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Breast cancer is the most frequently diagnosed cancer in American women and the second most frequent cause of cancer death.[1] Breast cancer growth is regulated by estrogen. Estrogen receptor (ER) status is used to predict prognosis and to determine which patients will benefit from antihormonal therapy.[2] Tamoxifen is the most commonly used antiestrogenic agent. Its long-term use leads to tumor resistance.[3] This has led investigators to search for antiestrogens that can be used as second-line therapy for patients who develop tamoxifen resistant tumors. This proposal investigates the effectiveness of rapamycin as an antiestrogenic agent. Four specific aims are described: 1) Flow cytometry was used to show that rapamycin can inhibit the estrogen-induced cell cycle progression of ER+ breast cancer cells. 2) Rapamycin was found to inhibit estrogen-mediated transcription in ER+ breast cancer cells which had been transiently transfected with the artificial construct 3XERE-TATA-Luc. 3) Since rapamycin could potentially be used clinically its efficacy at inhibiting estrogen-dependent breast cancer growth in a mouse xenograft tumor model is currently being tested. 4) Some cytotoxic agents are also antiangiogenic. Three different assays; a cellular migration assay, an aortic ring assay and a corneal pocket assay are being developed to determine if rapamycin has any antiangiogenic effects.

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Introduction

Abstract

Breast cancer is the most frequently diagnosed cancer in American women and the second most frequent cause of cancer death.[1] Breast cancer growth is regulated by estrogen. Estrogen receptor (ER) status is used to predict prognosis and to determine which patients will benefit from antihormonal therapy.[2] Tamoxifen is the most commonly used antiestrogenic agent. Its long-term use leads to tumor resistance.[3] This has led investigators to search for antiestrogens that can be used as second-line therapy for patients who develop tamoxifen resistant tumors. This proposal investigates the effectiveness of rapamycin as an antiestrogenic agent. Four specific aims are described: 1) Flow cytometry was used to show that rapamycin can inhibit the estrogen-induced cell cycle progression of ER+ breast cancer cells. 2) Rapamycin was found to inhibit estrogen-mediated transcription in ER+ breast cancer cells which had been transiently transfected with the artificial construct 3XERE-TATA-Luc. 3) Since rapamycin could potentially be used clinically its efficacy at inhibiting estrogen-dependent breast cancer growth in a mouse xenograft tumor model is currently being tested. 4) Some cytotoxic agents are also antiangiogenic. Three different assays; a cellular migration assay, an aortic ring assay and a corneal pocket assay are being developed to determine if rapamycin has any antiangiogenic effects.

Body

Specific Aims

Aim 1. A. To show that rapamycin inhibits ER+ breast cancer cellular proliferation. An XTT based cytotoxicity assay was used to measure the IC_{50} needed for MCF-7 and BT-474 inhibition by rapamycin. **B.** Fluorescence activated cell sorting (FACS) was used to follow the effect that rapamycin exerts on cell cycle progression of G_0-arrested ER+ breast cancer cell lines that have been stimulated with various mitogenic agents. Thymidine uptake assays will be performed to confirmed the FACS findings.

Aim 2. A. We have shown that rapamycin inhibits estrogen-mediated transcription of ER+ breast cancer cells in a transient transfection assay. The mechanism of this inhibition will be investigated. **B.** It has already been determined by Western blot analysis that the inhibition is not occurring via receptor degradation.
C. Immunohistochemistry and nuclear/cytoplasmic extraction will be used to follow shuttling of the ligand/receptor complex between the nucleus and the cytoplasm after exposure of ER+ breast cancer cells to estrogen and rapamycin. This will show if transcriptional inhibition is occurring because of the inability of the ligand/receptor complex to translocate to the nucleus. **D.** A gel binding assay will be employed to determine if transcriptional inhibition is occurring because rapamycin is preventing the translocated ligand/receptor complex from binding nuclear DNA.

Aim 3. It has been determined *in vitro* that rapamycin can inhibit the estrogen-mediated transcription of ER+ breast cancer cells. To establish rapamycin as a reasonable clinical option for the treatment of tamoxifen resistant tumors this finding must be confirmed *in vivo*. We have begun treating athymic nude mice, which have xenografted estrogen-dependent MCF-7 tumors, with intraperitoneal rapamycin. The tumor volumes of rapamycin treated mice will be compared to the tumor volumes of non-treated mice. Immunohistochemical staining will be performed on resected tumors to measure proliferation indices, ER status, apoptosis markers, and angiogenesis markers.

Aim 4. Rapamycin may have an inhibitory effect on vascular endothelial cells which would make it an even more attractive antitumor agent. Assays are being developed to determine if rapamycin has antiangiogenic properties. These include an *in vitro* cell migration assay, an *ex vivo* aortic ring assay and an *in vivo* corneal pocket assay.

Background and Significance

Breast cancer is the most common malignancy in American women, excluding skin cancer. In the United States in 1999, there will be more than 180,000 new cases and approximately 44,000 deaths from this disease. The activity of the estrogen receptor significantly influences the behavior and treatment of greater than two-thirds of human breast cancers.[36] Reducing estrogen secretion has been used historically to induce regression of breast cancer. This has been accomplished in the past with surgical measures such as oophorectomy, hypophysectomy and adrenalectomy. In the 1970's investigators began to learn much more about the structure and function of the estrogen receptor. This knowledge aided in the design of non-invasive agents which could bind to and block the estrogen receptor.

The Estrogen Receptor

The estrogen receptor (ER) was discovered in 1962 by Jensen and Jacobson who reported the presence of receptors for estrogens in breast and uterus.[37] Another major breakthrough came in the 1980's when ER-encoding cDNAs were cloned from several species which facilitated detailed molecular investigations about the function and modular structure of the receptor.[38-45]

Estrogen Receptor Domains. Five functional domains of the estrogen receptor have been defined. These domains have been designated A/B, C, D, E and F. The amino-terminal A/B region contains a ligand-independent transactivation domain called TAF-1. Region C contains two zinc finger structures which facilitate DNA binding. The DNA sequence, or estrogen response element (ERE), that is recognized by this DNA binding region is 5'GGTCANNNTGACC 3'. Region C also has a weak dimerization activity. The short D domain is regarded as a flexible hinge between the DNA binding domain (DBD) and the E domain. A nuclear localization signal (NLS) has been identified at the junction of the C and D domains. The E domain is large and regulates many functions. The most important of these is the ligand binding activity in the ligand binding domain (LBD). Other functions of the E domain include a ligand-dependent transactivation

domain (TAF-2), a dimerization interface, a heat shock protein 90 (hsp90) interacting region and an NLS. The carboxy-terminal F domain is involved in transcriptional activation.

Transactivation. The estrogen receptor requires the activation of at least one of the two transactivation domains (TAF-1 and TAF-2) for initiation of transcription. The activity of the TAFs appears to be cell and promoter specific. This was demonstrated in experiments which showed that a mutant ER, containing only TAF-1, activated transcription in chicken embryo fibroblasts but not in HeLa cells. In contrast, a TAF-2 containing receptor was shown to be active in both cell types. It was shown that TAF-2 only worked with certain promoters while TAF-1 activity was less sensitive to variations in the promoter context. It appears that TAF-1 and TAF-2 of the ER and some other nuclear receptors interact with the basal transcription machinery via different downstream targets.

There is a correlation between the conformation of the LBD of ER and its ability to activate transcription. The conformational changes induced by ligands are likely to expose regions of the receptor that are necessary for interactions with nuclear DNA. These changes are necessary for the receptor to interact with the transcriptional machinery and adaptor proteins (co-activators and co-repressors), thereby stabilizing the transcriptional apparatus and promoting transcriptional initiation by RNA polymerase II.

A Second Estrogen Receptor. In 1996 a novel transcription factor which showed high sequence homology to the ER was discovered.[49,50] This factor, named ERβ, has similar ligand and DNA binding properties as the original receptor, ERα. It is not clear if ERβ has overlapping or different physiological functions as ERα. The two ERs appear to have slightly different expression patterns suggesting that they might be involved in different biological processes. The observation that mice without functional ER are sterile and have a reduced bone density suggests that at least some of these functions are not rescued by ERβ.[51,52] Future experiments will certainly yield new information about specific ERβ functions or ERα/ERβ interactions.

Estrogen Receptor Associated Proteins

There are two proteins which are integral components of the ER macromolecular complex which lies dormant in the nucleus until activated by ligand.

HSP90. hsp90 is a highly conserved, ubiquitous, and abundant protein that is associated with the estrogen receptor in cytosol extracts. ER/hsp90 interactions are mediated through the ligand binding domain (E domain) of the receptor. The ER does not require hsp90 for ligand binding. hsp90 appears to dissociate from receptors treated with hormone *in vivo*, since subsequent receptor extracts lack bound hsp90. Although the actual function of hsp90 is not known, potential roles have been suggested based on how it functions in other systems. One of these suggested roles is folding the polypeptide chain into functional domains. It may assist in maintaining the proper oxidation/reduction state for the cysteines which coordinate with zinc ions to form the DNA binding finger domains. hsp90 may aid in phosphorylating the receptor through protein kinase activity. An autophosphorylation function has been reported in hsp90 since it is capable of phosphorylating its own serine and threonine residues. An ATPase activity, which may be involved in its interaction with other proteins, has also been

reported. Since the protein clearly maintains the receptor in an inactive state this function may also protect the receptor from degradation. Through interactions with microtubules and/or actin filaments hsp90 may assist in nuclear/cytoplasmic shuttling of the estrogen/ER complex.

p59. p59 is an ER associated protein that belongs to the immunophilin family of proteins that bind to the immunosuppressant drugs FK506 and rapamycin. Its receptor binding may be mediated by another ER associated protein, hsp90. p59 expression is induced in response to heat shock.[58] Cross-talk between steroid hormone and membrane signal transduction pathways may be mediated by p59. In its role as an immunophilin p59 is able to bind the calcium/calmodulin-dependent phosphatase, calcineurin. This may be one target in the disruption of cell signaling pathways. Since other FKBPs (**FK**-506 **B**inding **P**roteins) have functions that are important in signaling pathways for the activation of T-cells in the immune system, a regulatory function has also been postulated for p59. The responsiveness to heat shock and the observation that FKBPs contain peptidylprolyl isomerase activity suggest that p59 and other FKBPs may function as molecular chaperones.[58]

The Classes of Estrogen Receptor Ligands

The transcriptional activity of the ER is affected by each of three types of ligands; small molecules, target DNA and receptor-associated proteins. Classic models predict that the biological activity of a ligand is a direct reflection of its affinity for the receptor. This remains an important aspect of ER action. It is now only part of a larger picture in which high-affinity interactions of DNA and other proteins associated with the receptor must also be considered . Since target DNA and associated proteins demonstrate specific, high affinity interactions with ER they are by definition receptor ligands. It is the result of these three interactions which ultimately determines the biological activity of an ER ligand.[53]

Agonists. Estrogen modulates critical physiological processes, namely, differentiation, female reproductive function, osteoblast and osteoclast biology, lipid homeostasis and cholesterol homeostasis. The biological importance of estrogen is manifest in certain disease states. Osteoporosis is related to decreased ovarian function and decreased circulating estrogen levels. Prolonged estrogen exposure has been implicated in the progression of breast cancer and abnormalities of uterine function typically observed in endometriosis.

The agonist effects of estrogen are mediated by ER binding. The estrogen receptor (ER), like other members of the steroid hormone receptor superfamily, is a ligand-activated nuclear transcription factor. When not bound to hormone the ER lies in a transcriptionally inactive macromolecular complex within the nucleus of target cells. Interaction with estrogen results in a conformational change within the receptor which leads to the displacement of hsp90 and p59 and permits dimerization with another ER. The activation process promotes the interaction of the receptor dimers with specific estrogen response elements located within the regulatory regions of target promoters. Depending on the cellular milieu and the type of promoter, the ligand-activated receptor can interact with the general transcription apparatus directly or indirectly via adaptor proteins. These interactions stabilize the transcription preinitiation complex and enhance

RNA polymerase activity. Several rounds of phosphorylation of the receptor have been shown to occur during activation. It is not yet known if phosphorylation has a specific role in ER signaling.[36]

Compounds have been designed to interact with ER and interfere with its signaling pathway thus influencing the progression of certain disease states, particularly breast cancer.

Partial Agonists. Tamoxifen and its more potent metabolite 4-hydroxytamoxifen are nonsteroidal compounds that competitively block the estrogen receptor. Tamoxifen promotes high affinity binding of ER to DNA but inhibits transcriptional activation by the receptor.

Tamoxifen is a *partial* agonist of the estrogen receptor and thus has different physiologic effects depending on the organ system. This property proves beneficial to the cardiovascular and skeletal systems. Reductions in total cholesterol, low density lipoproteins and the incidence of myocardial infarction are seen with tamoxifen. Tamoxifen also preserves bone mineral density in postmenopausal women. Other organs are adversely affected by the partial agonist properties of tamoxifen. An increase in thromboembolic episodes and a decrease in antithrombin III levels have been seen with tamoxifen use. Tamoxifen may promote the growth of preexisting endometrial cancer.

Tamoxifen was initially used in the palliative treatment of advanced breast cancer in postmenopausal women. Numerous randomized trials now show that adjuvant tamoxifen improves relapse-free and overall survival for early node-negative and positive breast cancer. These trials have also demonstrated a reduction in contralateral breast cancers among women receiving tamoxifen compared to controls, and this has led to clinical trials evaluating tamoxifen as a preventive agent.

The limiting factor in the long-term use of tamoxifen is the development of drug resistance. Although the classical theory dictates that recurrence results from the development of estrogen receptor negative metastases, there are currently alternate explanations for the failure of tamoxifen. The estrogen-like properties of tamoxifen appear to encourage the growth of estrogen receptor positive tumors that are responsive to either tamoxifen or estradiol.

The development of resistance and potential adverse side effects have led to the development of novel agents which can interfere with estrogen receptor signaling. Raloxifene is a breast selective antiestrogen which functions as a partial agonist in bone and in the cardiovascular system but does not promote uterine proliferation.

Antagonists. The development of "pure antiestrogens" is an important advance in the endocrine treatment of breast cancer. In the mid-1970's Dr. Alan Wakeling and colleagues at Zeneca in England began a search for a compound which would recognize the estrogen receptor but *completely* antagonize its action. This led to the discovery of ICI 164,384 and ICI 182,780. The ICI compounds bind to the estrogen receptor with kinetics similar to estradiol and competitively inhibit estradiol binding.

There is general agreement that pure antiestrogens block transcriptional activity via the estrogen receptor; however the mechanism of how this is achieved has been the subject of debate. There are several theories. Some studies demonstrate that ICI 164,384 prevents the estrogen receptor from binding DNA, thus interfering with transcription. This interference is thought to be secondary to impaired receptor dimerization which is

required for DNA binding. A second mechanism of action is the reduction of cell and tissue levels of estrogen receptor. It has been shown that ICI treatment reduces nuclear and cytoplasmic ER protein in mouse uterine and human breast cancer cells in a dose-dependent manner. By comparison, estrogen and tamoxifen increase estrogen receptor levels. Thus ICI induces a disappearance of the estrogen receptor from target tissues either by reduced shuttling from cytoplasm to nucleus or by increased cytoplasmic degradation.

There have been promising results from clinical trials involving these compounds. They are effective against tamoxifen-resistant tumors. No serious side effects have been reported in healthy volunteers or in breast cancer patients participating in clinical trials. The ICI compounds are pure antiestrogens in every organ studied. They do not increase the endometrial or myometrial volume in monkeys and humans. They have no detrimental effects on the liver or the serum lipid profile.

TAFs influence ligand activity. The two activation functions of the ER are important in determining whether a compound acts as an agonist or an antagonist. It has been proposed that the partial agonistic activities of certain antiestrogens originate from the TAF-1 function. It has been shown that estradiol acts as an ER agonist in contexts where either or both TAFs are required. In contexts where TAF-1 alone is required, tamoxifen behaves as a partial agonist manifesting 30-40% the agonist activity of estradiol. Thus tamoxifen is a TAF-1 agonist. In contexts where TAF-2 is required either alone or with TAF-1, tamoxifen behaves as an ER antagonist. Thus tamoxifen has been defined as a TAF-2 antagonist. The pure anti-estrogen ICI 182,780 inhibits the activity of both TAF-1 and TAF-2 and may also be involved in receptor degradation.

The Effects of Estrogen on the Cell Cycle

Estrogen is mitogenic in breast cells because it accelerates progression through the G_1 phase of the cell cycle.[59,60] It has been documented, *in vivo*, that estrogen can recruit non-cycling G_0 cells into the cell cycle.[61] The cell cycle phase-specific effects of estrogens have focused attention on the role of estrogen and its receptor in the control of key regulatory processes controlling the entry into, progression through, and exit from the G_1 phase of the cell cycle.

Estrogen regulates c-myc expression. C-myc is a central regulator of cellular proliferation and apoptosis. It encodes a nuclear phosphoprotein of the basic helix-loop-helix family of transcription factors which is required for mitogenic signaling by growth factor receptors. The mitogenic, apoptotic and oncogenic functions of c-myc depend upon dimerization with the heterologous protein Max, DNA-binding and transactivation.[70] This suggests that c- myc transforms cells by activating genes involved in cell proliferation and /or apoptosis.

In fibroblasts inhibition of c-myc can cause G_1 cell cycle arrest. C-myc is rapidly upregulated by estrogen in breast cancer cells suggesting a similar importance in estrogen-regulated cell cycle progression.[67] Regulation of the proto-oncogene c-myc is one of the earliest detectable responses to estrogens and antiestrogens. The response is apparent within thirty minutes.[69] C-myc antisense oligonucleotides inhibit estrogen-stimulated breast cancer cell proliferation providing strong evidence that c-myc is likely to play a key role in estrogen action.[71]

Estrogen stimulates cyclin D1. Cyclin D1 which binds to and activates Cdk4 and Cdk6 has been implicated in estrogen-induced cell cycle progression. The D-type cyclins are induced as delayed-early response genes by a variety of mitogens in many cell types. Removal of growth factors during G_1 leads to rapid downregulation of D-type cyclins.[73] This response is consistent with the notion that these cyclins act as mitogenic sensors linking extracellular signals with cell cycle progression.

Cyclin D1 is essential in mammary gland development as is demonstrated by the absence of lobular-alveolar structures in mice with disruptions of the cyclin D1 gene.[74,75] In breast cancer cells D-type cyclins appear to play a role in mediating the effects of a diverse group of mitogens including growth factors and steroid hormones. The abundance of cyclin D1 declines rapidly following exposure to growth inhibitory antiestrogens.

Estradiol treatment of MCF-7 breast cancer cells that have been growth arrested by a variety of strategies is followed by pronounced increases in cyclin D1 protein expression beginning within three hours and attaining maximum levels after six to ten hours.[76-79]

Biochemical Effects of Rapamycin

Rapamycin is a macrocyclic triene antibiotic produced by Streptomyces hygroscopicus that was isolated from a soil sample collected from Easter Island (Rapa Nui). In addition to its potent antifungal activities, rapamycin is a potent antitumor and immuno-suppressive agent. Phase III clinical trials are currently underway to evaluate the use of rapamycin for the treatment of solid organ transplant rejection. Rapamycin has no known end organ toxicity.

Rapamycin has inhibitory effects on several different cell types. It inhibits cytokine-driven proliferation of activated T cells. Rapamycin inhibits the proliferation of B cells by decreasing IgM, IgG, and IgA production. It also blocks the effects of growth factors on nonlymphoid cells (i.e. smooth muscle cells, hepatocytes and fibroblasts).

Direct intracellular targets of rapamycin. To exert its effects rapamycin must bind to immunophilins (FKBPs). The target of the rapamycin/FKBP complex is mTOR (**m**ammalian **t**arget **o**f **r**apamycin). Inhibition of mTOR blocks IL2-mediated signal transduction pathways in T cells and prevents cell cycle progression from G_1 phase to S phase in T lymphocytes, osteosarcoma cells, myogenic cell lines and smooth muscle cells.

The FKBP/rapamycin/mTOR complex inhibits the mitogen-induced activation of the ribosomal protein kinase p70S6k. The inhibitory effects of rapamycin are highly specific as the closely related p90RSK and MAP kinase cascade are not inhibited. In addition, rapamycin has no effect on the early response genes c-fos, c-jun and c-myc.

Rapamycin inhibits translation. The rapamycin-mediated effects of p70S6k inactivation have dramatic effects on the translation of a particular class of mRNA transcripts that contain a polypyrimidine tract immediately after their 5' N7-methylguanosine cap (5'TOP). In one study rapamycin treatment inhibited only 10 to 15% of total protein synthesis in mitogen-stimulated 3T3 cells. Rapamycin-induced inhibition was selective for ribosomal proteins and the eEF-2 elongation factor for protein synthesis. The translation of non-5'TOP containing mRNAs coding for βactin, protein synthesis initiation factor eIF4A and β-tubulin were not affected. It has been proposed

that the inhibition of ribosomal protein synthesis by rapamycin results in the prolongation of G1 phase of the cell cycle.

Rapamycin inhibits transcription. Transcriptional events are also blocked by rapamycin as a consequence of p70S6k inhibition. p70S6k-mediated activation of CREMτ, a member of the cAMP response element binding (CREB/ATF) family of transcription factors, is blocked by rapamycin thereby affecting cAMP-induced late gene transcription.

Rapamycin effects on the cell cycle. Rapamycin dramatically reduces the kinase activity of the cdk4/cyclin D and cdk2/cyclinE complexes that normally peak in mid to late G1. This inactivation involves a change in their stoichiometry with the cyclin dependent kinase inhibitors, p21 and p27kip1. Rapamycin blocks the elimination of p27kip1. Consequently downstream events including hyperphosphorylation of the Rb:E2F complex are inhibited thereby resulting in decreased synthesis of the cell cycle proteins cdc2 and cyclin A.

Rapamycin as an antineoplastic agent

Rapamycin was first cited in the literature as an anti-tumor agent in 1984. The NCI conducted the initial studies and reported modest activity against lymphocytic leukemia, melanocarcinoma, colon carcinoma, ependymoblastoma, and breast carcinoma. They found that intramuscular, subcutaneous and intraperitoneal routes of administration were equally effective (93% tumor inhibition) and more effective than oral administration (65% tumor inhibition). It was also noted that injections close to the tumor (91% tumor inhibition) were more effective that distant injections (85% tumor inhibition). Treatments started the day after tumor implantation. The treatment schedule varied from a maximum of 9 injections to a minimum of 2 injections. Rapamycin was capable of inhibiting growth at any stage of tumor development, though it was more effective at earlier stages. Delay of treatment by 6 days, 13 days and 20 days showed 81%, 76% and 55% tumor growth inhibition respectively.

Nude mouse tumor xenograft model. Thus far rapamycin has not been tested as an antineoplastic agent *in vivo* in an estrogen-dependent tumor xenograft model. In 1980 Soule and McGrath published the results of experiments in which they injected MCF-7 cells into athymic nude mice and implanted subcutaneous estradiol pellets to stimulate tumor growth. In the late 1980's researchers began to use this system to study the effects of antiestrogens on tumor growth and the development of antiestrogen drug resistance. Studies by Osborne et al. showed that estrogen withdrawal or tamoxifen treatment resulted in cessation of tumor growth but not significant tumor regression. Extended antiestrogen treatment eventually results in the regrowth of tumors by mechanisms which are not clear.

Rapamycin as an angiogenesis inhibitor

The proliferation and survival of cells in a tumor are dependent on an adequate supply of growth factors and the removal of toxic molecules. Oxygen delivery to tumors is impaired when the distance from a capillary to a tumor cell exceeds 150 to 200 μm. When this distance is exceeded cell death is accelerated. Angiogenesis overcomes these

limitations and is required for tumor growth and metastasis. A tumor mass will expand to a diameter of only 1 or 2 mm if angiogenesis is disrupted.

Tumor cells and host cells (including endothelial cells, mesothelial cells and leukocytes) secrete factors that stimulate angiogenesis. Among these molecules are basic fibroblast growth factor (bfgf) and vascular endothelial cell growth factor (vegf). The extent of angiogenesis is determined by the balance between factors that stimulate and those, like thrombospondin, that inhibit new blood vessel growth. In many normal tissues the inhibitory influence predominates. Vascular endothelial cells are normally quiescent. In contrast, many neoplastic cells "switch" from an angiogenesis-inhibiting phenotype to an angiogenesis-stimulating phenotype. The predominant stimulating factors act on microvascular endothelial cells to generate capillary sprouts. The "activated" endothelial cells proliferate, migrate, and penetrate host stroma. The direction of endothelial cell migration points toward the angiogenic stimulus. The capillary sprout expands and undergoes morphogenesis to yield a capillary.

There is a two compartment model of tumor growth which has been advanced by Judah Folkman. Endothelial cells secrete factors which stimulate tumor cell growth. In a similar manner, endothelial cell survival is dependent on tumor-derived endothelial mitogens. If the endothelial cells are made unresponsive to angiogenic stimuli from the tumor cells by the administration of specific inhibitors both primary tumors and metastatic tumors can be held dormant. Therefore, both compartments can be targeted by one agent which is both antiangiogenic and cytotoxic or by two separate agents directed against each department.

This model explains the action of certain agents like taxol which are effective long after they should have induced drug resistance. After many cycles of administration taxol is still effective. This occurs because the tumor compartment has become drug resistant while the endothelial compartment remains sensitive. Taxol has been shown to inhibit angiogenesis *in vivo.* It also inhibits endothelial proliferation, chemotaxis and invasiveness *in vitro.* Various other reports have shown that epirubicin, doxorubicin, and mitoxantrone hydrochloride, cyclophosphamide are antiangiogenic. Cyclosporine, an immunosuppressant like rapamycin, has been shown to inhibit endothelial sprouting.

Experimental Methods/Preliminary Data

We have been studying the effects that rapamycin has on the proliferation, mitogenesis and transcription of the estrogen receptor positive breast cancer cell lines MCF-7 and BT474.

Aim 1. A. To show that rapamycin inhibits ER+ breast cancer cellular proliferation. An XTT based cytotoxicity assay was used to measure the IC_{50} needed for MCF-7 and BT-474 inhibition by rapamycin. **B.** Fluorescence activated cell sorting (FACS) was used to follow the effect that rapamycin exerts on cell cycle progression of G_0-arrested ER+ breast cancer cell lines that have been stimulated with various mitogenic agents. Thymidine uptake assays will be performed to confirm the FACS findings.

Aim 1A.

Purpose. Prior to determining what effect, if any, rapamycin would have on ER+ breast cancer cells it was important to determine the degree to which the drug was cytotoxic to the cell lines of interest (MCF-7 and BT474). From this data a working range of the drug to be used in subsequent experiments could be determined.

Research Design and Methods

XTT Assay. An XTT based cytotoxicity assay was used to measure the IC_{50} needed for MCF-7 and BT474 inhibition by rapamycin. We employed a commonly used colorimetric assay which was first described by Scudiero in 1988 for the measurement of drug sensitivity in tumor cell lines.[108] The assay utilizes a tetrazolium salt, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT), which is useful for the quantification of living metabolically active cells. XTT is metabolized by mitochondrial dehydrogenases to form an orange formazan dye which is soluble in aqueous solution. The addition of the electron-coupling agent, phenazine methosulfate (PMS), markedly enhances the cellular reduction of XTT. The absorbance strongly correlates with the cell number. An increase in the number of living cells increases the overall activity of mitochondrial dehydrogenase in the sample. This increase directly correlates with the amount of orange formazan formed as monitored by the absorbance as detected by an automated microtiter plate reader.

5×10^3 cells per well (in a volume of 200 µl) were seeded into a 96-well microtiter plate in RPMI 1640 (Gibco BRL) media with 10% fetal bovine serum (Gibco BRL) and allowed to attach over night in a humidified atmosphere (37°C, 5% CO2). Row A of the plate was not seeded with cells so that background absorbance could be measured. Rows B through G contained 10x dilutions of rapamycin in RPMI media beginning with 1000 ng/ml. The drug treatments were applied in quadruplicate. Row H contained no drug.

The plates were incubated at 37°C for 7 days at which time the cells had reached confluency. The media was not changed during the incubation period. After the incubation period the media was exchanged with 100 µl of serum-free, phenol red-free RPMI 1640 media and incubated under humidified conditions while the assay solution was being prepared.

XTT (Polysciences, Inc) was added to serum free, phenol red free RPMI 1640 media at a concentration of 1mg/ml. The mixture was warmed to 37°C for twenty minutes until it progressed from a cloudy solution to a clear solution. A 1.5 mg/ml stock solution of PMS (Sigma) in PBS was made. PMS was added to the XTT solution at a ratio of 10 µl of PMS per ml of XTT. 50 µl of the PMS/XTT solution were added to each well of the microtiter plate. The plate was incubated in a humidified atmosphere (37°C, 5% CO2) for four hours. Afterwards the plate was shaken on an orbital shaker for thirty minutes. The plate was read on a microtiter plate reader (Bio-Tek Instruments, Microplate Autoreader) at 540nm.

The background measurement was subtracted from each quadruplicate reading. The corrected quadruplicate readings were averaged.

Data/Preliminary Results.

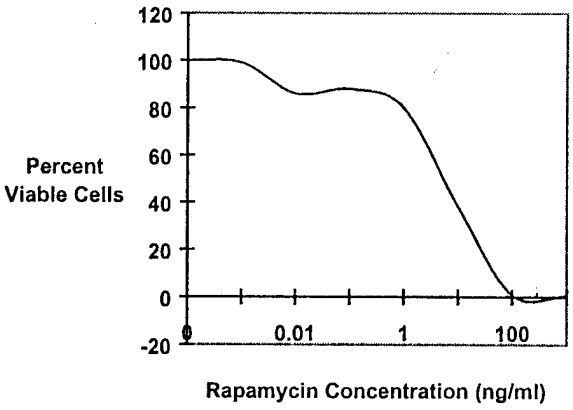

Figure 1. MCF-7 proliferation curve. MCF-7 cells were grown in the presence of tenfold dilutions of rapamycin for one week. The number of viable cells was determined by XTT assay.

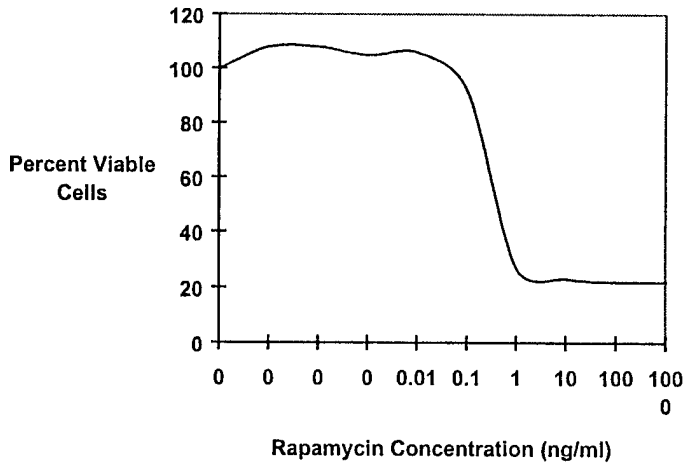

Figure 2. BT474 proliferation curve. BT474 cells were grown in the presence of tenfold dilutions of rapamycin for one week. The number of viable cells was determined by XTT assay.

Interpretation. *Rapamycin inhibits proliferation of ER+ breast cancer cells.* The IC_{50} for inhibition of MCF-7 cells by rapamycin is 10 ng/ml **(Figure 1)**. The IC_{50} for inhibition of BT474 cells with rapamycin is 0.5 ng/ml **(Figure 2)**. The BT474 cells are more sensitive to rapamycin than the MCF-7 cells.

From this assay we determined that at extremely high concentrations (> 1ng/ml) of rapamycin are cytotoxic to MCF-7 and BT474 cells. Lower concentrations of rapamycin

support 90-100% cell viability in both cell lines. Due to the high viability rates of both cell lines at .01 ng/ml we selected that concentration for our further studies.

Aim 1B.

Purpose. The effects of estrogen and rapamycin individually on the cell cycle are well documented and have been described in the background of this proposal. What is not well represented in the literature is the effect that rapamycin has on G_0-arrested estrogen receptor positive breast cancer cells which have been stimulated to proceed through the cell cycle by estrogen. After determining an appropriate concentration of rapamycin to use **(Aim 1A)** a FACS experiment was designed to address this question.

Research Design and Methods

Preparation of cells for FACS analysis. 3.5×10^5 cells per well were seeded into 6-well plates RPMI 1640 media containing 10% fetal bovine serum in a humidified incubator. After the cells attached, the media was exchanged for serum-free, phenol red-free media. The cells starved for twenty-four hours. The cells were then treated with 17β-estradiol (Cal Biochem) at 2.5×10^{-8} M or estradiol plus rapamycin (New Brunswick Scientific) at .01 ng/ml for an additional twenty-four hours. In subsequent studies estradiol was replaced with 10% serum or 1 ng/ml IGF-1. The treatments were applied in triplicate.

The cells were pulse labeled for 30 minutes with 10 μM BrdU (Boehringer Mannheim). A BrdU negative well was included as a negative control for autofluorescence. The cells were washed with PBS (37°C) and trypsinized (0.25% Trypsin, 1mM EDTA, Gibco BRL). The trypsin was inactivated with media containing 10% serum. The cells were pelleted by centrifugation (1500 x g for 2 minutes at 4°C). The cells were washed once with 3 ml of cold PBS by gentle vortexing. The cells were pelleted again. The cells were resuspended in 300 μl of cold PBS by gentle vortexing. 700 μl of cold (0-4 °C) ethanol were titrated slowly into the cell suspension. The cells were incubated at 4°C for thirty minutes.

The cells were pelleted and washed with PBS. The cells were resuspended in 1 ml of 2N HCl containing 0.2 mg/ml pepsin (Sigma) and incubated for thirty minutes at room temperature. 3 ml of 0.1 M sodium tetraborate pH 8.5 were added and the cells were pelleted by centrifugation (1500 x g for 2 minutes at 4°C). The cells were washed with PBS and pelleted again. The cells were washed with 0.5 ml of PBS/0.5% Tween 20 (Sigma)/2% mouse serum (Pierce) and pelleted. The cells were resuspended in 100 μl PBS/Tween 20/mouse serum containing an anti BrdU-FITC monoclonal antibody (Becton Dickinson) (15 μl antibody per 100 μl PBS/Tween 20/mouse serum) and incubated for 30 minutes at room temperature. 0.5 ml of PBS/Tween 20 was added to the cells which were centrifuged (1500 x g for 2 minutes at 4°C). The cells were washed with PBS and pelleted. The cells were counter-stained by resuspension in 0.5 ml of 50 ug/ml of propidium iodide (Boehringer Mannheim) /0.5 mg/ml Rnase (Sigma). The cells were incubated at 4°C for at least 30 minutes before being loaded onto the fluorescence activated cell sorter.

Data/Preliminary Results

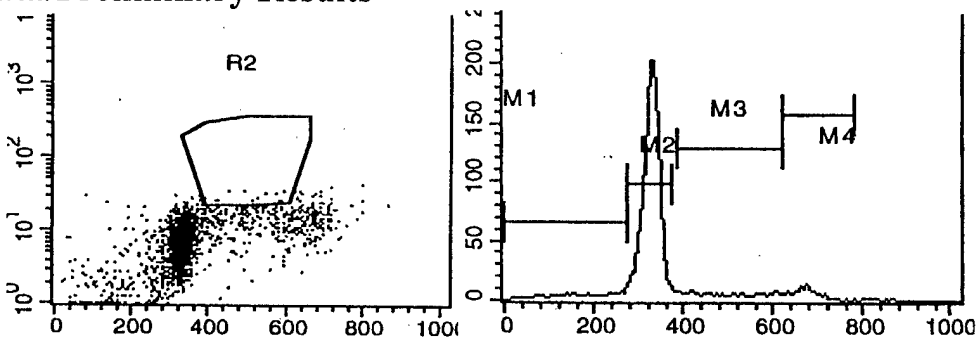

Figure 3A. MCF-7 cells arrested in G_0 by serum deprivation.

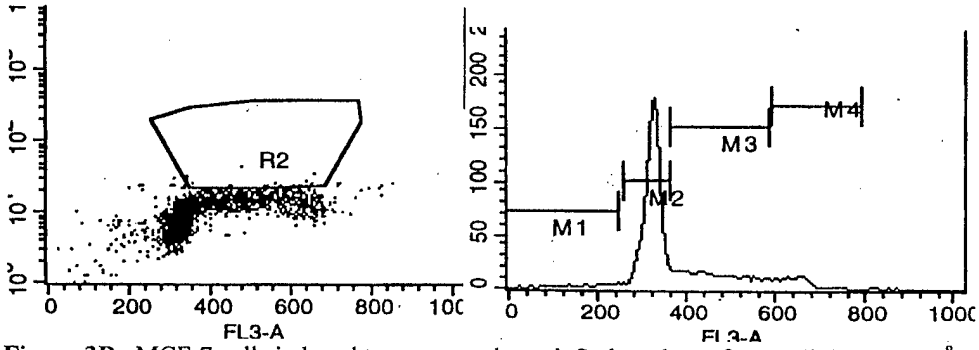

Figure 3B. MCF-7 cells induced to progress through S phase by 17β-estradiol (2.5×10^{-8} M).

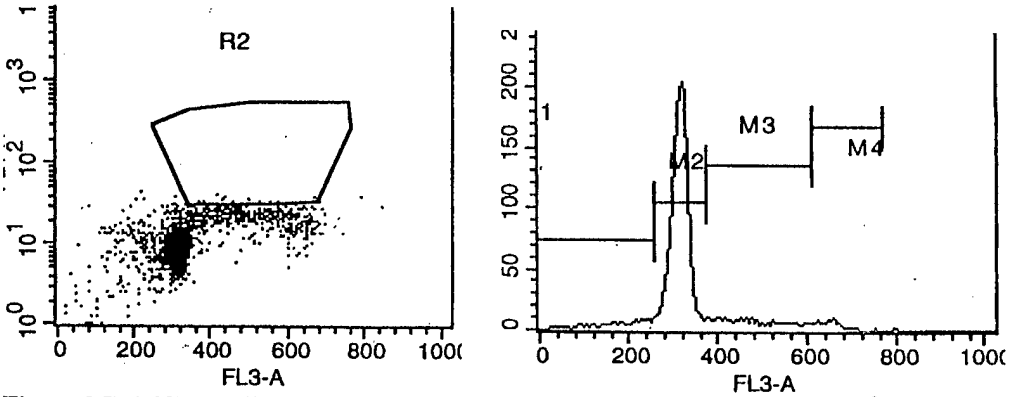

Figure 3C. MCF-7 cells arrested in G0 after exposure to 17β-estradiol (2.5×10^{-8} M) and rapamycin (.01 ng/ml).

Table 1. Percentages of MCF-7 cells in G0 and S after indicated treatment.

Treatment	%G0/G1	%S
Serum-starved	76	9.26
Estrogen-induced	65	25
Estrogen + Rapamycin	78	12

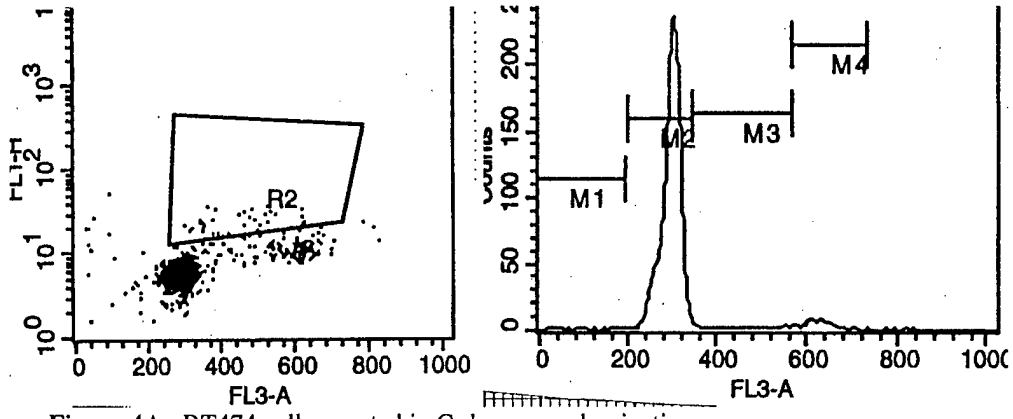

Figure 4A. BT474 cells arrested in G_0 by serum deprivation.

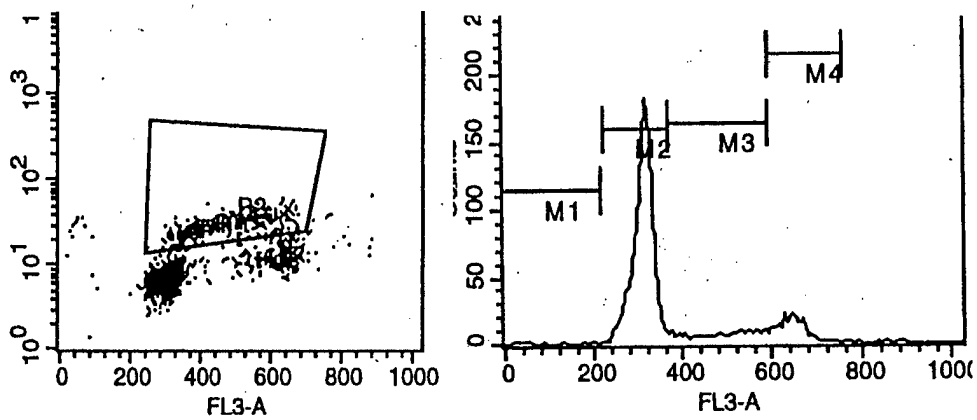

Figure 4B. BT474 cells induced to progress through S phase by 17β-estradiol (2.5×10^{-8} M).

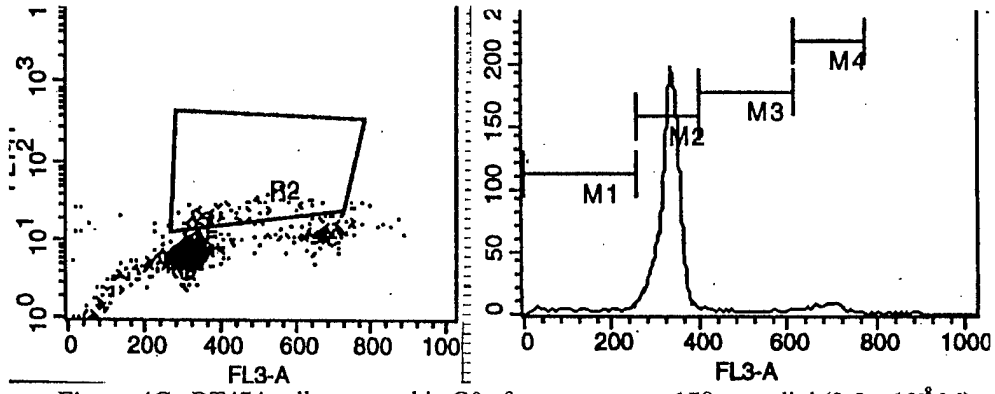

Figure 4C. BT474 cells arrested in G0 after exposure to 17β-estradiol (2.5×10^{-8} M) and rapamycin (.01 ng/ml).

Table 2. Percentages of MCF-7 cells in G0 and S after indicated treatment

Treatment	%G0/G1	%S
Serum-starved	86	3.75
Estrogen-induced	72	13
Estrogen + Rapamycin	87	6

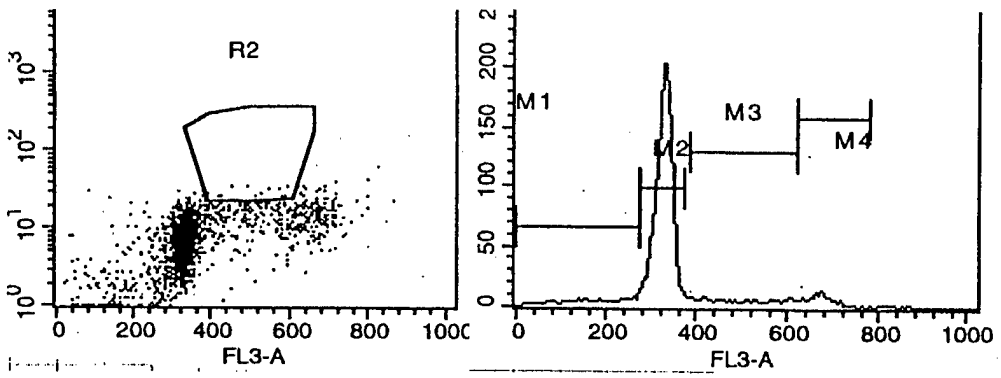

Figure 5A. MCF-7 cells arrested in G_0 by serum deprivation.

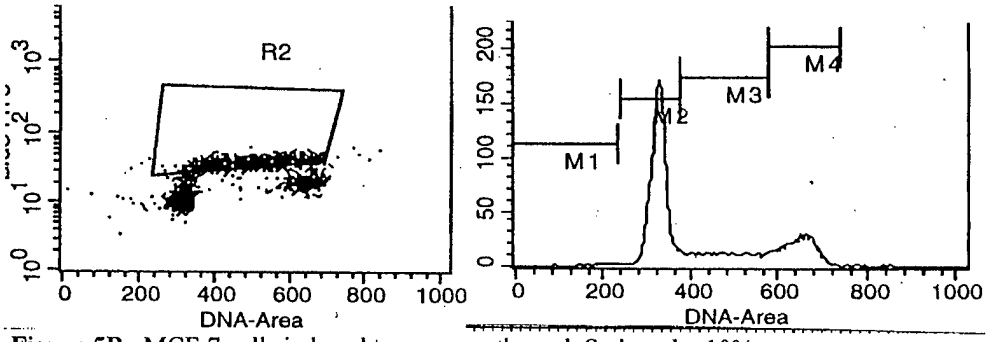

Figure 5B. MCF-7 cells induced to progress through S phase by 10% serum.

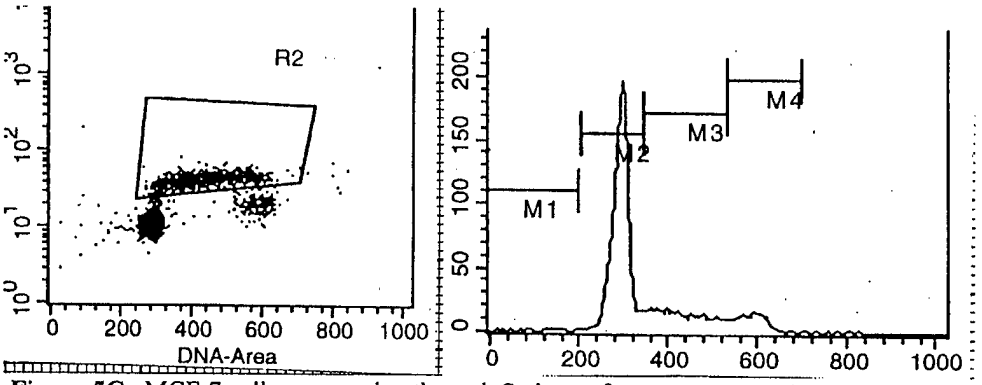

Figure 5C. MCF-7 cells progressing through S phase after exposure to 10% serum and rapamycin (.01 ng/ml).

Table 3. Percentages of MCF-7 cells in G0 and S after indicated treatment

Treatment	%G0/G1	%S
Serum-starved	76	9.26
Serum-induced	41.8	48
Serum + Rapamycin	51	41

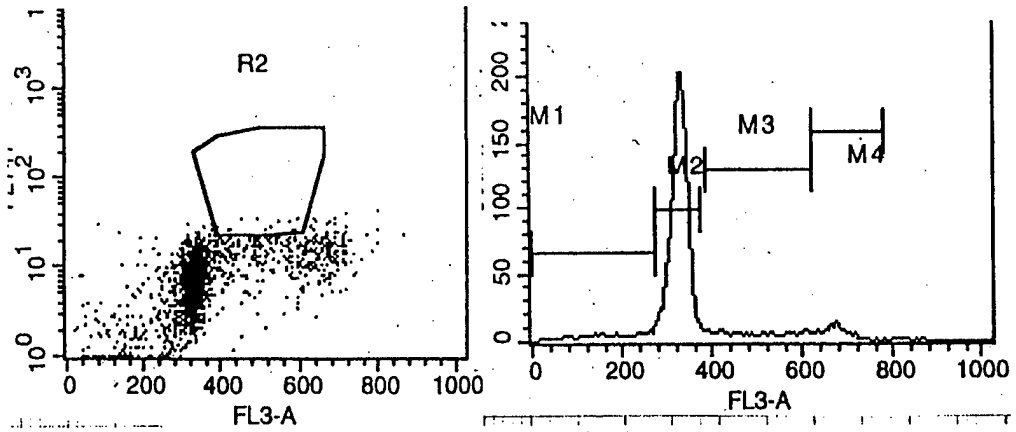

Figure 6A. MCF-7 cells arrested in G_0 by serum deprivation.

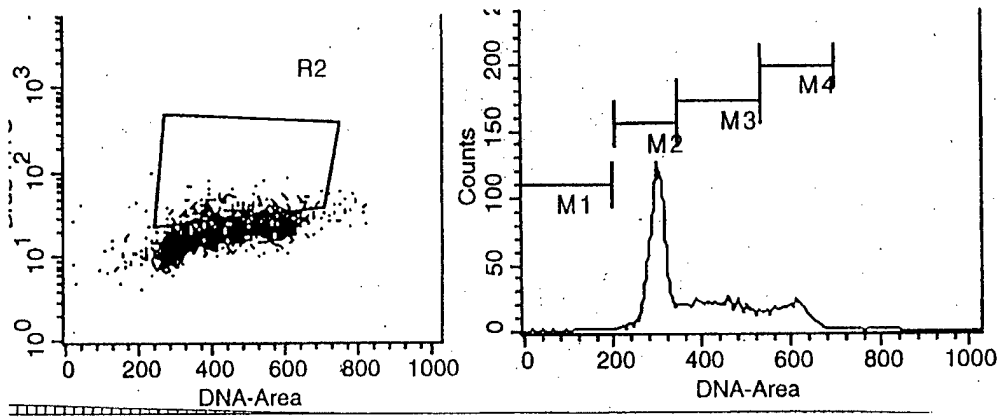

Figure 6B. MCF-7 cells induced to progress through S phase by IGF-1 (1ug/ml).

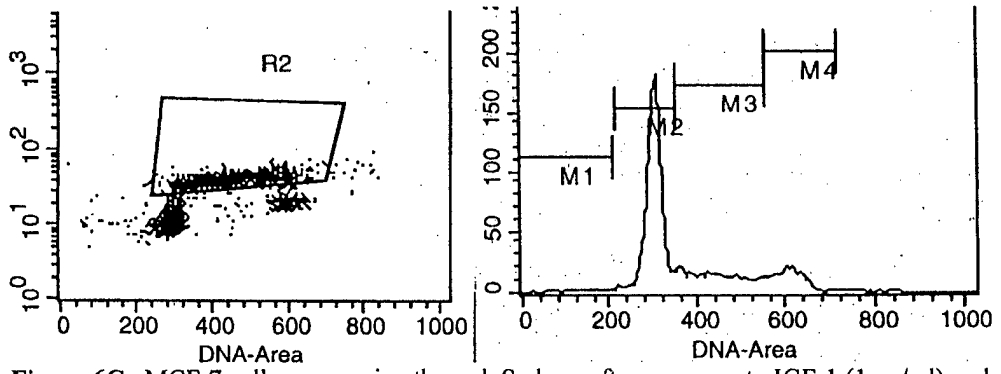

Figure 6C. MCF-7 cells progressing through S phase after exposure to IGF-1 (1 ug/ml) and rapamycin (.01 ng/ml).

Table 3. Percentages of MCF-7 cells in G0 and S after indicated treatment

Treatment	%G0/G1	%S
Serum-starved	76	9.26
IGF1-induced	44.2	33
IGF1 + Rapamycin	64.5	20.6

Interpretation. *Rapamycin inhibits estrogen-induced mitogenesis in ER+ breast cancer cells.* By FACS analysis MCF-7 cells which have been arrested in G_0 by serum deprivation (76 % of cells in G_0, 9.26% of cells in S phase, **Figure 3A and Table 1**) progress through S phase after the addition of 2.5 $x10^{-8}$ M 17β-estradiol (65% of cells in G_0, 25% of cells in S phase, **Figure 3B and Table 1**). The addition of .01ng/ml of rapamycin in the presence of estradiol prevents S phase progression (78% of cells in G_0, 12% of cells in S phase, **Figure 3C and Table 1**). BT474 cells which have been arrested by serum deprivation (86% of cells in G_0, 3.75% of cells in S phase, **Figure 4A and Table 2**) also progress through S phase after exposure to 17β-estradiol (72% of cells in G_0, 13% in S phase, **Figure 4B and Table 2**). Rapamycin in the presence of estradiol prevents S phase progression (87% of cells in G_0, 6% in S phase, **Figure 4C and Table 2**). These results support the hypothesis that rapamycin has antiestrogenic effects on breast cancer cell lines *in vitro.*

Rapamycin does not inhibit serum-induced mitogenesis or IGF-1- induced mitogenesis in ER+ breast cancer cells. MCF-7 cells which have been arrested in G_0 by serum deprivation (76% of cells in G_0, 9.26% in S phase, **Figure 5A and Table 3, Figure 6A and Table 4**) can be induced to progress through S phase by the addition of 10% fetal calf serum (41.8% of cells in G_0, 48% of cells in S phase, **Figure 5B and Table 3**) and 1 μg/ml insulin-like growth factor-1 (IGF-1) (44.2% of cells in G0, 33% in S phase, **Figure 6B and Table 4**). The addition of rapamycin in the presence of these non-estrogenic growth factors *does not inhibit* progression through S phase. These data support the hypothesis that rapamycin specifically inhibits estrogen-mediated mitogenesis.

Future Direction. More confirmatory data is required to support the finding that the inhibitory effects of rapamycin on ER+ breast cancer cells are specific for estrogen-mediated mitogenesis. Documenting the effect of rapamycin on mitogenesis in an ER negative breast cancer cell line (e.g. SKBR3) would contribute toward this objective. The FACS results will be confirmed by measuring ^{3}H-thymidine incorporation in ER+ breast cancer cell lines in the presence of estrogen, estrogen + rapamycin, as well as other mitogenic factors. This will allow progression through S phase to be followed and the exact duration of S phase to be measured.

The effect of rapamycin on cell cycle mediators will be investigated in further detail to determine if rapamycin has the same effects on the cell cycle in breast cancer cells as it has in lymphocytes, the cell type in which it has been most widely studied. Preliminary western blot data shows that, as expected, rapamycin causes a delay in the accumulation of cyclin D1, which is rapidly induced by estrogen in cycling cells **(Figure 7)**. Western blots were also used to show that $p27^{kip1}$ protein levels are stabilized in breast cancer cells treated with rapamycin, as would be expected in cells that are arrested in G_0 **(Figure 8).**

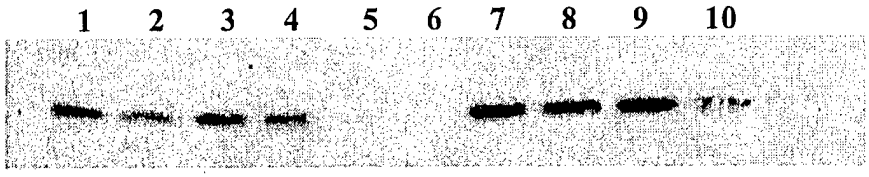

Figure 7. *Rapamycin delays the increase in cyclin D1 seen during estrogen-mediated mitogenesis.* Western blot of cyclin D1 protein levels in MCF-7 cells that were serum starved for 24 hours and treated with either 17β-estradiol or 17β-estradiol and rapamycin for 24 hours. Cells were harvested for protein extraction at 6 hour intervals. **Lane 1** Estradiol, 6 hrs. **Lane 2** Estradiol + rapamycin, 6 hrs. **Lane 3** Estradiol, 12 hrs. **Lane 4** Estradiol + rapamycin, 12 hrs. **Lanes 5 and 6**, empty. **Lane 7** Estradiol, 18 hrs. **Lane 8** Estradiol + rapamycin, 18 hrs. **Lane 9** Estradiol, 24 hrs. **Lane 10** Estradiol + rapamycin, 24 hrs. Estrogen causes an increase in cyclin D1 levels over time which allows the cells to progress through the cell cycle. Rapamycin inhibits cell cycle progression by causing a delay in the increase of cyclin D1. During the earliest time points, six hours and twelve hours, the cyclin D1 protein levels were lower in the cells treated with estrogen and rapamycin when compared with the cells treated with estrogen alone. At the later time points the levels appear to have equalized.

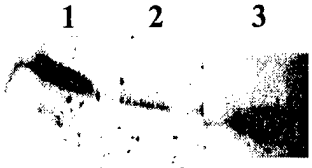

Figure 8A.

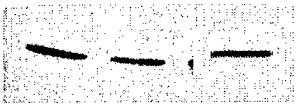

Figure 8B.

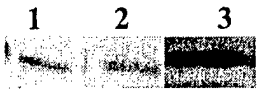

Figure 8C.

Figure 8D.

Figures 8. *Rapamycin stabilizes p27kip1 levels.* Western blot of $p27^{kip1}$ protein levels in BT474 cells **(A)** and MCF-7 cells **(B)**. Actin levels are shown in **C** and **D**. Cells which have been arrested in G_0/G_1 via growth factor deprivation or treatment with a cell cycle inhibitor have a stabilization of p27kip1 levels. Cycling cells which have been stimulated with estrogen have decreased levels of p27kip1 **(Lane 2)**. Cells which have been starved **(Lane 1)** or treated with rapamycin **(Lane 3)** have increased levels of $p27^{kip1}$. **Lane 1** Serum starvation, 24 hrs. **Lane 2** Serum starvation for 24 hrs followed by estradiol treatment for 24 hrs. **Lane 3** Serum starvation for 24 hrs followed by estradiol and rapamycin treatment for 24 hrs.

Northern blots will be used to measure the appearance of the early response g enes c-fos, c-myc, and c-jun upon exposure of ER+ cells to estradiol and estradiol plus rapamycin. As described in the background of this proposal, estrogen rapidly upregulates these genes while rapamycin reportedly has no effect on them. Northern blot analysis will show if rapamycin is inhibiting estrogen-mediated mitogenesis by downregulating the early response genes or if it is acting via another pathway.

Aim 2. A. We have shown that rapamycin inhibits estrogen-mediated transcription of ER+ breast cancer cells in a transient transfection assay. The mechanism of this inhibition will be investigated. **B.** It has already been determined by Western blot analysis that the inhibition is not occurring via receptor degradation. **C.** Immuno-histochemistry and nuclear/cytoplasmic extraction will be used to follow shuttling of the ligand/receptor complex between the nucleus and the cytoplasm after exposure of ER+ breast cancer cells to estrogen and rapamycin. This will show if transcriptional inhibition is occurring because of the inability of the ligand/receptor complex to translocate to the nucleus. **D.** A gel binding assay will be employed to determine if transcriptional inhibition is occurring because rapamycin is preventing the translocated ligand/receptor complex from binding nuclear DNA.

Aim 2A.

Purpose. It has been documented in the literature that immunosuppressants have either stimulatory or inhibitory effects on steroid receptor-mediated transcription depending on the type of receptor (e.g. progesterone and glucocorticoid). Given the findings described in **Aim 1** that rapamycin exerts an inhibitory effect on cell cycle progression, the effect of rapamycin on estrogen-mediated transcription in ER+ breast cancer cells was investigated.

Research Design and Methods

Transient Transfection Assay. Cells were seeded at 2.5 x 10^5 cells per well and allowed to attach overnight in RPMI 1640 media with 10% fetal calf serum. Transfections were performed with the artificial construct, 3XERE-TATA-Luc, which consists of three estrogen response elements upstream of a minimal promoter driving the transcription of the luciferase gene. Transfections were performed with the Qiagen SuperFect Transfection Reagent at a ratio of 1μg plasmid:2 μl of reagent. The construct CMV-Luc was co-transfected to determine the transfection efficiency. Cells were incubated with the plasmids and the transfection reagent for five hours. Afterwards the media was changed to one of three conditions: 1. serum-free, phenol red-free RPMI 1640 media 2. serum-free, phenol red-free RPMI 1640 media with 17β-estradiol (2 x 10^{-8} M), or 3. serum-free, phenol red-free RPMI 1640 media with 17β-estradiol and rapamycin (.01 ng/ml). The treatments were applied in triplicate. The cells were incubated in the treatment for 48 hours (at 37°C, 5% CO2). The cells were harvested in Passive Lysis Buffer (Pro-Mega) and analyzed for luciferase activity in a luminometer (Turner Designs) utililizing a dual luciferase assay system (Pro-Mega).

Data/ Preliminary Results.

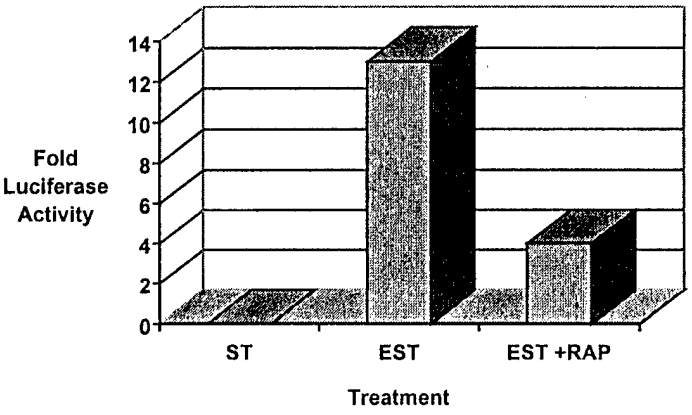

Figure 9. Rapamycin inhibits estrogen-mediated transcription by threefold in MCF-7 cells which have been transfected with the artificial construct 3XERE-TATA-Luc. **ST**=starved, **EST**=estrogen, **RAP**=rapamycin.

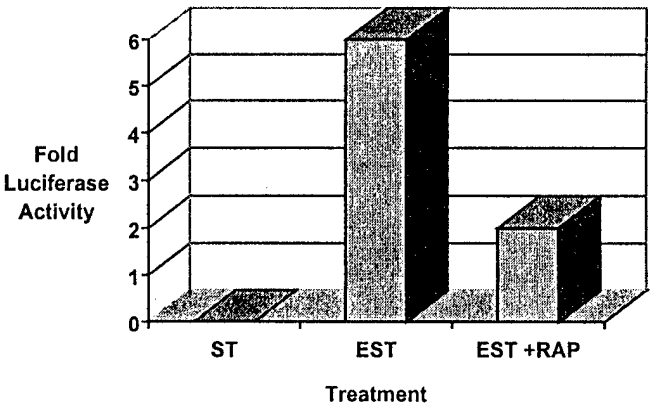

Figure 10. Rapamycin inhibits estrogen-mediated transcription by threefold in BT474 cells which have been transfected with the artificial construct 3XERE-TATA-Luc. **ST**=starved, **EST**=estrogen, **RAP**=rapamycin.

Interpretation. *Rapamycin inhibits estrogen-mediated transcription in ER+ breast cancer cells.* MCF-7 cells which have been transfected with the estrogen-responsive plasmid, 3XERE-TATA-Luc respond to estrogen treatment with a twelvefold increase of luciferase activity when compared with cells which have been starved after transfection **(Figure 9)**. Transfected, estrogen-treated BT474 cells show a sixfold increase of luciferase activity when compared with starved transfected cells **(Figure 10)**. When the transfected cells are exposed to estrogen in the presence of rapamycin the luciferase

activity is fourfold higher than starvation levels and threefold less than estrogen induced levels in MCF-7 cells **(Figure 9)**. In BT474 cells the luciferase activity in transfected cells treated with estrogen and rapamycin is only twofold higher than the starvation level and threefold less than the estrogen treated levels **(Figure 10)**.

Future Direction. An interesting question that follows from this finding is what, if any, co-repressors or co-activators might be binding to the estrogen/ER transcriptional apparatus? Several ER co-repressors and co-activators have been described (e.g. SRC-1, NCoR and SMRT). Ligand-induced conformational changes regulate the interaction of the receptor with co-activators and/or co-repressors. Antagonists allow the strongest association with co-repressors while agonists allow a minimal interaction. A mammalian two hybrid assay has been designed to determine which proteins might interact with the estrogen receptor at this level.

The mammalian matchmaker two-hybrid assay kit by (Clontech) will be used to investigate the possible association of co-repressors with the rapamycin/estrogen/ER complex. Two-hybrid assays are based on the fact that many eukaryotic transcriptional activators consist of two physically and functionally separable domains. These units are a DNA binding domain (DNA-BD) that binds to a promoter and an activation domain (AD) that directs RNA polymerase II to transcribe the gene downstream of the DNA-binding site. These domains can also function as two separate proteins as long as the AD is tethered to the promoter-bound DNA-BD. In two-hybrid assays that tether is created by the interaction of two additional proteins that have been fused to the AD and DNA-BD.

A pM cloning vector will be used to generate fusions of NCoR and SRC-1 to the GAL4 DNA-BD. The PVP16 plasmid will be used to construct fusions of ER to an AD derived from the VP16 protein of herpes simplex virus. $(GAL4)_5$-LUC is a reporter vector which contains the luciferase gene downstream of five consensus GAL4 binding sites. All three plasmids will be co-transfected into ER+ breast cancer cell lines. 48-72 hours later the interaction between proteins X and Y will be assayed by measuring luciferase gene expression in the presence of estradiol +/- rapamycin.

Aim 2B

Purpose. Rapamycin has been shown in the previous aim to inhibit estrogen-mediated transcription of ER+ breast cancer cells. The level at which this inhibition occurs in the ER signaling pathway will be investigated. Published studies have demonstrated that steroidal antiestrogens accelerate estrogen receptor destruction. Since rapamycin is a nonsteroidal compound it would most likely not have any effect on estrogen receptor protein levels or the estrogen receptor turnover rate. To confirm this hypothesis Western blotting was used to investigate what happens to estrogen receptor protein levels at specific intervals upon prolonged exposure to rapamycin *in vitro*.

Research Design and Methods.

Protein extraction and Western blotting. 5×10^6 MCF-7 cells were seeded in eight 10-cm plates and allowed to attach overnight in RPMI 1640 media containing phenol red and 10% fetal bovine serum. The next morning the cells were synchronized by serum deprivation in serum-free, phenol red-free RPMI 1640 media for 24 hours. Four of the

plates were then induced with 17β-estradiol (2 x 10^{-8} M) for 24 hours. The four remaining plates were treated with 17β estradiol and rapamycin (.01 ng/ml) for 24 hours.

At six hour time points over 24 hours (6,12,18 and 24 hours) the cells were harvested for protein extraction. The cells were washed in cold PBS and collected by scraping in a final volume of one ml PBS. The cells were centrifuged in a microcentrifuge (Eppendorf) at 3000 rpm for fifteen minutes at 4°C. The supernatant was aspirated and the pellet was lysed in 200 µl of cold lysis buffer. The lysis buffer consisted of 50mM Tris pH 8.0, 5 mM EDTA, 10mM NaCl and 0.5% NP40 (all reagents from Sigma). The following reagents were added to the buffer: 100mM PMSF, 3mg/ml leupeptin, 2 mg/ml aprotonin, 1M benzamidine and 2 mg/ml trypsin inhibitor (all reagents from Sigma). The lysed cells were centrifuged again in a microcentrifuge at 3000 rpm for fifteen minutes at 4°C. The supernatant (protein lysate) was saved for protein concentration determination by Biorad assay.

100 µg of protein from each time point were separated on a 10% SDS-polyacrylamide gel. 100 µg of protein from the ER negative breast cancer cell line SKBR3 were used as a negative control. The gel was transferred to a nitrocellulose membrane (Optitran) and probed with polyclonal anti-ERα (Santa Cruz). The gel was developed with a chemiluminescence reagent (NEN). The membrane was stripped of the antibody by incubating it with a buffer c
onsisting (100mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7) of for thirty minutes at 50°C with agitation. The stripped membrane was re-probed twice, first with monoclonal anti-cyclin D1 (Santa Cruz) then with monoclonal anti-actin (Boehringer Mannheim).

Data/Preliminary Results

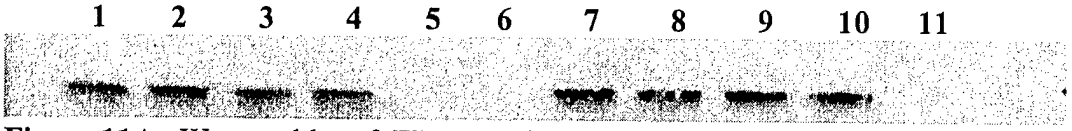

Figure 11A. Western blot of ERα protein levels in MCF-7 cells that were serum starved for 24 hours and treated with either 17β-estradiol or 17β-estradiol and rapamycin for 24 hours. Cells were harvested for protein extraction at 6 hour intervals. **Lane 1** Estradiol, 6 hrs. **Lane 2** Estradiol + rapamycin, 6 hrs. **Lane 3** Estradiol, 12 hrs. **Lane 4** Estradiol + rapamycin, 12 hrs. **Lanes 5 and 6,** empty. **Lane 7** Estradiol, 18 hrs. **Lane 8** Estradiol + rapamycin, 18 hrs. **Lane 9** Estradiol, 24 hrs. **Lane 10** Estradiol + rapamycin, 24 hrs. **Lane 11** SKBR3 protein (ER- cell line).

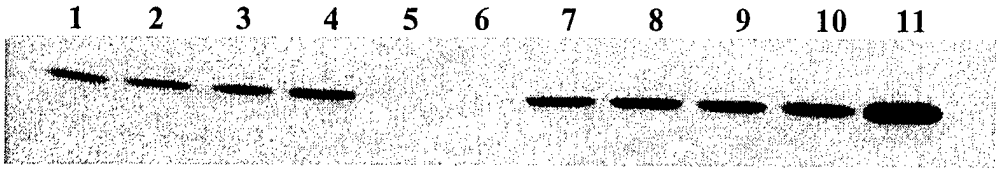

Figure 11B. Actin probe of previous Western blot .

Interpretation. *Rapamycin does not degrade the estrogen receptor at the protein level.* Protein levels of ERα are not diminished by exposure of ER+ breast cancer cells to rapamycin **(Figure 11A)**. Cells exposed to estrogen for six hours when compared with cells exposed to estrogen and rapamycin for six hours equivalent amounts of ER **(Lanes 1 and 2)**. After a longer exposure of 12 hours the protein levels remain equivalent **(Lanes 3 and 4)**. Upon treatment for 18 hours **(Lanes 7 and 8)** and 24 hours **(Lanes 9 and 10)** the levels of ER protein in the rapamycin treated cells are not markedly diminished. The actin blot in **Figure 11B** shows the relative amounts of protein loaded in each lane. These findings support the idea that as a nonsteroidal compound with antiestrogenic properties rapamycin does not interfere with receptor integrity or turnover. Its inhibitory effect on estrogen-mediated transcription must be mediated through another mechanism.

Aim 2C

Purpose. To determine if rapamycin inhibits transcription in ER+ cells by preventing cytoplasmic/nuclear shuttling by the receptor/ligand complex. This would keep the estrogen/ER complex locked in the cytoplasm and prevent any interaction with nuclear DNA.

Research Design and Methods.

Immunohistochemistry. Falcon 8 chamber cultureslides were pre-treated with poly-D-lysine for ten minutes and washed well with sterile water. The chambers were seeded with 4 x 10^4 cells (MCF-7 or BT-474) per chamber in RPMI 1640 media containing 10% fetal bovine serum and allowed to attach overnight in a humidified incubator. The next day the slides were washed with PBS and fixed by incubating in 3% paraformaldehyde (Kodak) in PBS at room temperature for 30 minutes. The slides were washed in PBS containing 10mM glycine (Sigma). The cells were incubated for 5 minutes in PBS containing 1% Triton (Sigma) and 10% goat serum (Gibco BRL). The slides were washed in PBS/glycine. The cells were incubated with antibody buffer (0.25 M NaCl, 10% goat serum, and 0.1% Tween 20 in PBS) containing a 1:100 dilution of polyclonal anti-ERα (Santa Cruz) for 2 hours. The cells were washed with PBS/glycine. The cells were incubated with secondary antibody (1:50 dilution of FITC conjugated goat anti-rabbit or anti-mouse IgG from Jackson Laboratories) in antibody buffer in the dark at room temperature for 30 minutes. The slides were washed extensively in PBS/glycine then mounted using Fisher mounting reagent and coverslips. Mouse or rabbit IgG (depending on the type of primary antibody, Sigma) is used in one well instead of the primary antibody as a negative control for non-specific binding.

Pitfalls. Several different antibodies have been used in this technique without success; rabbit polyclonal anti-ERα (Santa Cruz), mouse monoclonal anti-ERα (Santa Cruz), mouse monoclonal ERα (Cal Biochem) and mouse monoclonal anti-ERα carboxy-terminus (Cal Biochem). Each antibody has shown non-specific fluorescence when compared to the negative control well. A recently acquired highly specific monoclonal antibody (H222, Abbott Laboratories) will be tested next.

Future Directions

Nuclear/Cytoplasmic Extracts. Another approach to answering this question is through the preparation of nuclear and cytoplasmic protein extracts for Western blot analysis. Four plates of approximately 4 x 10^7 MCF-7 cells were grown under four separate conditions. One plate was incubated in serum-free, phenol red-free RPMI 1640 media for 24 hours, a second plate was grown in RPMI 1640 media containing 10% serum and phenol red for 24 hours, a third plate was grown in RPMI 1640 media containing 10% serum, phenol red and 17β-estradiol (2 x 10^{-8} M), a fourth plate was grown in RPMI 1640 media containing 10% serum, phenol red, estradiol and rapamycin (.01 ng/ml) for 24 hours. The cells were washed twice with cold PBS, harvested by scraping and spun in a microcentrifuge for 15 minutes at 1000 rpm at 4°C. The pellet was resuspended in 5 pellet volumes of CE buffer (10 mM Hepes pH 7.6, 60 mM KCl, 1mM EDTA, 0.075% NP40, 1mM DTT and 1 mM PMSF, all reagents from Sigma). The cells were incubated on ice for 20 minutes. The cells were spun at 1000 rpm for 4 minutes. The supernanant (cytoplasmic extract) was removed and stored in a new tube. The nuclei were washed with 100 μl CE buffer lacking NP40 and resuspended gently. The nuclei were spun at 1000 rpm for 4 minutes at 4°C. One pellet volume of NE buffer (20 mM Tris pH 8.0, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA 0.5 mM PMSF, 25% Glycerol, all reagents from Sigma) was added to the nuclear pellet. The salt concentration of the nuclei solution was adjusted to 400 mM with 5 M NaCl. One more pellet volume of NE buffer was added to the nuclei and the pellet was gently resuspended. The nuclei were incubated on ice for 10 minutes with periodic vortexing to resuspend the pellet. The nuclei were spun at maximum speed for 10 minutes at 4°C in a microcentrifuge. The supernatant was transferred to a new tube. The nuclear extract and the cytoplasmic extract were analyzed for protein content with the Biorad protein assay. Glycerol was added to the cytoplasmic extract to a final concentration of 20% before storage. Both extracts were stored at -70°C. 100 μg of each extract were resolved in a 10% SDS polyacrylamide gel and transferred to nitrocellulose. The blot was initially probed with polyclonal USF-1 (Santa Cruz), which only appears in the nuclear extracts. The blot was stripped and re-probed with polyclonal GST (Santa Cruz), which should only appear in the cytoplasmic extracts. From those two Westerns it appears as if the procedure worked well and the two compartments were separated cleanly. The blot was then serially stripped and re-probed with polyclonal anti-ERα (Santa Cruz) and polyclonal anti-HSP90 (Santa Cruz).

Thus far it has been difficult to discern any patterns in the appearance of the bands in the two compartments at 24 hours. The experiment will be repeated using fifteen minute time points to assess early ER and HSP90 localization.

Aim 2D

Purpose. If Aim 2C shows that rapamycin does not inhibit the free movement of the estrogen/ER complex to the nucleus the next question to be addressed is what happens to the complex once it reaches the nucleus. Gel binding assays have been used to study DNA binding proteins including receptors. The assay is based on the fact that complexes of DNA and protein will migrate more slowly through a polyacrylamide gel than uncomplexed DNA. In the presence of estrogen, the estrogen receptor should form a complex with the ERE. Several classical antiestrogens are known not to inhibit binding.[113,114] This aim will determine if rapamycin inhibits estrogen-mediated transcription by preventing binding of translocated estrogen/ER complexes to nuclear DNA.

Research Design and Methods.

Gel Binding Assay. MCF-7 cells at 30-50% confluence were grown in 10-cm plates for 48 hours in RPMI 1640 media containing 10% fetal bovine serum and phenol red. The cells were incubated for 1 hour prior to harvesting with either 17β-estradiol (2×10^{-8} M) or estrogen plus rapamycin (.01 ng/ml). Cells were washed with chilled PBS, collected and homogenized with a glass homogenizer in binding buffer (20 mM Tris-HCl pH 7.5, 2 mM DTT, 20% glycerol and 0.4 M KCl, all reagents from Sigma). After centrifugation at 105,000 x g for 1 hour at 2°C, the supernatant (whole cell extract, WCE) was collected and protein concentration was determined by Biorad assay.

WCEs (12 μg) were incubated with 2 μg of poly (dI-dC) (Sigma) at 0°C for 15 minutes. The binding reaction, initiated by adding a [^{32}P] 5' end-labeled oligonucleotide probes (~5 fmol, 4×10^4 cpm) was incubated at 20°C for 15 minutes.
The sequence of the double stranded oligonucleotide wild type ERE probe is:

```
                    ←       →
5' GTCCAAAGTCAGGTCACAGTGACCTGATCAAAGTT 3'
3' CAGGTTTCAGTCCAGTGTCACTGGACTAGTTTCAA 5'
```

The sequence of the double stranded oligonucleotide mutant ERE probe is:

```
                    ←       →
5' GTCCAAAGTCAGGTCACAGTGACCTGATCAAAGTT 3'
3' CAGGTTTCAGTCCAGTGTCACTGGACTAGTTTCAA 5'
```

The final concentration of buffer components in a 16 μl binding reaction was as follows: 10 mM Tris-HCl pH 7.5, 1 mM DTT, 100 mM KCl and 10% glycerol. A pre-electrophoresed (10 mA for 30 minutes) 4% polyacrylamide gel (acrylamide to bisacrylamide ratio of 30:1) containing 6.7 mM Tris-HCl pH 7.5, 3.3 mM sodium acetate, and 1 mM EDTA was used to separate the receptor-DNA complexes at 30 mA in TEA buffer at 4°C. After suitable separation was achieved, the gel was vacuumed dried for autoradiography. For the competition assays, a 500-fold excess of competitor oligonucleotide (either wild type or mutant) was added to the binding reaction.

Data/Preliminary Results.

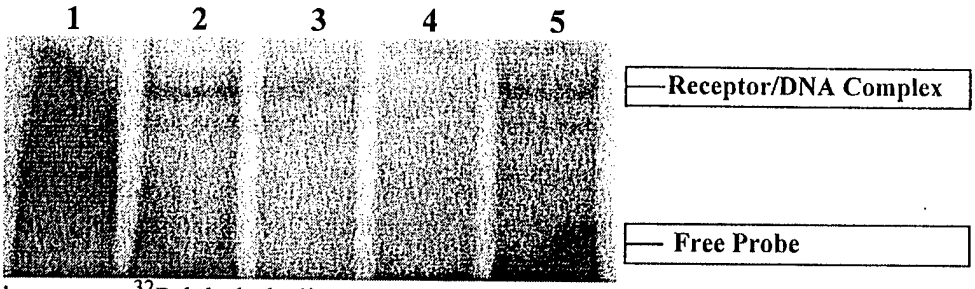

Figure 12. Gel binding assay. ^{32}P-labeled oligonucleotide containing the ERE sequence +/- unlabeled competitor incubated with whole cell extract (WCE) of MCF-7 cells. **Lane 1** Labeled probe alone. **Lane 2** Labeled ERE and WCE. **Lane 3** Unlabeled mutant ERE, labeled ERE and WCE. **Lane 4** Unlabeled ERE, labeled ERE and WCE. **Lane 5** Labeled ERE and WCE which has been treated with rapamycin.

Interpretation. *Rapamycin does not prevent binding of the estrogen/ER complex to nuclear DNA.* When whole cell extract is incubated with the labeled wild type ERE oligonucleotide a band appears because the ER is able to bind to the response element **(Lane 2)**. When an unlabeled mutant competitor oligonucleotide is added to the mixture the band remains because the ER does not recognize the mutated response element **(Lane 3).** When an unlabeled wild type competitor oligonucleotide is added to the binding reaction the band disappears because the excess competitor binds the ER. When WCE which has been incubated with rapamycin is placed in the binding reaction with labeled wild type ERE the band appears. Thus it appears that rapamycin does not prevent the estrogen/ER complex from binding nuclear DNA.

Pitfalls/Future Direction. So far the preliminary results support the idea that after brief (1 hour) exposure of MCF-7 cells to rapamycin the estrogen/estrogen receptor complex is capable of binding DNA The experiment should be repeated using a longer exposure time of the cells to rapamycin. The technique as described in Dr. Chambon's paper requires only a one hour exposure to each treatment. However, more time may be required for rapamycin to have an effect on DNA binding. To determine if the band in the estrogen plus rapamycin lane is a result of estrogen alone it will be compared with cells which have been exposed to rapamycin and estrogen for a longer period of time.

Aim 3. It has been determined *in vitro* that rapamycin can inhibit the estrogen-mediated transcription of ER+ breast cancer cells. To establish rapamycin as a reasonable clinical option for the treatment of tamoxifen resistant tumors this finding must be confirmed *in vivo*. We have begun treating athymic nude mice, which have xenografted estrogen-dependent MCF-7 tumors, with intraperitoneal rapamycin. The tumor volumes of rapamycin treated mice will be compared to the tumor volumes of non-treated mice. Immunohistochemical staining will be performed on resected tumors to measure proliferation indices, ER status, apoptosis markers, and angiogenesis markers.

Purpose. To simulate a clinical scenario in a mouse model which would demonstrate the efficacy of rapamycin as a growth inhibitory agent for estrogen responsive breast tumors.

Research Design and Methods

Toxicity Study. In the first experiment four nude mice were injected with 400mg of rapamycin/kg/ip dose once per day for five days. The drug was solubilized in ethanol, propylene glycol and sterile water. There were no matched controls in the first trial. During the second trial three nude mice were treated with 200 mg of rapamycin/kg/ip dose for five days. Another group of three mice were treated with vehicle (ETOH/propylene glycol/dH2O) for five days. During the third trial four nude mice were treated with 100 mg/kg/ip dose for five days. In this trial the drug was solubilized in DMSO and Cremophor which greatly enhanced its solubility. The control group of four nude mice was treated with vehicle alone (10% DMSO/90% Cremophor EL) for five days.

Data/Preliminary Results

Table 5. Results of toxicity trial of nude mice treated with rapamycin

Rapamycin Dose	# of Surviving Mice/Total Mice (Treatment Group)	# of Surviving Mice/Total Mice (Control Group)
400 mg/kg/ip	0/4	---
200 mg/kg/ip	0/3	3/3
100 mg/kg/ip	4/4	4/4

Interpretation. *Rapamycin is tolerated by nude mice at a concentration of 100mg/kg/ip injection (x 5 days).* The 400 mg/kg/ip dose was lethal to all four mice in the first trial by day five. The 200 mg/kg/ip dose was lethal to all three mice in the treatment arm of the trial by day five while all of the mice treated with injection vehical alone survived. The 100 mg/kg/ip dose was tolerated by all four mice who survived to the end of the trial. Therefore a rapamycin dose of 100 mg/kg/ip or less should be well tolerated by nude mice for a least five injections.

Research Design and Methods

Nude mice xenograft treatment trial. For the pilot study MCF-7 tumors were created in nude mice which would serve as tumor donors for 16 athymic nude mice (at 4 weeks of age). The breast cancer cell line MCF-7 was cultured in flasks and harvested at log phase. An inoculation of 10^7 cells in 0.2-0.3 ml media /site was made into the flank of the donor mice. The cell inoculate developed into solid tumors under the stimulation of subcutaneously implanted estradiol pellets. Tumors were minced aseptically and 1-mm^2 pieces were implanted into the flanks of the 16 recipient mice with a trochar. Sustained release pellets of estradiol were co-implanted under the skin on the backs of animals. After 4 weeks the tumors became established in the recipient mice.

Intraperitoneal injections were started 28 days after implantation. The first dose given was 100mg/kg/ip. After a death in the treatment group was discovered 48 hours later, the

dose was decreased to 50 mg/kg/ip. The 8 mice in the treatment group were treated with this dose 2 times a week. The 8 mice in the control group were treated with an equal volume of vehicle (10% DMSO and 90% Cremophor EL) alone. Tumors were measured twice a week with calipers in two dimensions and the tumor volume calculated with the formula W^2 x L/2.

Data/Preliminary Results

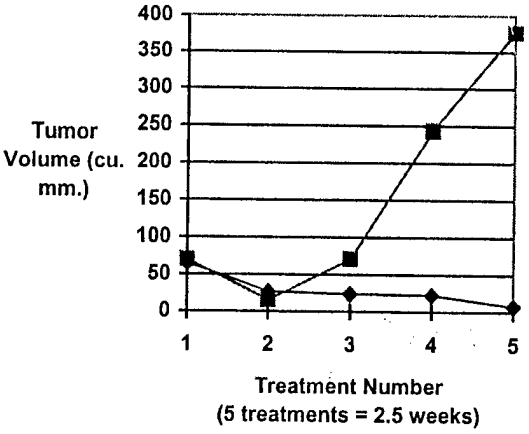

Figure 13. Tumor volume of rapamycin-treated mice versus tumor volume of control-treated mice. Square= control group, Diamond=treatment group.

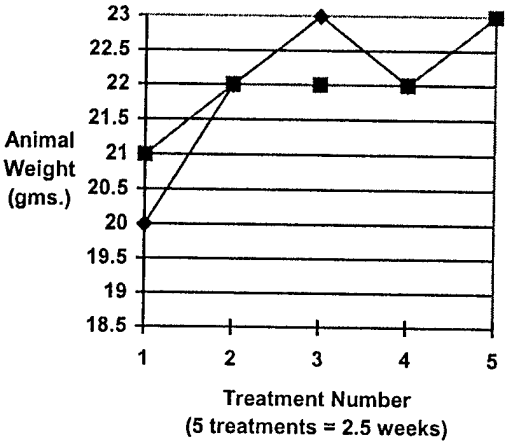

Figure 14. Weights of rapamycin-treated mice versus control-treated mice. Square=control group, Diamond=treatment group.

Interpretation. *Rapamycin inhibits growth of ER+ breast cancer tumors in vivo.* At the beginning of the experiment the average tumor volume of the rapamycin group was 66 mm^3 and the average tumor volume for the control group was 70 mm^3. After five treatments over two and a half weeks the average tumor volume in the rapamycin treated group was 7.54 mm^3 **(Figure 13).** The average tumor volume of the control (intraperitoneal injection of 10% DMSO, 90% Cremophor EL) of group was 377 mm^3 **(Figure 13).** The weights of the animals remained constant over the treatment period **(Figure 14).** At the beginning of the experiment the average weight in the rapamycin group was 20g and the average weight in the control group was 21g. By treatment five the average weight in both groups was 23g. At this point in the study it appears that rapamycin does have a growth inhibitory effect on MCF-7 tumors *in vivo.*

Pitfalls and Future Direction. The potential problems with any antiestrogen is the development of tumor resistance. This will be addressed by withdrawing rapamycin for a period of time to see if the tumors begin to grow. Once growth resumes the mice will be given a second course of rapamycin to see if the tumors stop growing. Another problem, which cannot be addressed in the nude mouse, is determining a dose which will be effective against tumors while not suppressing the immune system in an immunocompetent host.

At the end of the study we will stain the tumors for estrogen receptors, proliferation indices, apotosis markers, and angiogenesis markers.

Aim 4. Rapamycin may have an inhibitory effect on vascular endothelial cells which would make it an even more attractive antitumor agent. Assays are being developed to determine if rapamycin has antiangiogenic properties. These include an *in vitro* cell migration assay, an *ex vivo* aortic ring assay and an *in vivo* corneal pocket assay.
Purpose. To determine is rapamycin is an effective antiangiogenesis agent.

Research Design and Methods

In vitro angiogenesis assays. Assays are currently being developed to determine if rapamycin has antiangiogenic properties. The initial *in vitro* approach will include an XTT assay, FACS analysis and a cell migration assay. The technique for the XTT and FACS assays have been described in Aim 1. Instead of the breast cancer cell lines we will be using the human vein endothelial cell line (huvec). The cell migration assay requires that cells be grown in 150mm plates. After reaching confluency a small area of cells will be injured with a sterile razor blade. The media will be exchanged for media containing various concentrations of rapamycin. The cells will be cultured for another 24 hours and the number of cells migrating into a 1cm square area will be counted. The positive control will be complete media, the negative control will be serum-deprived media.

Aortic ring assay. The *ex vivo* approach to the angiogenesis question will utilize an aortic ring assay. Aortas are harvested from 1-2 month old Fisher rats and cut into rings. Fibrinogen (3 mg/ml in PBS) is mixed with 60 µl of 300 mg/ml EACA. 300µl of

fibrinogen/EACA are overlayed into each well of a 24 well plate. Aortic rings are placed in the middle of the gel and 30-50 μl of thrombin (final concentration at least 100u/ml) are added. The gel is allowed to polymerize for 1-2 hours at 37°C before adding EGM media.

Media containing rapamycin at several different dilutions is added to the wells in triplicate. Sprouting from each ring is quantitated at Day 5.

Corneal pocket assay. The *in vivo* effects of rapamycin on angiogenesis will be investigated with a corneal pocket assay. Dr. Mark Dewhirst's lab has developed a corneal pocket screening assay in Fischer 344 rats that will be used to test the effects of rapamycin on *in vivo* angiogenesis. Corneal pockets are created in anesthetized rats. The corneal pockets of the control animals are implanted with polymers containing nothing (negative control) and bfgf (positive control). The test animals will be treated with intraperitoneal injections of rapamycin. The rats are monitored up to day 14 and the degree of hyperemia of the cornea pockets is quantitated.

Data/Preliminary Results

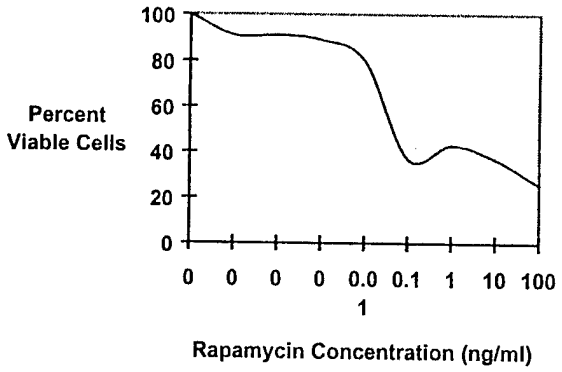

Figure 11. HUVEC proliferation curve. Huvec cells were grown in the presence of tenfold dilutions of rapamycin for one week. The number of viable cells was determined by XTT assay.

Interpretation. *Rapamycin inhibits the proliferation of human vein umbilical endothelial cells.* We determined that the IC_{50} for huvec inhibition by rapamycin is 0.1 ng/ml.

Pitfalls and Future direction. Thus far the aortic ring assay results have been equivocal. The fibrinogen gel has been lysing early and not providing a good substrate for endothelial cell growth. The positive controls do not consistently sprout despite being treated with bfgf and vegf. I am currently repeating the experiment using a new growth matrix, matrigel.

Key Research Accomplishments

1. Demonstrated that rapamycin inhibits cellular proliferation in estrogen receptor positive breast cancer cell lines.

2. Demonstrated that rapamycin inhibits estrogen-mediated transcription of estrogen-receptor positive breast cancer cell lines.

3. Demonstrated that rapamycin inhibits growth of breast cell line tumors implanted in nude mice.

4. Demonstrated that rapamycin has potential antiangiogenic properties which may make it an even more powerful antineoplastic agent.

Reportable Outcomes

1. Abstract: Rapamycin Inhibits Estrogen-Mediated Transcription in Breast Cancer Cell Lines. Presented at AACR meeting in Philadelphia 1999.

2. Funding: UNCF/Merck Graduate Dissertation Fellowship, 2000-2001.

3. Currently applying for fellowships in academic vascular surgery programs.

4. PhD in pathology expected 2002.

Conclusions

1. Rapamycin inhibits proliferation of ER+ breast cancer cells.
2. Rapamycin inhibits estrogen-induced mitogenesis in ER+ breast cancer cells.
3. Rapamycin inhibits estrogen-mediated transcription in ER+ breast cancer cells.
4. Rapamycin does not degrade the estrogen receptor at the protein level.
5. Rapamycin does not prevent binding of the estrogen/estrogen receptor complex to nuclear DNA.
6. Rapamycin inhibits growth of ER+ breast cancer tumors in vivo.
7. Rapamycin inhibits the proliferation of human vein umbilical endothelial cells.

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