin D2, Twist, RASSF1A, and HIN-1) which are categorical and continuous respectively. Measurements of the baseline and change in gene methylation profile will be measured. The methylation silencing data for each gene will be tabulated and proportions will be estimated and compared using Fisher's exact test. For gene expression, exploratory plots, means and 95% confidence intervals will be calculated. We will use t-tests and signed-rank tests to determine whether or not the data shows evidence of change from baseline.

12.5.3 For the pharmacokinetic study, summary statistics (mean, standard deviation, median) and graphical techniques (boxplots, scatter-plots, histograms) will be used to characterize the behavior of pharmacodynamic (PD) and pharmacokinetic (PK) measurements. Regarding the exploratory endpoints of correlation between entinostat exposure and PD endpoints (i.e. acetylation of histone H3 and H4), A Mann-Whitney U test and logistic regression will be used to evaluate the association between entinostat exposure and PD endpoints as well as the change in Ki67 and ER expression as a categorical variable (i.e. response or no response). The entinostat exposure parameters explored will be plasma concentrations.

12.5.4 For the global gene expression analysis, we will have tissue samples from between 12-41 women enrolled in this study. Each of the tissue samples will be preprocessed using DASL assay from Illumina as described above. The resulting CEL files will be imported and stored on the Gene Traffic database in the Biostatistics Core (Core 4). Gene Traffic software will be used for quality control and identification of any experimental artifacts, including spatial patterns on chips. GCRMA will be applied to the CEL file results using the "Affy" library within the Bioconductor suite using R statistical software.[48] The GCRMA method summarizes probe set information to obtain one gene expression measurement per probe set on the array. Significance analysis of microarrays (SAM) will then be applied to the gene expression data to find genes which are differentially expressed between before and after treatment tissues.[49] The SAM statistic is similar to performing a two sample t-test for each gene under consideration, but it includes an adjustment to stabilize the coefficient of variation of the SAM statistic as a function of the standard deviation of the gene expression (across genes). As part of the SAM approach, a permutation method will be used to determine a cutoff for significance. Specifically, the permutation method takes the original data (the G x 6 matrix of expressions, where G is the number of genes) and permutes the data within each row of the expression matrix. The SAM analysis is then repeated on the permuted data. The distribution of the SAM statistics from the permuted analysis is called the "null" distribution, meaning that it is the distribution of SAM statistics we would expect to see if there were no relationship between gene expression and treatment (or, treatment modality). We then compare the distribution of the SAM statistics based on original data to the null distribution and choose a cutoff such that our false-discovery rate is 5%. That is, we choose a cutoff so that we would only expect that about 5% of the genes we claim to be differentially expressed would not be.

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APPENDIX A: ECOG Performance Status Scale

Grade	***Descriptions***
0	Normal activity. Fully active, able to carry on all pre-disease performance without restriction.
1	Symptoms, but ambulatory. 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).
2	In bed <50% of the time. Ambulatory and capable of all self-care, but unable to carry out any work activities. Up and about more than 50% of waking hours.
3	In bed >50% of the time. Capable of only limited self-care, confined to bed or chair more than 50% of waking hours.
4	100% bedridden. Completely disabled. Cannot carry on any self-care. Totally confined to bed or chair.
5	Dead.

APPENDIX B: American Joint Committee on Cancer Staging (AJCC)

T – Primary Tumor	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ
Tis (DCIS)	Ductal carcinoma in situ
Tis (LCIS)	Lobular carcinoma in situ
Tis (Paget)	Paget's disease of the nipple with no tumor Note: Paget's disease associated with a tumor is classified according to the size of the tumor.
T1	Tumor ≤ 2 cm in greatest dimension
T1mic	Microinvasion ≤ 0.1 cm in greatest dimension
T1a	Tumor > 0.1 cm but not > 0.5 cm in greatest dimension
T1b	Tumor > 0.5 cm but not > 1 cm in greatest dimension
T1c	Tumor > 1 cm but not > 2 cm in greatest dimension
T2	Tumor > 2 cm but not > 5 cm in greatest dimension
T3	Tumor > 5 cm in greatest dimension
T4	Tumor of any size with direct extension to (a) chest wall or (b) skin, only as described below
T4a	Extension to chest wall, not including pectoralis muscle
T4b	Edema (including peau d'orange" or ulceration of the skin of the breast, or satellite skin nodules confined to the same breast
T4c	Both T4a and T4b
T4d	Inflammatory carcinoma

N – Regional lymph nodes	
NX	Regional lymph nodes cannot be assessed (e.g., previously removed)
N0	No regional lymph node metastasis
N1	Metastasis in movable ipsilateral axillary lymph node(s)
N2	Metastases in ipsilateral axillary lymph nodes fixed or matted, or in clinically apparent ipsilateral internal mammary nodes in the absence of clinically evident axillary lymph node metastasis
N2a	Metastasis in ipsilateral axillary lumph nodes fixed to one another (matted) or to other structures
N2b	Metastasis only in clinically apparent ipsilateral internal mammary nodes and in the absence of clinically evident axillary lymph node metastasis
N3	Metastasis in ipsilateral infraclavicular lymph node(s), or in clinically apparent ipsilateral internal mammary lymph node(s) and in the presence of clinically evident axillary lymph node metastasis; or metastasis in ipsilateral supraclavicular lymph node(s) with or without axillary or internal mammary lymph node involvement
N3a	Metastasis in ipsilateral infraclavicular lymph node(s) and axillary lymph node(s)
N3b	Metastasis in ipsilateral internal mammary lymph node(s) and axillary lymph node(s)
N3c	Metastasis in ipsilateral supraclavicular lymph node(s)

APPENDIX B (Continued)

pN – Regional lymph nodes	
pNX	Regional lymph nodes cannot be assessed (e.g., previously removed or not removed for pathologic study)
pN0	No regional lymph node metastasis histologically, no additional examination for isolated tumor cells
pN0(i-)	No regional lymph node metastasis histologically, negative IHC
pN0(i+)	No regional lymph node metastasis histologically, positive IHC, no IHC cluster > 0.2 mm
pN0(mol-)	No regional lymph node metastasis histologically, negative molecular findings (RT-PCR)
pN0(mol+)	No regional lymph node metastasis histologically, positive molecular findings (RT-PCR)
pN1mi	Micrometastasis (> 0.2 mm, none > 2.0 mm)
pN1	Mestastasis in one to three axillary lymph nodes and/or in internal mammary nodes with microscopic disease detected by sentinel lymph node dissection but not clinically apparent
pN1a	Mestastasis in one to three axillary lymph nodes
pN1b	Metastasis in internal mammary nodes with microscopic disease detected by sentinel lymph node dissection but not clinically apparent
pN1c	Mestastasis in one to three axillary lymph nodes and in internal mammary lymph nodes with microscopic disease detected by sentinel lymph node dissection but not clinically apparent
pN2	Metastasis in four to nine axillary lymph nodes, or in clinically apparent internal mammary lymph nodes in the absence of axillary lymph node metastasis
pN2a	Metastasis in four to nine axillary lymph nodes (at least one tumor deposit > 2.0 mm)
pN2b	Metastasis in clinically apparent internal mammary lymph nodes in the absence of axillary lymph node metastasis
pN3	Metastasis in 10 or more axillary lymph nodes, or in infraclavicular lymph nodes, or in clinically apparent ipsilateral internal mammary lymph nodes in the presence of one or more positive axillary lymph nodes; or in more than three axillary lymph nodes with clinically negative microscopic metastasis in internal mammary lymph nodes; or in ipsilateral supraclavicular lymph nodes
pN3a	Metastasis in 10 or more axillary lymph nodes (at least one tumor deposit > 2.0 mm), or metastasis to the infraclavicular lymph nodes
pN3b	Metastasis in clinically apparent ipsilateral internal mammary lymph nodes in the presence of one or more positive axillary lymph nodes; or in more than three axillary lymph nodes and in internal mammary lymph nodes with microscopic disease detected by sentinel lymph node dissection but not clinically apparent
pN3c	Metastasis in ipsilateral supraclavicular lymph nodes

M – Distant metastasis	
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

APPENDIX C: Study Drug Diary

Study Drug Diary: Entinostat

Please complete this diary every time you take your entinostat. You should also use this diary to record any side effects that you experience and medications that you take other than your study drugs (entinostat and anastrozole or tamoxifen). Please be sure to bring this diary with you to your next doctor's visit.

You will take entinostat once a week until you have your surgery (i.e. lumpectomy or mastectomy). Entinostat is a tablet either in light brown (1 mg) or intense yellow (5 mg). Entinostat should be taken with a meal after the first two or three bites.

You are currently taking:

☐ 3 mg of entinostat weekly

☐ 5 mg of entinostat weekly

Please contact your study doctor or nurse with any new complaints or symptoms that you have after starting to take entinostat. If you have severe symptoms, your dose of entinostat may be changed or you may be told to stop taking entinostat. Please do not make any changes in your entinostat dose without speaking with your study doctor or nurse.

Dose #	Due Date	Due Time	Date Taken	Time Taken	# of Pills Taken	**Comments** (Side effects, complaints, other medications)
1						
2						
3						
4						
5						

Note: For any time that you did not take your entinostat (Example: You forgot to take it), write the reason that you did not take it in the "Comments" field.

Completed by: _____ Date: _____
Signature of Patient

Reviewed by: _____ Date: _____
Signature of Study Staff

APPENDIX C (Continued)

Study Drug Diary: Anastrozole or Tamoxifen

Please complete this diary every time you take your anastrozole or tamoxifen. You should also use this diary to record any side effects that you experience and medications that you take other than your entinostat and anastrozole/tamoxifen. Please be sure to bring this diary with you to your next doctor's visit.

You will take one tablet of anastrozole (1 mg) or tamoxifen (20 mg) by mouth once a day started on day 4 after the first dose of entinostat. Anastrozole and tamoxifen are white tablets that can be taken with or without food. You should take anastrozole or tamoxifen at around the same time every day.

Please contact your study doctor or nurse with any new complaints or symptoms that you have after starting to take anastrozole or tamoxifen. If you have severe symptoms, your dose of anastrozole or tamoxifen may be changed or you may be told to stop taking anastrozole or tamoxifen. Please do not make any changes in your anastrozole or tamoxifen dose without speaking with your study doctor or nurse.

Dose #	Due Date	Due Time	Date Taken	Time Taken	# of Pills Taken	Comments (Side effects, complaints, other medications)
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						
12						
13						
14						
15						
16						
17						
18						
19						
20						

APPENDIX C (Continued)

Study Drug Diary: Anastrozole or Tamoxifen

Dose #	Due Date	Due Time	Date Taken	Time Taken	# of Pills Taken	**Comments** (Side effects, complaints, other medications)
21						
22						
23						
24						
25						
26						
27						
28						
29						
30						
31						
32						

Note: For any time that you did not take your anastrozole or tamoxifen (Example: You forgot to take it), write the reason that you did not take it in the "Comments" field.

Completed by: _____ Date: _____
Signature of Patient

Reviewed by: _____ Date: _____
Signature of Study Staff