Presurgical treatment with letrozole in patients with early-stage breast cancer

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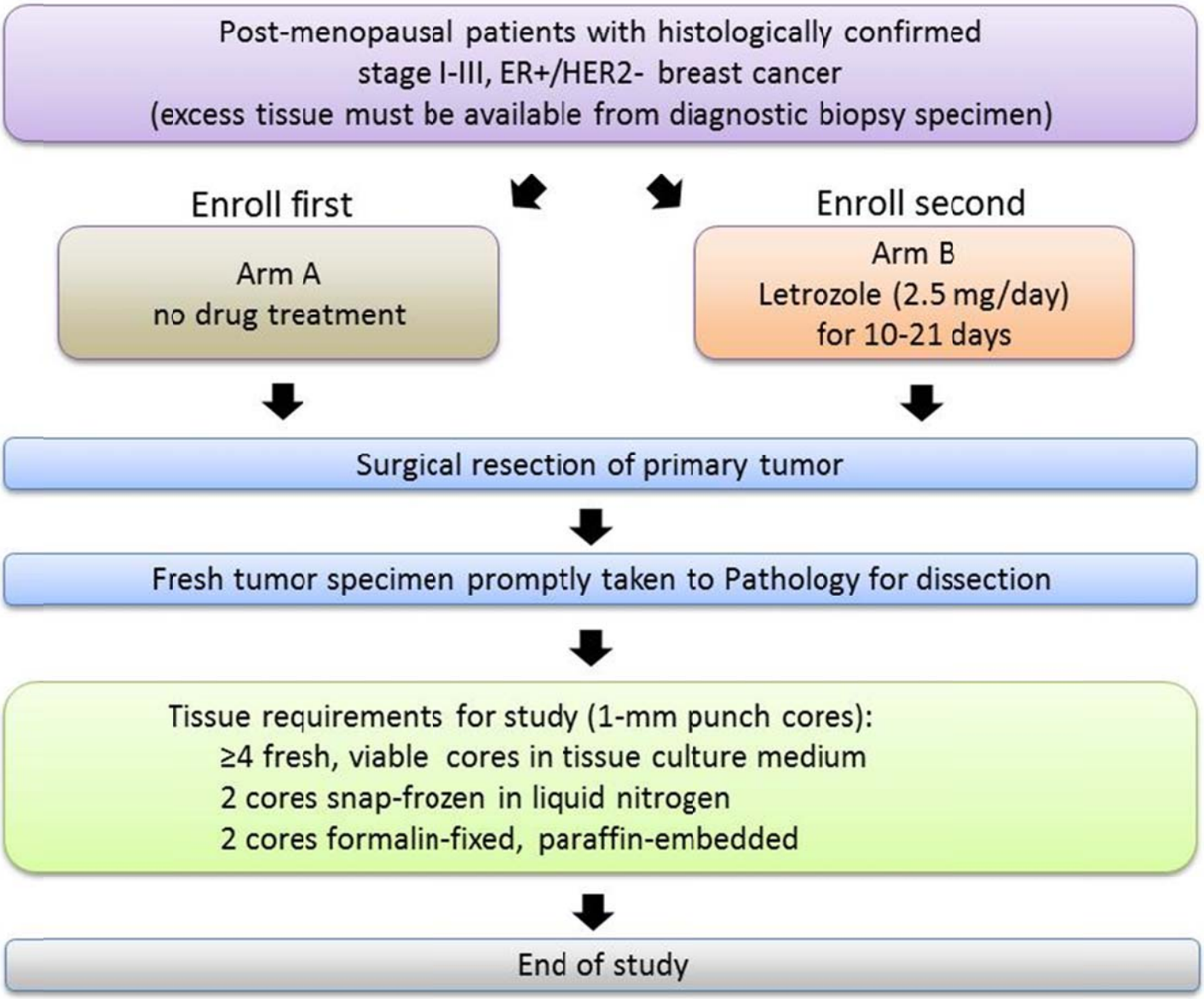
Schema


Post-menopausal patients with histologically confirmed
stage I-III, ER+/HER2- breast cancer
(excess tissue must be available from diagnostic biopsy specimen)
Enroll first
Arm A
no drug treatment
Enroll second
Arm B
Letrozole (2.5 mg/day)
for 10-21 days
Surgical resection of primary tumor
Fresh tumor specimen promptly taken to Pathology for dissection
Tissue requirements for study (1-mm punch cores):
≥4 fresh, viable cores in tissue culture medium
2 cores snap-frozen in liquid nitrogen
2 cores formalin-fixed, paraffin-embedded
End of study

ABBREVIATIONS

CPHS	Committee for the Protection of Human Subjects at Dartmouth College
DMBA	7,12-Dimethylbenz(a)anthracene
DSMAC	Data Safety Monitoring and Accrual Committee of the Norris Cotton Cancer Center
ER	estrogen receptor alpha
FDA	Food & Drug Administration (U.S.)
FFPE	formalin-fixed, paraffin-embedded
HER2	HER2 / ERBB2 proto-oncogene
IGF-1R	insulin-like growth factor receptor
IHC	immunohistochemistry
InsR	insulin receptor
IRS1	insulin receptor substrate 1
IRS2	insulin receptor substrate 2
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
PHI	protected health information
PI	primary investigator
PI3K	phosphatidylinositol 3-kinase
PR	progesterone receptor
OPRR	Dept. of Health and Human Services Office for Protection from Research Risks (U.S.)

1.0 INTRODUCTION

1.1. Breast cancer

Most patients are diagnosed with Stage I-III breast cancer, which is localized to the breast (and adjacent lymph nodes), and is surgically resectable and potentially curable. Seventy-five percent of primary breast tumors express estrogen receptor alpha (ER). Patients with Stage I-III ER-positive breast cancer are typically treated with adjuvant anti-estrogen therapy for 5 years after surgical removal of the primary tumor. Among the cases of ER+ breast cancer, ~10% harbor genomic amplification of the *ERBB2* (HER2) proto-oncogene; patients with HER2+ cancer are treated with HER2-directed therapy, and will not be part of this study. This study focuses on patients with Stage I-III ER+/HER2- breast cancer.

1.2. The presurgical study model

An initial diagnosis of breast cancer is typically made by histological confirmation of a tissue biopsy specimen. Patients with Stage I-III cancer will then typically undergo surgery within a few weeks to remove the primary tumor(s). Therefore, it is common to have a paired tumor biopsy specimen and a surgical tumor specimen. The availability of such paired specimens has prompted a large initiative in recent years to treat patients (*e.g.*, with drug) during the intervening period between the time of diagnostic biopsy and the time of surgery. We can then compare the biopsy tissue (baseline, pre-treatment) to the surgical tissue (post-treatment) to assess biological changes (*e.g.*, changes in mRNA and protein expression). This study design has provided a wealth of information on the effects of systemic therapies on tumor biology [reviewed in ref. (*1*)], and it requires no additional biopsy procedure.

1.3. Therapeutic inhibition of mTORC1

mTORC1 is a molecular hub that integrates signaling inputs from an array of sources, including growth factor receptor signaling pathways, nutrient sensing pathways, and energy sensing pathways. Activated mTORC1 promotes cap-dependent protein translation, and this pathway is hyperactivated in many cancer types. As such, mTORC1 is an attractive therapeutic target. The mTORC1 inhibitor RAD001 (Afinitor, Everolimus) was recently approved for the treatment of patients with advanced/metastatic ER+/HER2- breast cancer in combination with the steroidal aromatase inhibitor Exemestane following progression on a non-steroidal aromatase inhibitor (Letrozole, Anastrozole). Everolimus is also approved for the treatment of other non-breast malignancies. While the phase 3 BOLERO-2 study demonstrated that Exemestane/Everolimus increased progression-free survival compared to Exemestane/Placebo (*2*), nearly all patients eventually progressed. Genetic analysis has not identified a clear, individual biomarker that predicted benefit from Everolimus (*3*). Furthermore, it is unclear

whether continued treatment with Exemestane (in the context of Everolimus) is beneficial, given that the 3 major aromatase inhibitors in clinical use have been shown to be equally efficacious on a population level (*4, 5*); this issue is being addressed in the ongoing BOLERO-6 study (NCT01783444; testing Everolimus vs. Exemestane/Everolimus vs. Capecitabine; same patient population as in BOLERO-2).

In some cell types, including ER+ breast cancer, mTORC1-induced activation of p70-S6-kinase (p70S6K) elicits negative feedback whereby p70S6K phosphorylates the insulin-like growth factor-1 receptor (IGF-1R)/ insulin receptor (InsR) effector IRS-1 to promote IRS-1 degradation (Fig. 1). Thus, mTORC1 inhibition (with RAD001) blocks this negative feedback, inducing upregulation of IGF-1R/InsR/IRS-1/2 signaling that in turn activates PI3K/AKT and MEK/Erk to promote cell survival (*6-9*). As such, mTORC1 inhibitors may stimulate cancer cell survival pathways. Circumventing these effects of mTORC1 inhibitors may increase therapeutic efficacy.

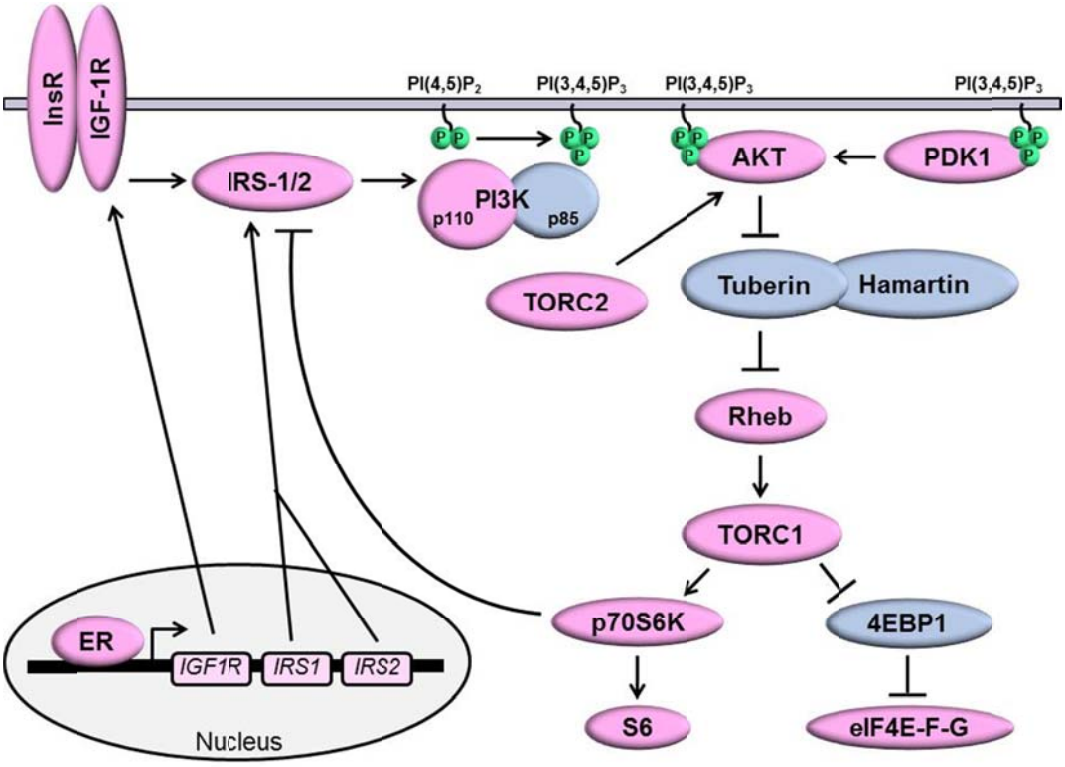


Fig. 1. mTORC1 inhibition blocks negative feedback signaling from p70S6K to IRS-1/2. This promotes IRS-1/2 activation, which in turn promotes PI3K/AKT activation and cell survival. The genes encoding IRS1, IRS2, and IGF-1R are transcriptionally activated by ER. Hence, anti-estrogen-mediated suppression of IGF-1R, IRS1, and/or IRS2 expression blunts activation of PI3K/AKT induced by mTORC1 inhibition.

1.4. Preclinical findings

We observed that mTORC1 inhibition with RAD001 in ER+ breast cancer cell lines induces upregulation of PI3K/AKT signaling (Fig. 2). Pretreatment with the anti-estrogen fulvestrant (which blocks ER transcriptional activity) suppressed RAD001 -induced PI3K/AKT signaling. *IGF1R*, *IRS1*, and *IRS2* are ER-induced genes, and studies have demonstrated crosstalk between the ER and IGF-1R pathways at several levels [reviewed in (*10*)]. Fulvestrant treatment decreased the levels of IGF-1R, IRS-1, and IRS-2 at the protein and/or mRNA levels, which likely suppressed RAD001-induced PI3K/AKT activation. These findings suggest that continued anti-estrogen therapy would be beneficial in the context of treatment with RAD001 in patients.

Presurgical treatment with the aromatase inhibitor Letrozole for 3 days is sufficient to significantly suppress plasma estrogen levels. Gene expression profiling of pre-treatment (baseline, diagnostic biopsy) and post-two-week-treatment (surgical) ER+ tumor specimens revealed that estrogen deprivation via Letrozole induced changes in expression of ER-regulated genes. *IRS1* was one of the most significantly and consistently downregulated genes when comparing pre- and post-treatment samples. *IGF1R* was also significantly downregulated by Letrozole (*11*).

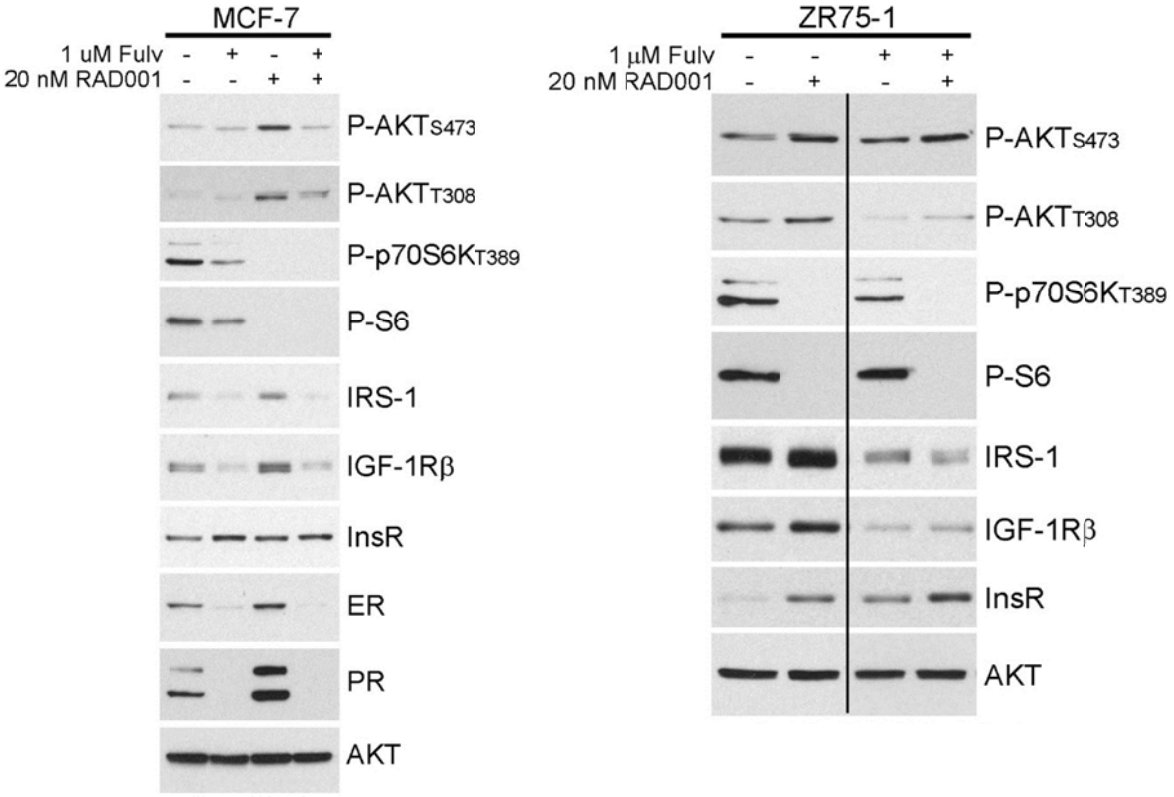


Fig. 2. Anti-estrogen-mediated inhibition of ER with fulvestrant abrogates PI3K/AKT activation induced by mTORC1 inhibition with RAD001. *Left*: MCF-7 cells were pre-treated with medium containing 10% FBS ± 1 μM fulvestrant for 24 hours. Then cells were treated with medium ± 1 μM fulvestrant ± 20 nM RAD001 for 24 hours before lysis. Lysates were analyzed by immunoblotting using the indicated antibodies. *Right*: ZR75-1 cells were pre-treated with medium containing 10% FBS ± 1 μM fulvestrant for 3 days. Then cells were treated with medium ± 1 μM fulvestrant ± 20 nM RAD001 for 24 hours before lysis. Lysates were analyzed as in (A). Note that RAD001 treatment decreases P-S6 (marker of mTORC1 activity) but increases P-AKT. Fulvestrant treatment decreases IRS-1 and IGF-1R levels, which are encoded by ER-inducible genes.

1.5. Presurgical endocrine therapy

Letrozole is appropriate neoadjuvant therapy for postmenopausal women with hormone receptor positive breast cancer. Ellis et al randomized 250 post-menopausal women with locally-advanced hormone receptor positive breast cancer requiring mastectomy, to four months of neoadjuvant endocrine therapy with either letrozole or tamoxifen. Sixty percent of subjects receiving presurgical letrozole had an objective radiologic response and 48% were able to undergo breast-conserving surgery, while only 41% of subjects receiving tamoxifen had an objective response and 36% were able to undergo breast-conserving surgery (*12*). The Ki-67 proliferation biomarker can be assayed at diagnosis and again after a short run-in period of treatment with letrozole, and Ellis showed that suppression of Ki-67 with persistent expression of ER in a node negative patient with a primary tumor under 5 cm in size is predictive of such a low risk of recurrence that adjuvant chemotherapy is obviated (*13*). 377 post-menopausal women with clinical Stage II-III hormone receptor positive breast cancer were randomized to receive letrozole, anastrozole, or exemestane for 16 to 18 weeks prior to surgery. The clinical response rate to letrozole was 75% versus 69% for anastrozole and 63% for exemestane. 51% of the patients who were judged to require a mastectomy at the time of study registration were able to achieve breast preservation (*14*). Neoadjuvant endocrine therapy can therefore increase the likelihood of achieving breast preservation in a woman with a locally advanced hormone receptor positive breast cancer, and biopsies after a brief treatment interval have provided biomarkers which were predictive of prolonged disease-free survival with endocrine therapy alone. Letrozole is an active agent in the neoadjuvant setting and can be considered an appropriate standard of care as neoadjuvant therapy for locally advanced breast cancer.

1.6. Study rationale

We propose a presurgical study in patients with Stage I-III ER+/HER2- breast cancer to determine whether anti-estrogen therapy (Letrozole) suppresses mTORC1 inhibitor (RAD001)-induced activation of PI3K/AKT signaling. As the primary objective is to determine whether anti-estrogen therapy alters mTORC1 inhibitor-induced signaling that can be assessed in a short time frame *ex vivo*, we will treat patients with or without Letrozole for 10-21 days prior to surgery; this time frame will allow sufficient time for maximal estrogen suppression and downstream changes in the levels of ER-regulated transcripts and encoded

proteins. Viable core samples of surgically resected tumors will be used for *ex vivo* culture to assess the effects of RAD001 on signaling, to avoid exposing subjects to potentially adverse side effects of RAD001. Findings from this study will provide mechanistic insight into the effects of mTORC1 and ER inhibition on oncogenic signaling pathways in cancer cells.

2.0 OBJECTIVES

2.1 Primary Objectives

1. Arm A: To determine whether mTORC1 inhibition with RAD001 increases IRS-1/PI3K/AKT pathway activation in ER+/HER2- breast tumors *ex vivo*.
2. Arm B (comparison with Arm A): To determine whether presurgical treatment of patients with ER+/HER2- breast cancer with Letrozole suppresses IRS-1/PI3K/AKT pathway activation compared to tumors from untreated patients.

2.2 Secondary objectives

1. To determine whether presurgical treatment with Letrozole suppresses IRS-1/PI3K/AKT pathway activation compared to baseline.
2. To determine whether the level of IRS-1/PI3K/AKT pathway activation is correlated with rate of tumor cell proliferation as determined by Ki67 score.
3. To determine whether RAD001-induced changes in IRS-1/PI3K/AKT signaling *ex vivo* are correlated with rate of tumor cell proliferation as determined by Ki67 score.
4. To identify changes in mRNA levels of ER-regulated gene induced by presurgical treatment with Letrozole.

3.0 ELIGIBILITY CRITERIA

Inclusion Criteria:

3.1. Histologic documentation of invasive breast cancer by core needle or incisional biopsy. Excess baseline biopsy tumor tissue sufficient to make three 5-micron sections must be available for molecular analyses as part of this study.

3.2. The invasive cancer must be estrogen receptor alpha (ER)-positive, with ER staining present in greater than 50% of invasive cancer cells by IHC

3.3. The invasive cancer must be HER2-negative (IHC 0-1+, or with a FISH ratio of <1.8 if IHC is 2+ or if IHC has not been done).

3.4. Clinical Stage I-III invasive breast cancer with the intent to treat with surgical resection of the primary tumor. Tumor must be ≥2 cm to provide adequate tissue.

3.5 Patients with multicentric or bilateral disease are eligible if the target lesions meet the other eligibility criteria. Samples from all available tumors are requested for research purposes.

3.6. Women ≥ age 18, for whom adjuvant treatment with an aromatase inhibitor would be clinically indicated. Women must be either post-menopausal, or pre-menopausal having undergone oophorectomy.

3.7. Patients must meet the following clinical laboratory criteria:

- Absolute neutrophil count (ANC) ≥ 1,000/mm^3 and platelet count ≥ 75,000/mm^3.
- Total bilirubin ≤ 1.5 × the upper limit of the normal range (ULN).
- Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) ≤ 3 × ULN.

3.8. Ability to give informed consent.

Exclusion Criteria:

3.8. Prior endocrine therapy for any histologically-confirmed cancer is not allowed. Prior endocrine therapy that was administered ≥5 years ago for the prevention of breast cancer in patients with no history of breast cancer is allowed.

3.9. Systemic drug treatment to induce ovarian suppression if woman is pre-menopausal.

3.10. Any other neoadjuvant therapy for breast cancer [*i.e.*, treatment with any other anti-cancer agent besides Letrozole (10-21 days) before surgical resection of the primary breast tumor].

4.0 REGISTRATION

Patients will be registered by the Clinical Research Associate through the Norris Cotton Cancer Center Clinical Trials office following completion of pre-treatment evaluations and informed consent. At the time of registration, the following information will be recorded:

- Patient's name and subject identification number.
- Patient's date of birth.
- Date of treatment start.

Eligibility criteria will be verified at the time of registration. A case number will be assigned following registration.

5.0 TREATMENT PLAN

5.1 Screening Period

Subjects will provide written informed consent, be evaluated for eligibility criteria, and undergo standard baseline clinical and tumor assessments and staging. Subjects will undergo our standard pre-treatment surgical evaluation. To determine our ability to provide breast conservation, patients at DHMC have imaging of the breast prior to neoadjuvant chemotherapy to include mammography, ultrasound, and breast MRI, to determine the suitability of breast conservation, and to guide the surgical oncologist on surgical planning. This period will be up to 4 weeks prior to the start of presurgical treatment with Letrozole for subjects enrolled onto Arm B (this time period does not apply to Arm A).

5.2 Treatment Period

Patients will receive either A) no presurgical drug treatment, or B) presurgical treatment with Letrozole for 10-21 days prior to surgery. Arm A will enroll patients first until accrual is full (n=10). Following successful completion of molecular analyses to support the first primary objective (To determine whether mTORC1 inhibition with RAD001 increases IRS-1/PI3K/AKT pathway activation in ER+/HER2- breast tumors *ex vivo*), we will proceed to enroll patients in Arm B (presurgical Letrozole, as below in Section 5.2.2). An estimated 10 subjects will be enrolled into Arm B to obtain the necessary evaluable number of subjects (n=10).

5.2.1. Surgery and tumor specimens

The patient will undergo definitive surgical resection of the primary tumor.
Management of the axilla will be left to the judgment of the surgical oncologist and the patient.

It is strongly preferred that surgery be scheduled between Monday and Thursday for optimal timing of tissue fixation and processing.

The Pathologist will promptly dissect the surgical primary tumor specimen. ASCO-CAP guidelines require initiation of dissection of a surgical specimen within 1 hour of removal from the patient; for this study, minimal “cold ischemia time” is preferred.

The Pathologist will assess whether the tumor specimen is large enough to procure:

A. tissue for routine diagnostics

B. ≥8 core punch biopsies that are 1 mm in diameter and ≥0.5 cm long for research

C. ≥4 core punch biopsies that are 3 mm in diameter and ≥0.25 cm long for research

A 2-cm tumor is expected to provide sufficient material for all needs.

When the tumor is sufficiently large to provide adequate tissue (*e.g.*, >3 cm), the Pathologist will first remove core punch biopsies for this research study using 1-mm and 3-mm punch biopsy devices. **All research samples must be acquired within 1.5 hours of tissue removal from the patient; a shorter lag time is strongly preferred to maximize tissue viability.** The Pathologist will then acquire tissue samples for routine diagnostics.

If the tumor specimen is approximately 2 cm, the Pathologist may opt to first procure tissue for routine diagnostics. Then if adequate tumor tissue remains to provide punch biopsies for research purposes, these samples will be taken from the tumor. If the tumor is too small to provide adequate tissue for research, the tumor will be considered non-evaluable and the patient will need to be replaced.

Punch biopsy tumor specimens required for this research study are listed below in order of priority.

A) Fresh tissue (n=8) - Using a **1-mm** punch biopsy device with plunger (provided by Dr. Miller), remove 8 tumor samples ≥0.5 cm long. These samples will be **immediately** placed into cold (0-4°C) tissue culture medium (serum-free DMEM + Pen/Strep; provided by Dr. Miller to be kept on-hand in Pathology lab) and kept on ice or refrigerated (0-4°C). These tissues will be transported **within 30 minutes** of dissection to Dr. Miller's lab on the 6th floor of Rubin Research Building. Jennifer Bean, Wei Yang, and Todd Miller (members of the Miller lab) will be responsible for transporting tissues, and need to be contacted early on the date of surgery with notification that samples will be available the same day. They will be paged when tumor tissues are available for pick-up.

B) Frozen tissue (n=2) - Using a **3-mm** punch biopsy device with plunger (provided by Dr. Miller), remove 2 tumor samples ≥ 0.25 cm long, put into cryotubes (1 core per tube), and snap-freeze in liquid nitrogen. Liquid nitrogen can be provided in a small container by the Miller lab, if they are notified early on the day of surgery.

C) Formalin-fixed (FFPE) tissue (n=2) - Using the same **3-mm** punch biopsy device, remove 2 tumor samples ≥ 0.25 cm long, fix in formalin (3 hours to overnight), and paraffin-embed the next day as per standard tissue processing procedures in Pathology lab.
Alternatively, if Pathology will be processing diagnostic FFPE tumor samples that will provide sufficient excess tissue for molecular analyses,

and tissues are fixed for less than 24 hours, then such samples may be used in lieu of punch biopsies.

D) Extra tumor tissue- If tissue requirements for categories A, B, and C are filled and excess tumor tissue is available, more 1-mm cores of fresh viable tissue for tissue culture are requested (up to 20 more). Such extra cores should be placed into tissue culture medium as in section A (above).

Histological qualification of tumor samples- The Pathologist will perform routine histological analysis of the diagnostic FFPE surgical tumor specimen. If the diagnostic tumor sample is found to have <50% tumor cellularity, the tumor samples procured for research will be considered non-evaluable, and this patient will need to be replaced.

5.2.2. Presurgical Letrozole

Patients enrolled on Arm B will be treated with Letrozole (2.5 mg/day, administered orally) for 10-21 days prior to surgery. This time period has been and continues to be used as a standard time frame in presurgical studies with aromatase inhibitors in patients with ER+ breast cancer. This drug and dose are given as standard adjuvant therapy for up to 5 years following surgical removal of a primary ER+/HER2- breast tumor in patients with Stage I-III disease. The final dose of Letrozole should be taken on the morning of surgery.

It is strongly preferred that surgery be scheduled between Monday and Thursday for optimal timing of tissue fixation and processing.

The primary tumor will be surgically resected as in Section 5.2.1. The Pathologist will promptly dissect the surgical tumor specimen, and obtain punch biopsies for this study as in Section 5.2.1.

5.2.3. Letrozole drug information

Letrozole (4,4'-(1H-1,2,4-Triazol-1-ylmethylene)bis-benzonitrile) is a synthetic achiral benzydryltrizole derivative. It is an orally active highly selective, non-steroidal competitive inhibitor of the aromatase enzyme system (*15, 16*). Aromatase inhibitors block the aromatase enzyme, consequently lowering estrogen levels and thereby deprive the tumor of its growth stimulus. Letrozole effectively inhibits the conversion of androgens to estrogens in both *in vitro* and *in vivo* (*15*). This property makes it in particular suitable for postmenopausal women whose main source of estrogen is via peripheral aromatization of androgen precursors.

Preclinical and pharmacodynamic data in humans

In vivo, in female rats, the aromatase inhibition manifests itself in a reduction in uterine weight, an increase in body weight, a decrease in serum estradiol concentration, an increase in plasma LH concentration

(*16, 17*) and suppression of the growth of DMBA-induced rat mammary tumors (*15, 18*). The ED50 values for some of these effects lie in the range of 0.001 to 0.03 mg/kg with maximal endocrine and anti-tumor efficacy in the dose range of 0.3 to 1.0 mg/kg po (*18*).

Comparison to Aminoglutethimide (AG)

Letrozole is up to 150-250 times more potent than the first generation aromatase inhibitor AG, *in vitro* and more than 10,000 times as potent as AG in inhibiting aromatase *in vivo* (*15*). The high potency of letrozole is not accompanied by any significant effect on adrenal steroidogenesis *in vitro* or *in vivo* over its maximally effective dose range (*16, 17*). Inhibition of adrenal steroidogenesis resulting in adrenal hypertrophy does occur with therapeutic doses of AG. The high potency and selectivity of letrozole explains its pharmacological profile and high therapeutic index.

Activity in breast cancer patients

In postmenopausal patients with advanced breast cancer, daily doses of 0.1 to 5 mg letrozole suppressed plasma levels of estradiol, estrone and estrone sulfate to 75-95% from baseline (*15, 19*). Estrogen suppression was maintained throughout the treatment period of 28 days in all patients.

Selectivity of letrozole

Letrozole is a highly selective inhibitor of the aromatase enzyme. No clinically relevant changes in the plasma levels of cortisol, aldosterone, 11-deoxycortisol, 17-hydroxyprogesterone, ACTH or plasma renin activity were found in postmenopausal patients treated with a daily dose of letrozole ranging from 0.1 to 5 mg (*15, 20, 21*). Synacthen7 tests performed after 6 weeks of treatment with daily doses of 0.1, 0.25, 0.5, 1, 2.5 and 5 mg letrozole indicated no attenuation of aldosterone or cortisol production (*22*).

Toxicities of letrozole

Most adverse reactions to letrozole in the literature are mild to moderate in severity and are indistinguishable from the consequences of the patient's metastatic breast cancer, the effects of estrogen deprivation, or other concurrent illness.

Reported events in greater than 5% of subjects include chest pain, hypertension, edema, hyperlipidemia, cough, dyspnea, nausea, vomiting, abdominal pain, anorexia, increased appetite, constipation, diarrhea, weight gain, weight loss, bone fractures, arthralgias, arthritis, back pain, bone pain, breast pain, myalgias, weakness, asthenia, dizziness, depression, fatigue, headache, insomnia, lethargy, malaise, rash, hot flashes, sweats, vaginal bleeding, vaginal dryness, and infections.

Letrozole will be prescribed by the subject's oncology provider and the subject will be asked to fill the prescription at her commercial pharmacy.

5.3 Study exit

Following surgery, the patient's role in the study will end. The patient will then be treated as per standard-of-care by her treating oncologist. Subjects who begin letrozole in the pre-surgical setting may receive chemotherapy followed by resumption of letrozole or letrozole alone following the definitive surgical resection.

5.4. Evaluable patients

Subjects who proceed to a definitive surgical procedure with A) tumor tissue sufficient to provide punch biopsy specimens for research purposes, and B) histologically-confirmed tumor tissue in the surgical specimen with ≥50% tumor cellularity, will be considered evaluable. Subjects whose surgical specimens do not contain adequate tumor tissue to assess the primary endpoint will not be considered evaluable, and will need to be replaced.

5.5. Analyses

5.5.1. *Ex vivo analysis of RAD001 effects on IRS-1/PI3K/AKT signaling.*

Fresh viable tumor cores in tissue culture medium (serum-free DMEM + Pen/Strep) will be placed into medium containing 20 nM RAD001 (4 cores) or DMSO control (4 cores) and cultured at 37°C for 1 hour. If extra tissue cores are available, we will treat +/- 20 nM RAD001 for 2-8 hours to evaluate delayed changes in signaling, and treat +/- 1 uM OSI-906 (IGF-1R/InsR inhibitor) for 1-8 hours to determine whether PI3K/AKT signaling is IGF-1R/InsR-dependent in these tumors. Tissue cores will then be snap-frozen in liquid nitrogen and stored at -80°C.

Immunoblotting- Two cores each of DMSO- and RAD001-treated tumor will be homogenized and lysed on ice in RIPA buffer containing protease and phosphatase inhibitors. Samples will be sonicated and centrifuged, and supernatant will be used to quantify protein concentration. Protein lysates for immunoblotting will then be mixed with reducing sample buffer and stored at -80°C. Lysates will be heated at 95°C for 1 min. just before SDS-PAGE following by immunoblot analysis using antibodies against:

IRS-1
phospho-IRS-1-Ser636/639 (marker of p70S6K activity)
IRS-2
AKT
phospho-AKT-S473 (marker of mTORC2 activity)
phospho-AKT-T308 (marker of PIP_3 levels and PI3K/PDK1 activity)
4E-BP1
phospho-4E-BP1-Thr37/46 (marker of mTORC1 activity)

p70S6K
phospho-p70S6K-Thr389 (marker of mTORC1 activity)
S6
phospho-S6-Ser240/244 (marker of p70S6K activity)
phospho-S6-Ser235/236 (marker of p70S6K and RSK activity)
Erk1/2
phospho-Erk1/2-Thr202/Tyr204 (marker of MEK activity)
IGF-1Rb
InsRb

If excess protein lysate is available, we will immunoprecipitate the p85 subunit of PI3K, and immunoblot for IRS-1 and IRS-2 to assess drug induced changes in IRS-1/2-induced PI3K activation. We will also immunoprecipitate using phospho-Tyrosine antibody, and immunoblot for IGF-1Rb, InsRb, IRS-1, and IRS-2 to assess protein activation.

The primary goal of this analysis is to determine whether RAD001 treatment *ex vivo* globally decreases mTORC1 signaling (indicated by a decrease in levels of phospho-p70S6K, phospho-S6, and phospho-4E-BP1), increases IRS-1/2 levels and phosphorylation, and increases AKT phosphorylation. Secondary goals are to assess changes in IGF-1R and InsR activation, and compensatory signaling through the MEK/Erk pathway.

Immunohistochemistry (IHC) on frozen tissues- Two cores each of DMSO- and RAD001-treated tumor will be sectioned and processed for IHC in the Pathology Translational Research Shared Resource. Tissue sections will be immunostained using antibodies against phospho-S6-Ser240/244, phospho-AKT-S473, phospho-AKT-T308 and phospho-IGF-1Rb/InsR- Tyr1131/Tyr1146. Samples will be scored using a Histoscore scale that incorporates staining intensity and frequency (*12*).

The primary goal of this analysis is to determine whether RAD001 treatment *ex vivo* decreases mTORC1 signaling (indicated by a decrease in levels of phospho-S6) and increases AKT phosphorylation uniformly throughout tumor cores, and whether drug treatment alters IGF-1R/InsR phosphorylation (which cannot be assessed by immunoblot analysis of protein lysates). Staining intensities in the center of each core will be compared to the outer edges to assess uniformity of signal; non-uniformity may be due to time of exposure of cells to drug or medium.

5.5.2. *Immunohistochemical analysis of pre- vs. post-Letrozole tumors.*
Baseline (diagnostic core biopsy specimen) and post-Letrozole (surgical specimen) tumor samples will be sectioned and processed for IHC in the Pathology Translational Research Shared Resource. Tissue sections will be immunostained using antibodies against ER, PR, and Ki67. ER and PR

will be scored using a Histoscore scale (*23*); we expect Letrozole treatment to decrease PR levels, while ER may be unaffected. Ki67 will be scored by counting the number of positively-stained cells in 10 high-power (400x magnification) microscopic fields (*24, 25*). Pre- and post-Letrozole Ki67+ cell counts will be compared by t-test; Letrozole treatment should induce an overall decrease in Ki67 score.

The primary goal of this analysis is to determine whether changes in signaling induced by RAD001 treatment *ex vivo* are associated with Ki67 score. A higher post-anti-estrogen Ki67 score, and a smaller decrease in Ki67 score in response to presurgical treatment with an anti-estrogen, are associated with shorter recurrence-free survival (*26*). Hence, it is thought that a higher level of residual tumor cell proliferation (higher Ki67 score) following anti-estrogen treatment, or a weaker biological response (change in Ki67 score) to anti-estrogen treatment, are indicative of less dependence of tumor cells on ER for growth and survival. Our preclinical data indicate that ER drives IRS-1 and IGF-1R expression, which facilitate RAD001-induced PI3K/AKT activation. Therefore, in tumors that are less dependent upon ER, Letrozole treatment is expected to have less of an effect on IRS-1/PI3K/AKT signaling. Such findings would support the use of combinations of anti-estrogens and mTORC1 inhibitors in patients with ER-driven tumors.

6.0 DATA COLLECTION, HANDLING AND RECORD KEEPING

6.1. Confidentiality

Information about study subjects will be kept confidential and managed according to the requirements of the Health Insurance Portability and Accountability Act of 1996 (HIPAA). Participants will sign an authorization that includes the following:

- What protected health information (PHI) will be collected from subjects in this study
- Who will have access to that information and why
- Who will use or disclose that information
- The rights of a research subject to revoke their authorization for use of their PHI.

In the event that a subject revokes authorization to collect or use PHI, the investigator, by regulation, retains the ability to use all information collected prior to the revocation of subject authorization.

Loss of patient confidentiality is a risk of participation. Study participant identities will be kept confidential except as required by law. Subjects' samples will be identified by code only (i.e., linked, but de-identified). Patient samples will be de-identified at the time and site of collection. Participant and study information will be kept in the Velos eResearch password-protected database or its equivalent. Additionally, documents containing participant identifiers, such as those from the medical record to confirm eligibility, will be filed in binders and

kept in a locked, secure location in the Office of Clinical Research at the Norris Cotton Cancer Center.

6.2. Data retention

Following closure of the study, the investigator will maintain all site study records in a safe and secure location. The records are maintained to allow easy and timely retrieval, when needed (e.g., audit or inspection) and, whenever feasible, to allow any subsequent review of data in conjunction with assessment of the facility, supporting systems, and staff. Upon completion of study analysis, research information is stored in Dartmouth College Records Management off-site storage located at 6218 Etna Road, Hanover, NH 03755. Documents are shredded on site after 50 years of storage.

Participant and study information will be kept in the Velos eResearch password protected database (or its equivalent) indefinitely.

7.0 STUDY MONITORING, AUDITING, AND INSPECTING

7.1 Safety and Data Monitoring

This study will be monitored by the Data Safety Monitoring and Accrual Committee (DSMAC) of the Norris Cotton Cancer Center. The Committee meets quarterly to review accrual rates and information of all studies that have accrued participants. The DSMAC has the authority to suspend or terminate all research activities that fall within its jurisdiction. In the event that a study is suspended or terminated by the DSMAC, that information will be forwarded to the CPHS office.

7.2 On-Site Monitoring

Clinical research monitoring will be conducted by appropriately trained staff of Dartmouth-Hitchcock Medical Center Clinical Trials Office. This monitoring will include periodic assessment of the regulatory compliance, data quality, and study integrity. Study records will be reviewed and directly compared to source documents and the conduct of the study will be discussed with the investigator, Monitors may request access to all regulatory documents, source documents, CRFs, and other study documentation for on-site inspection. Direct access to these documents is guaranteed by the investigator, who must provide support at all times for these activities.

7.3 Auditing and Inspecting

Participation as an investigator in this study implies acceptance of potential inspection by government regulatory authorities and applicable Dartmouth compliance and quality assurance offices. The investigator will permit study-

related audits and inspections by the Dartmouth CPHS, government regulatory bodies, and Dartmouth compliance and quality assurance groups of all study related documents (e.g. source documents, regulatory documents, data collection instruments, study data etc.) The investigator will ensure the capability for inspections of applicable study-related facilities (e.g. pharmacy, diagnostic laboratory, etc.)

8.0 ADVERSE EVENT REPORTING

Patients on Arm B will be instructed to report the occurrence of any adverse event (this will not apply to participants on Arm A). An adverse event is any undesirable event associated with the administration of letrozole. Adverse events will be graded according to the NCI Common Toxicity Criteria Version 4.0. A copy of the CTC version 4.0 can be downloaded from the CTEP home page (http://ctep.info.nih.gov). All appropriate treatment areas should have access to a copy of the CTC version 4.0.

This trial will be independently monitored by the Norris Cotton Cancer Center Data Safety and Accrual Monitoring Committee.

The following definitions will be used to assess causality:

No: The clinical adverse event is definitely unrelated to the study medications (e.g., does not follow a reasonable temporal sequence from study medication administration, present prior to receiving study medication, etc.)

Unlikely: The study medications do not have any reasonable association with the observed experience; however, relationship cannot be definitely excluded.

Possibly: The connection with study medication administration appears feasible, but cannot be excluded with certainty (e.g., follows a reasonable temporal sequence from medication administration, but may also be related to other known factors).

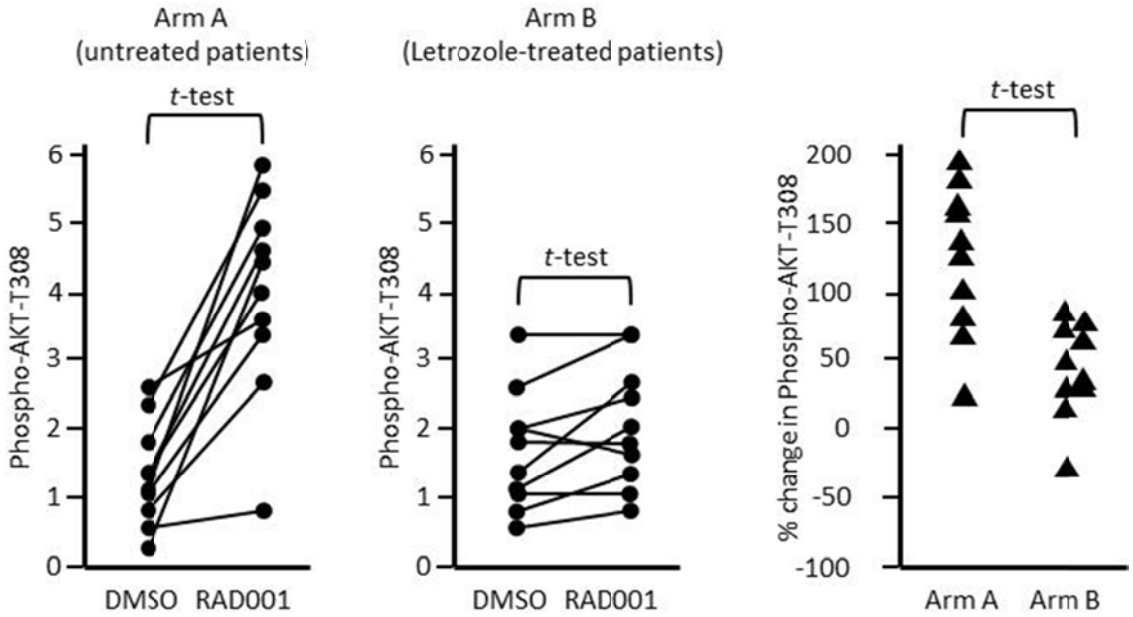
Probably: The clinical experience appears related to the study medications with a high degree of certainty (e.g., follows a reasonable temporal sequence from medication administration and abates upon discontinuation of the medication, cannot be reasonably explained by known characteristics of the patient's clinical state or other modes of therapy administered to the patient, etc.)

9.0. STATISTICAL CONSIDERATIONS

9.1 PRIMARY ENDPOINT – The primary endpoint of this trial is the level of IRS-1/PI3K/AKT pathway activation in primary tumors, measured by densitometric analysis of immunoblot signals of markers of pathway activation (IRS-1, phospho-IRS-1-Ser636/639, phospho-AKT-T308, phospho-AKT-S473).

9.2 SECONDARY ENDPOINT – The secondary endpoint is Ki67 score, as determined by the percentage of Ki67+ tumor cells by IHC.

9.3 PRIMARY ANALYSIS PLAN - The primary analyses determine whether RAD001 treatment of tumor samples *ex vivo*, and/or presurgical treatment of patients with Letrozole, alter IRS-1/PI3K/AKT pathway activation in breast tumors. Signal values of IRS-1, phospho-IRS-1-Ser636/639, phospho-AKT-T308, phospho-AKT-S473 will be determined by densitometric analysis of immunoblots. Below is an illustration of projected data and relevant comparisons. Signal values of control (DMSO)-treated and RAD001-treated tumor samples will be compared using a 2-sided paired t-test. These tests will be performed separately in tumors from each arm of the study (*i.e.*, untreated, or letrozole-treated, as shown below in *left* and *center* panels). An additional test will compare the magnitude of change in signal values in Arm A vs. Arm B (2-sided independent t-test), to determine whether Letrozole treatment alters response to RAD001 (shown below in *right* panel).



9.4 SECONDARY ANALYSIS PLAN – Secondary analysis to determine whether presurgical treatment with Letrozole suppresses IRS-1/PI3K/AKT activation compared to baseline will be achieved using a paired t-test comparing IRS-1/PI3K/AKT pathway markers scored by IHC analysis of matching tumor samples from patients acquired A) at baseline (diagnostic biopsy) and B) at surgery (post-Letrozole). We will also determine whether tumor Ki67 IHC score is associated with the level of IRS-1/PI3K/AKT pathway activation, or with RAD001-induced changes in IRS-1/PI3K/AKT signaling, by Spearman correlation between Ki67 score and densitometric signal values of pathway markers as determined by immunoblot analysis or IHC Histoscore.

9.5 STATISTICAL TESTING - All statistical tests will be 2-sided, and the overall type I error will be 0.05.

9.6 SAMPLE SIZE – The primary analyses determine whether RAD001 treatment of tumor samples *ex vivo*, and/or presurgical treatment of patients with Letrozole,

alter IRS-1/PI3K/AKT pathway activation in breast tumors. RAD001-induced pathway activation will be defined as a ≥50% increase in phospho-AKT densitometric signal value compared to DMSO-treated control; this scoring system provides a binary metric for sample size determination for Arm B (below). Given that this is a pilot study, we do not know what fraction of ER+/HER2- breast tumors will exhibit RAD001-induced IRS-1/PI3K/AKT activation. Hence, tumors from 10 untreated patients in Arm A will be used to determine the frequency with which RAD001 induces AKT activation.

The fraction of tumors in Arm A (untreated patients) that exhibit RAD001-induced AKT activation will be used to determine the number of patients that need to be enrolled in Arm B (presurgical Letrozole treatment) to detect a significant effect of presurgical Letrozole treatment on RAD001-induced AKT activation. We estimate that ≤15% of Letrozole-treated tumors will exhibit RAD001-induced AKT activation. If we observe that RAD001 increases phospho-AKT in _X_ of 10 tumors in Arm A, we will enroll _Y_ patients in Arm B (presurgical Letrozole) to provide a sufficient number of tumors to detect a significant effect ($p \leq 0.05$) of presurgical Letrozole on RAD001-induced changes in phospho-AKT levels with 80% power (calculated using uncorrected chi-squared statistic). If we observe that ≤3 tumors from untreated patients in Arm A exhibit RAD001-induced AKT activation, the study will be terminated at this point, and Arm B will not enroll patients. To account for interpatient/intertumor variability, we will enroll a minimum of 10 patients in Arm B.

X (n of tumors from untreated patients that exhibit a ≥50% increase in P-AKT levels in response to RAD001 treatment *ex vivo*)	**Y (n of patients to be enrolled in Arm B, presurgical Letrozole)**
0-3	Terminate study
4-6	30
7-8	14
9-10	10

10.

10. HUMAN SUBJECTS

10.1 Subject patient population

Patients with potentially resectable Stage I-III biopsy-proven ER+/HER2- breast cancer.

10.2 Consent Procedures

Patients must give a statement of informed consent. The informed consent must be approved by the Committee for the Protection of Human Subjects at Dartmouth College, the Dartmouth-Hitchcock Medical Center IRB. Before initiating a trial, the investigator will have written and dated approval from the CPHS of Dartmouth-Hitchcock Medical Center for the trial protocol, amendment(s), written informed consent form, consent form updates, subject recruitment procedures (e.g., advertisements) and written information to be provided to subjects. The investigators, or a person designated by the investigators, will explain the benefits and risks of participation in the study to each subject (and impartial witness when applicable) and obtain written informed consent prior to the subject entering the study (before initiation of non-routine tests and administration of study drug). The final form must be agreed to by the CPHS of Dartmouth-Hitchcock Medical Center and must contain all elements in the sample form, in language readily understood by the subject. Each subject's original consent form, signed and dated by the subject and by the person who conducted the informed consent discussion, will be retained by the investigator. In addition, a copy of the signed informed consent will be given to the subject.

10.3 Potential Risks and Benefits

The physical risk of study participation is low. Subjects in Arm A are receiving no study-related treatment. Subjects in Arm B are receiving 10-21 days of treatment with 2.5 mg/day Letrozole prior to surgery; adjuvant therapy with this drug and dose are standard-of-care for this patient population, and will be administered for up to 5 years after surgery. The results of this investigation may provide novel information on ER and PI3K/mTOR signaling pathways in breast cancer, which may shape the development of future treatment regimens to benefit future patients with breast cancer. Loss of patient confidentiality is a risk of participation. Participation in this study is of low overall risk and has no potential for direct clinical benefit.

10.4 Confidentiality

The pathology specimens from the patient biopsies will be labeled only with the patient case number. The key matching patient identification with the patient case number will be kept in a locked drawer in the PI's office. Only the PI and his designated representatives will have access to this key. No one will have access to the study records but for the Investigators and CRA's, the Dartmouth-Hitchcock CPHS, the FDA, and OPRR.

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