The mammalian target of rapamycin inhibitor RAD001 (everolimus) in early breast cancer

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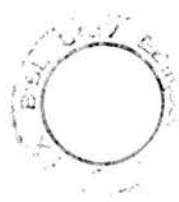

Abstract

The mammalian target of rapamycin (mTOR) plays a key role in tumour cell cycle control, proliferation and survival, and has been implicated in resistance to endocrine therapy in breast cancer. RAD001 (everolimus) is a novel macrolide that inhibits mTOR and its downstream substrates in vitro. This study explores the use of RAD001 at a dose of 5mg daily in women with early breast cancer.

31 postmenopausal women were given RAD001 for 14 days prior to primary surgical intervention for early breast cancer. RAD001 was well tolerated in most patients, 5 did not complete treatment due to drug adverse effects.

Tumour samples before (pre) and after (post) 14 days treatment were assessed for changes in proliferation and markers of the mTOR pathway. Significant reductions in proliferation (Ki67) and oestrogen receptor (ER) expression were seen, and the downstream effects of the mTOR pathway inhibited (p-S6 (ser235/236 and ser 240/244) and nuclear expression of p-Akt). Gene expression profiling from these tumour samples has confirmed these findings, demonstrating reduction in expression of proliferative genes and oestrogen dependence genes with RAD001 treatment.

The mTOR protein exists in two distinct complexes, raptor and rictor, and it has previously been thought that mTOR inhibitors such as RAD001 only have effects upon raptor. The implication of this if correct would be upregulation of Akt (Protein Kinase B), which has been shown to be present in more aggressive and resistant tumour types. The cell line study described herein has demonstrated no upregulation in p-Akt expression with RAD001 treatment, and in one cell line inhibition of p-Akt was sustained with prolonged cell treatment.

To further assess the role of Akt in resistance to endocrine treatment, tumour samples with known endocrine resistance or sensitivity were assessed for p-Akt expression before and after treatment. The results suggest an increase in nuclear expression of p-Akt in endocrine resistant tumours.

The mTOR inhibitor RAD001 is therefore effective at reducing proliferation of early breast cancer, inhibiting mTOR in both raptor and rictor complexes, and may be of particular use in highly proliferative or resistant tumours that overexpress p-Akt.

Declaration

This thesis has been composed by myself, the candidate.

The work relating to the clinical study and the immunohistochemical study of RAD001 treated and endocrine treated tumour samples is my own. The cell line study work was carried out in conjunction with the research group of Professor John Bartlett, Endocrine Cancer Group, Edinburgh Cancer Research Centre, and a substantial contribution was made by myself, the candidate to this. The transcriptional profiling was performed upon tumour samples collected by the candidate as part of the clinical study described herein, and the profiling carried out by members of the research group of Professor John Bartlett. The statistical analysis of the clinical and biological studies was performed in conjunction with Dr Linda Williams, Centre for Population Health Sciences, University of Edinburgh.

This work has not been submitted for any other degree or professional qualification.

The work was carried out within South East Scotland.

Signed:_

E. Jane Macaskill

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Introduction

Normal Structure and Function of the Breast

Discussion of the normal structure and function of the breast is necessary in order to understand the pharmacology of the medical treatment of breast cancer.

The breasts exist in both sexes but are rudimentary in the male throughout life, comprising small ducts without alveoli, supported by fibrous tissue and fat. The male breast is still prone to the same major diseases that affect the female breast, and 240 men were diagnosed with breast cancer in the UK in 2000.[1] The studies carried out as part of this thesis recruited only women, and for this reason, reference to breast cancer in this text applies to female breast cancer unless otherwise specified.

Anatomy of the breast

In the female, each of the two breasts lies within the superficial fascia overlying the second to sixth ribs, extending superolaterally toward the axilla to form the axillary tail.[2, 3] The deep fascia separates the breast from the underlying pectoralis major, serratus anterior and, at the lower medial edge, the rectus sheath. The dissection of the breast from the deep fascia during mastectomy is facilitated by a zone of loose areolar tissue, the retromammary space. The breast consists of glandular tissue in 15-20 lobules, each of which is drained by lactiferous ducts to the nipple. This glandular tissue is embedded in fat, which accounts for the smooth shape and variation in size of the breasts between individuals. Fibrous septa known as the suspensory ligaments (of Cooper) run from the subcutaneous fascia to the nipple separating the lobules. If infiltrated by malignancy, these ligaments can become contracted resulting in tethering and retraction of the skin.

The nipple commonly lies at the level of the 4th intercostal space, and is a pink or light brown eminence that projects from just below the centre of the anterior of the breast containing numerous circumferential and longitudinal arrangements of unstriped muscle fibres. These muscle arrangements result in erection and contraction of the nipple. The base of the nipple is encircled by a coloured area of skin called the areola, underneath which the lactiferous ducts draining the breast lobes form dilatations known as lactiferous sinuses, which act as reservoirs for milk during pregnancy and lactation. At the base of the nipple these ducts perforate it at small orifices at the tip.

After the menopause, the mammary gland atrophies as most of the cellular elements of the alveoli and ducts degenerate, although the breast size remains variable due to amount of fat.

The blood supply of the breast comes from two primary sources: the axillary artery, via lateral thoracic and acromiothoracic branches, and from the internal thoracic artery via perforating branches. The lateral perforating branches of the intercostal arteries provide additional supply.

Venous drainage is via an anastomotic circle, the circulus venosus around the base of the nipple, which branches drain blood to the circumference of the breast, and thence into the axillary and internal thoracic veins.

The sensory nerve supply to the breast is from T4-6, from the anterior and lateral cutaneous branches. These nerves convey sympathetic fibres to the breast, but its secretory fibres are under hormonal control as discussed below.

The lymphatic drainage of the breast is of much interest in oncological surgery, and is a topic of debate with the development and increased use of techniques to identify

the 'sentinel' node. This will be discussed in more detail under the section on the surgical management of breast cancer.

Physiology of the breast

The action of drugs to combat cancer can only fully be comprehended with knowledge of the normal function of the organ concerned and its related physiology, and an understanding of the change that is undergone by the cancer process.

In the non-pregnant female the breasts have a rudimentary duct system as described above. Each lobule contains sac-like epithelial-lined alveoli from which milk is synthesised and then secreted into the alveolar lumen. Milk then drains via the ducts to the surface of the nipple. The process of duct development during pregnancy takes place due to high oestrogen concentrations in pregnancy, and high levels of progesterone stimulate the alveolar-lobular formation. From the anterior pituitary prolactin is secreted in response to high oestrogen contributing to mammary gland development in pregnancy. Until birth, the stimulatory effect of prolactin is blocked by progesterone and oestrogen, which dramatically decrease after parturition resulting in prolactin stimulation of lactation. In conjunction with oxytocin from the hypothalamus, milk ejection is stimulated by prolactin. Release of both these hormones is triggered by the suckling neuroendocrine reflex.

Breast cancer

Incidence

Breast cancer is the most common cancer in the UK, although it is a rare disease in men. More than 100 new cases of breast cancer are diagnosed each day in the UK.[1] In Scotland in 2006, 4079 people were diagnosed with breast cancer, an increase in incidence of 9.6% from ten years previous.[4] In England and Wales between 1971 and 2002, the incidence rate for breast cancer (after adjustment for age) increased by around 70 per cent - with a steep increase in the period following the introduction of the national programme of mammographic screening of women aged 50-64 in 1988.[5] Although diagnosis and treatment have improved significantly over the last thirty years with a corresponding fall in mortality, breast cancer remains the third most common cancer death in the Scotland after cancers of the lung and large bowel, with around 1000 deaths per year.[4]

Risk Factors

The incidence of breast cancer diagnosis increases with age in women.[4] Hormone dependent factors are likely to be involved in this increase, and lifetime exposure to endogenous sex hormones affects the risk of breast cancer, dependent on many variables such as age at menarche, age at first pregnancy, number of pregnancies and age at menopause.[6] Use of exogenous hormones such as hormone replacement therapy after the menopause are associated with increased breast cancer risk with prolonged use, as well as current or recent use of combined preparations or progesterone only oral contraceptives.[7-9] Obesity in postmenopausal years, tall stature and increased alcohol intake, as well as increased intake of saturated fat are

associated with increased risk.[10, 11] High mammographic density is also associated with higher rates of breast cancer.[7]

For some patients there is an increased risk of breast and other cancers due to family history or specific genetic mutations, in particular those associated with BRCA1 or BRCA2. From a study amongst Ashkenazi Jews, who have specific inherited mutations of BRCA1 or BRCA2, the estimated increased risk of developing breast cancer is 56%, ovarian cancer 16% and prostate cancer 16%.[12] These mutations have been implicated in association with other malignancies such as non-melanoma skin cancers (BRCA1), thyroid (BRCA1), leukaemia (BRCA1 and BRCA2), colorectal, pancreatic, gallbladder and bile duct, stomach and malignant melanoma (BRCA2).[13, 14]

The options for prevention of breast cancer in these women include chemoprevention or prophylactic surgery in the form of bilateral oophorectomy or bilateral mastectomy. In patients with a strong family history who are known to be at high or moderate risk, a retrospective study has shown a risk reduction of greater than 90% with bilateral prophylactic mastectomy after 14 years.[15] For patients who have known BRCA1 or BRCA2 mutation, the risk reduction of bilateral prophylactic mastectomy has been reported as being between 85-89.5% at 13.4 years.[16]

Diagnosis

The main referral routes through which patients are diagnosed with breast cancer in the UK are through symptomatic clinics or through the National Breast Screening Programme. Patients present symptomatically most commonly with a lump in the breast, with skin changes or distortion, nipple changes or discharge, or an axillary lump. A triple assessment approach is used, incorporating clinical, radiological and

cytological or histological examination, thereby increasing the accuracy of diagnosis. [17-19] In many centres in the UK, core biopsy is performed to confirm clinical and radiological suspicion of malignancy and may be combined with fine needle aspiration cytology, which can be reported at the one stop clinic. Histological correlation between core biopsy and excision specimens have been assessed in a number of studies and found to have comparable results for ER, PR, p53 and proliferation (MIB-1). [20-22]

Management Options in Breast Cancer

The National Cancer Plan published by the UK government in 2000 stressed the importance of multidisciplinary team (MDT) working in the management of cancer patients, stating that patients treated by specialist teams are more likely to survive by improvement of co-ordination and continuity of care for patients. [23] The Plan advised that all patients with cancer should be formally reviewed by a specialist team. The National Institute for Clinical Excellence (NICE) published a report recommending meetings of the core team on a regular basis (usually weekly) to discuss each patient with confirmed breast cancer both after initial diagnosis and after surgery to plan and monitor treatment. [24] Decisions about future treatment should also be discussed at these meetings, taking account of clinical practice guidelines and protocols agreed by the team. These recommendations should form the backbone of the way in which breast cancer care is delivered, and management decisions discussed fully by the MDT and with the patient and her family. With increasing methods of obtaining information available to patients, it can be bewildering for patients recently diagnosed to understand the array of potential options for treatment.

The clinical management of the newly diagnosed patient with breast cancer can be subdivided into local and systemic approaches. The management of patients with advanced disease will not be discussed as it is outwith the scope of this thesis, which addresses drug development within the setting of early breast cancer.

Local management of breast and axilla

Surgery

In patients with large operable or locally advanced breast cancer where it is felt that surgery is either not technically feasible, would require mastectomy or where the patient is considered unfit for anaesthetic and extensive surgery, neoadjuvant treatment has emerged as a preferred choice of treatment. This will be discussed below with the options for systemic treatment.

Radical mastectomy remained the surgical treatment of choice for breast cancer from when first it was described by Halsted in 1898 until the 1970s. [25] Following results from randomised, controlled studies which were commenced to determine the potential benefits of less extensive surgery for early breast cancer, less extensive surgery is now recommended. One of the first trials to publish comparative results was the NSABP-06, for which there are now 20-year follow-up data available, these data being consistent with the five year data originally published. [26] Patients with tumours <4cm which were either node positive or negative were randomised to three groups to receive either lumpectomy alone or lumpectomy followed by radiotherapy or total mastectomy each combined with axillary dissection (modified radical mastectomy). Results for 1851 randomised patients showed no difference in overall survival or disease-free survival or distant disease-free survival between the three groups. There was, however, a statistically significantly greater number of local recurrences in the group of patients that had lumpectomy alone (39.2%) compared to those who had lumpectomy plus radiotherapy (14.3%; $p<0.001$).

The Milan trials of quadrantectomy and radiotherapy versus Halsted mastectomy have reported results similar to those of NSABP-06. [27] Twenty-year follow-up data

published for 701 patients with T1, N0, M0 breast cancer randomised to one of two arms: quadrantectomy, axillary dissection and radiotherapy or radical mastectomy have shown no difference between the two arms for distant metastases, breast cancer death, overall survival or contralateral breast cancer. There was a higher number of local recurrences in the quadrantectomy arm (8.8% versus 2.3%; $p<0.001$). An EORTC study of 868 patients randomised to either breast-conserving surgery with radiotherapy or mastectomy showed no difference at ten years in overall survival or distant metastases-free survival, but there was a significantly lower local recurrence rate in the mastectomy group (12.2%) compared with breast-conservation and radiotherapy (19.7%; $p=0.0097$). [28] The conclusions from these studies were that breast-conserving surgery is appropriate when followed by radiotherapy for patients with small tumours, provided that the margins of the resection are free of tumour and an acceptable cosmetic result can be achieved.

As part of the traditional approach to the surgical management of breast cancer, axillary surgery has been performed as part of standard treatment for all patients both for diagnostic and therapeutic purposes. [25] Axillary dissection or removal of all the axillary lymph nodes is associated with morbidity in terms of arm and shoulder function, pain, lymphoedema and numbness. [29] The effectiveness of 'sentinel node mapping' in melanoma has led to randomised trials of this technique in early breast cancer. [30] A trial from Milan randomised 516 female patients with <2cm tumours and clinically negative axillary lymph nodes to either sentinel lymph node biopsy (SLNB) followed by axillary dissection or to SLNB with no further surgery if the sentinel node was negative on frozen section or to axillary dissection if positive. [31] Results showed an overall accuracy of the sentinel-node technique of 96.9%, with

sensitivity of 91.2% and specificity of 100%. It was not stated in this paper what was the mean number of sentinel nodes removed per patient. A comparative side effect analysis for a subgroup of patients was favourable for patients who had only SLNB in terms of length of hospital stay, pain, numbness, arm mobility and swelling. In the intention to treat population, there was no difference in breast cancer related events or overall survival at a median 46 months of follow-up. A more detailed study of quality of life outcomes with SLNB and axillary dissection has been published by the Axillary Lymphatic Mapping Against Nodal Axillary Clearance (ALMANAC) trialists. [32] This was a multicentre randomised trial of 954 male and female patients <80 years with early stage breast cancer who were clinically node negative and proceeding to either breast-conserving surgery or mastectomy, and were randomised to either SLNB or standard axillary treatment. In contrast to the Milan study, patients with any tumour size were entered, and patients who were found to have one or more positive sentinel lymph nodes were subsequently offered either delayed axillary dissection or radiotherapy to the axilla. Outcomes relating to quality of life (QoL) and side effects were assessed in both groups at 1, 3, 6 and 12 months. In the SLNB group there was significantly less moderate/severe lymphoedema, less sensory deficit, better arm functioning and better QoL scores at all time points. There was significantly less arm swelling at each but the 12 month timepoint and better shoulder function at 1 month. The duration of surgery in both groups was the same, with a shorter hospital stay in the SLNB group, possibly related to less axillary drain usage, and there was a trend to fewer infections in the SLNB group. Local recurrence and survival data are awaited from this and other ongoing studies, but early data suggest no increase in axillary recurrence with sentinel node biopsy alone. [33, 34] The

use of sentinel lymph node biopsy has been established as a minimally invasive staging procedure for the axilla, and a national training programme has been started in the UK for this procedure.

Radiotherapy

As discussed above, there have been a number of studies showing that radiotherapy should be used in conjunction with breast-conserving surgery for it to be equivalent in long term local disease control and survival to mastectomy. This was confirmed in the most recent meta-analysis by the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) of radiotherapy in early breast cancer published in 2005, which analysed local recurrence and 15-year survival on 42,000 women in 78 randomised trials. [35] For patients receiving radiotherapy after breast-conserving surgery, there was a highly significantly lower rate of local recurrence (7%) compared to those who did not receive radiotherapy (26%) at 5 years. In those allocated radiotherapy there was a significant reduction in breast cancer mortality (absolute reduction 5.4%). For patients who underwent mastectomy and axillary clearance who were node negative there was a low risk of local recurrence (6%) without radiotherapy to chest wall, axilla, supraclavicular fossa and internal mammary chain. The local recurrence risk was significantly lower when radiotherapy was given after mastectomy even to the node negative group, but with an absolute five-year gain of only 4%, and with no difference in mortality between the two groups. For patients with node-positive disease, however, there was a significant and clinically important reduction in local recurrence from 23% without radiotherapy to 6% with radiotherapy (absolute 5-year gain 17%). In node-positive disease there was also an improvement in breast cancer

mortality with radiotherapy (54.7% versus 60.1% without; absolute reduction 5.4%; p=0.0002).

Radiotherapy is not without its risks. In the above meta-analysis, it was found that there was an excess incidence of cancers in the contralateral breast in those patients who received radiotherapy (p=0.002) and an excess incidence of lung cancers (p=0.0007), as well as an increase in mortality from heart disease (p=0.0001) and lung cancer (p=0.0004).

Current practice is in keeping with the above findings: patients who undergo breast-conserving surgery are recommended to have radiotherapy, as well as patients who have undergone mastectomy and axillary surgery and are shown to be node-positive.

Systemic non-hormonal therapy

It is outwith the scope of this thesis to explore in any depth the use of chemotherapy in the management of early breast cancer. Chemotherapy has, however, a key role in improving overall survival, as emphasised in the results of the most recent Early Breast Cancer Trialists' Collaborative Group meta-analysis of the effects of chemotherapy and hormone therapy in early breast cancer. [36] These data show that although single-agent regimens significantly reduce recurrence rates, polychemotherapy regimens, most commonly anthracycline based, result in an even better reduction in recurrence rates, and improved breast cancer specific and overall survival rates at 15 years. The reduction in mortality benefits of polychemotherapy compared with no chemotherapy were three times greater for women age <50 years (15-year gain 10%) compared with women aged 50-69 years (15-year gain 3%). The greatest reduction in mortality was seen in younger patients who were node positive (15-year gain 14.6%), or had oestrogen receptor (ER) poor tumours (15-year gain

13.2%). From trials directly comparing anthracycline-based regimens versus CMF-based regimens, usually given for about 6 months, there was a significant advantage for anthracyclines for recurrence (ratio 0.89; p=0.001) and for breast cancer mortality (ratio 0.84; p<0.00001). The absolute difference between the two regimens for probability of recurrence, breast-cancer mortality and overall mortality was 3% at 5 years and 4% at 10 years, irrespective of age of the patient. There was no significant increase in mortality, heart disease or haemopoietic neoplasms in groups who received anthracyclines.

Current recommendations in the UK are for anthracycline combinations to be prescribed in favour of non-anthracycline regimens in the adjuvant setting, with epirubicin also recommended due to its lower risk of cardiotoxicity. [37]

Five large trials have been performed to assess the role of taxanes (docetaxel and paclitaxel) in the adjuvant setting. [38-42] Three studies have been in node positive disease with taxanes given either concurrently or following chemotherapy. The CALGB 9344 study randomised 3121 node-positive women who had completed surgical management to 4 courses of AC (doxorubicin at three doses 60, 75, 90 mg/m2 plus cyclophosphamide) followed by 4 cycles of paclitaxel or nothing. [38] At a median follow-up of 69 months there was no significant difference in disease-free survival (DFS) or overall survival (OS) for different doses of doxorubicin, but there was a significant benefit favouring the addition of paclitaxel for DFS (HR 0.83; 95%CI 0.73, 0.94; p=0.0023) and OS (HR 0.82; 95%CI 0.71, 0.95; p=0.0064). This equals an improvement of 5% in disease-free and 3% in overall survival at 5 years with paclitaxel. 92% of the patients who started paclitaxel completed all four cycles.

In a similar study design, the NSABP B-28 trial randomised 3060 patients who had undergone initial surgical management for node-positive breast cancer to 4 cycles of doxorubicin and cyclophosphamide alone or followed by 4 cycles of paclitaxel. [39] After a median follow-up of 64 months there was a significant improvement in DFS (HR 0.83; 95%CI 0.73, 0.95; p=0.008) but no significant difference in OS (HR 0.94; 95%CI 0.78, 1.12; p=0.46). No difference in DFS, relapse-free survival (RFS) or OS was seen between ER positive (ER+) and ER negative (ER-) tumours. In both of the above studies patients received adjuvant tamoxifen. Baseline characteristics were similar for both studies, although the NSABP B-28 had a higher proportion (70%) of patients with less than 4 nodes positive than in the CALGB (46%), which could in part account for lack of OS benefit for taxanes in NSABP B-28, although in the French study disussed below both node negative and positive disease had a benefit with a taxane.

The BCIRG 001 trial randomised 1491 women with node-positive tumours who had undergone primary surgery to with 6 cycles of docetaxel plus doxorubicin plus cyclophosphamide (TAC) or to 6 cycles of fluorouracil plus doxorubicin plus cyclophosphamide (FAC). [40] At a median follow-up of 55 months there was a significant improvement in DFS (HR 0.72; 95%CI 0.59, 0.88; p= 0.001) in favour of the TAC regimen. A significant improvement in OS was also found for TAC compared with FAC (HR 0.70; 95%CI 0.53, 0.91; p=0.008). In planned subgroup analyses, there was a benefit for TAC regardless of lymph node status, ER status, menopausal status or HER-2 status.

In a French study of 1999 women with node negative and node positive disease, randomisation was to six cycles of FEC (5FU/ epirubicin/ cyclophosphamide) or to

FEC three cycles followed by docetaxel for 3 cycles. [41] Patients received concurrent tamoxifen. At a median follow-up of 60 months, there was a significant improvement in DFS in patients who received docetaxel (HR 0.83; p=0.012) and a significant improvement in OS (HR 0.77; p=0.014).

In the largest of the primary adjuvant taxane studies, the UK based TACT trial (Taxanes as Adjuvant ChemoTherapy), 4162 patients with node positive or high risk node negative operable early breast cancer were randomised to 4 cycles of 5-FU plus epirubicin plus cyclophosphamide (FEC) followed by docetaxel or 8 cycles of FEC or 4 cycles of epirubicin followed by 4 cycles of CMF, with the primary endpoint disease-free survival. [42] Results at median follow-up 62 months for ITT population did not show a DFS (HR 0.95; 95%CI 0.85, 1.08; p=0.44) or OS (HR 0.99; 95%CI 0.86, 1.14; p=0.91) benefit for taxanes. There were significantly greater numbers of acute grade 3 or 4 adverse events reported in the taxane arm of the study, and nearly 20% of the patients did not complete the scheduled four cycles of docetaxel in the experimental group.

Given the preceding results from taxane studies this lack of benefit in the TACT trial was surprising. A meta-analysis of adjuvant taxanes in early breast cancer has shown an absolute 5-year risk reduction of 5% for DFS and 3% for OS with taxanes, with no difference between agent used, or whether taxane given sequentially or in combination. [43]

Current UK guidelines are that docetaxel, but not paclitaxel should be offered as part of adjuvant chemotherapy regimen in node-positive early breast cancer. [44]

Hormonal treatment of breast cancer

Ovarian ablation

Historically, the earliest description of successful hormonal manipulation in the treatment of breast cancer was by Beatson in 1896. [45] He described the effective use of oophorectomy in 2 premenopausal women with locally advanced breast cancer to reduce tumour bulk, after observing during the time of writing up his MD thesis a similar practice carried out in sheep in order to continue milk production. He proposed that there was evidence of the ovaries and testicle having control in the human body over local proliferation of epithelium, challenging the understanding at that time of "cancer-bodies" as a parasitic entity. This discovery served as the basis for the use of ovarian ablation until randomised trials began in 1948. The combined analysis of these early trials conducted by the Early Breast Cancer Trialists' Collaborative Group (EBCTCG) confirmed that ovarian ablation is associated with improvement in recurrence-free and overall survival in premenopausal women. [46] The method of choice can be tailored to the needs of the woman in terms of whether temporary or permanent ablation is required, and whether surgical or medical treatment is preferred. Methods include potentially reversible use of luteinising-hormone releasing hormone (LH-RH) agonists such as goserelin, or permanent methods such as surgical oophorectomy or ovarian ablation by radiotherapy.

Oestrogen antagonists

The most commonly used oestrogen antagonist in therapeutic use is tamoxifen. Tamoxifen functions through its ability to compete with oestradiol at an allosteric site on the oestrogen receptor (ER) with reduction of nuclear expression of the ER. [47]

Tamoxifen is a classical example of a partial agonist with tissue specificity for inducing either agonist or antagonist responses. In the clinical context, this results in antagonism in breast tissue and thus reduction in the incidence of primary breast cancer development in high risk women and a reduction in contralateral breast cancer, but agonist effects in bone, leading to maintenance of bone density and agonist effects on the endometrium, causing an increase in incidence of endometrial cancer in women who take this drug. [48] A number of other drugs have been developed in this class known as selective oestrogen receptor modulators (SERMs), and are under investigation for adjuvant and preventative therapeutic use. [49-51] Tamoxifen is currently licensed for use as a first line agent in early and advanced breast cancer in pre-, peri- and post-menopausal women, and for chemoprevention in women at high risk of breast cancer. The use of tamoxifen in the neoadjuvant setting is discussed below. For early breast cancer current recommendations are five years of tamoxifen, which has been shown to lower recurrence rates and breast cancer mortality compared to a shorter 1-2 year period of treatment in patients who have ER-positive disease, [36] although this benefit in survival is not apparent until after five years treatment is completed. [52] In randomised studies patients who had ER-positive disease and received 5 years tamoxifen had a significantly lower recurrence rate (33.2% versus 45%; $p<0.00001$) compared to control groups at 15 years, and a lower mortality rate (25.6% versus 34.8%; $p<0.00001$) compared with control groups. The relative reduction in recurrence was even greater for patients under 40 years or with node positive disease. [36] The use of tamoxifen for longer than 5 years is not currently advised, as prolonged treatment in one study actually decreased disease-free survival (tamoxifen 78% versus 82%; $p=0.03$), with no improvement in overall survival. [53]

The large multicentre trial ATLAS (Adjuvant Tamoxifen- Longer Against Shorter) and the national aTTom (adjuvant tamoxifen-offer more?) trials are currently ongoing to address the issue of prolonged tamoxifen treatment by randomising patients to 5 years or ten years tamoxifen. The most recent data from these trials suggest that earlier results indicating prolonged tamoxifen is detrimental are wrong and show an approximate 12% reduction in events with 10 rather than 5 years of treatment. [54, 55]

A newer generation of pure oestrogen antagonists have been developed which by their nature do not have any of the oestrogen agonist effects of a drug such as tamoxifen. Fulvestrant is one example of such drugs, and this agent has been shown in premenopausal and postmenopausal women to be as effective in reducing proliferation and to have similar anti-oestrogenic properties as tamoxifen, [56] and has a clinical benefit rate of 35% in postmenopausal patients who have progressed on aromatase inhibitors. [57] This drug is currently available only as an intramuscular injection, which is well tolerated by patients, and serves as an alternative in patients where there may be compliance issues. [56]

Aromatase inhibitors

Over three quarters of breast cancers are dependent on oestrogen for growth, and virtually all breast cancers that respond to endocrine treatment express significant levels of oestrogen receptor (ER) alpha. As will be discussed, despite high levels of ER, some cancers do not respond and others develop resistance to treatments that block ER such as tamoxifen. A class of drugs called the aromatase inhibitors have been developed that inhibit oestrogen production. In the premenopausal woman, the majority of oestrogen is produced in the ovaries by negative feedback loops

involving the anterior pituitary. In the postmenopausal woman, however, oestrogen biosynthesis occurs mainly in tissues such as subcutaneous fat, skin, liver, muscle and breast cancer cells. From the starting point of cholesterol, there are a series of conversions through progestogens and corticoids to androgens to oestrogens. The final step is the conversion of androgens to oestrogens catalysed by the aromatase enzyme, the target for aromatase inhibitors. [58] The aromatase inhibitors can be sub-classed on the basis of their structure as steroidal (exemestane and formestane) or non-steroidal (letrozole, anastrozole and aminoglutethimide).

The three most commonly used aromatase inhibitors in current clinical practice are letrozole, anastrozole and exemestane, all three having had a licence for use in metastatic breast cancer for some time. More recently letrozole and anastrozole have been licensed as first-line adjuvant therapy for early breast cancer, and letrozole is licensed for use in the extended adjuvant setting. Anastrozole and exemestane also are licensed for use after 2-3 years tamoxifen (switching). The evidence base for these therapeutic applications is discussed below.

Adjuvant and extended adjuvant aromatase inhibitors

The ATAC (Anastrozole or Tamoxifen Alone or in Combination) trial randomised 9366 postmenopausal women with invasive breast cancer (ER positive or unknown) to 5 years of adjuvant anastrozole, tamoxifen or a combination of the two. [59] Anastrozole achieved a significant disease free survival benefit at 100 months with a hazard ratio (HR) of 0.85 (95%CI 0.76, 0.94; p=0.003) in the ER+ group compared with tamoxifen or the combination, but no difference in OS (HR 0.97; 95%CI 0.86, 1.11; p=0.7). There was a reduction in contralateral breast cancer in the anastrozole

treated group compared with tamoxifen or the combination (HR 0.60; 95%CI 0.42, 0.85; p=0.004).

The BIG1-98 study directly compared letrozole with tamoxifen as primary adjuvant therapy in 8010 women with early breast cancer randomised to one of four study arms: tamoxifen for 5 years, letrozole for 5 years, tamoxifen for 2 years followed by letrozole for 3 years, or letrozole for 2 years followed by tamoxifen for 3 years. After a median overall follow up period of 25.8 months patients receiving immediate treatment with letrozole rather than tamoxifen had an improved disease free survival (HR0.81; 95%CI 0.70, 0.93; p=0.003), a better breast cancer relapse rate (absolute benefit 3.4% at 5 years, p=0.0002) and a significant improvement in time to development of metastases (HR=0.73), but no difference in overall survival. [60] At a median 40.4 months the disease-free survival benefit of letrozole over tamoxifen was sustained for the 4922 patients in the two monotherapy arms. [61]

In both the ATAC and BIG 1-98 studies there were more fractures, less thromboembolic events and less endometrial cancer in women receiving anastrozole or letrozole. There was a trend toward increased cardiac events (4.1% v 3.8%, p=0.61) in the letrozole group, with significantly more grade 3-5 cardiac events (2.1% v 1.1%; p<0.001).

A number of studies assessing optimum effects of switching between tamoxifen and aromatase inhibitors are ongoing, but three have reported results. In a combined report from the ABCSG8/ARNO95 study, results have shown that a switch to anastrozole after 2 years of tamoxifen therapy improves event free survival compared to continuing on tamoxifen (HR 0.60; 95%CI 0.44, 0.81) at a median of 28 months follow-up. [62] There was no difference in overall survival between those who

continued on tamoxifen and those that switched to anastrozole. Patients switching to anastrozole had significantly more fractures (2% versus 1%; p=0.015), and less thrombotic events (p=0.034), although the numbers of these events were small. In the Italian Tamoxifen Anastrozole trial (ITA), 448 women were randomised to continue tamoxifen after 2-3 years or switch to anastrozole. [63] After a median follow-up of 64 months in the intention to treat population there was a significant benefit in terms of event-free survival (HR 0.57; 95%CI 0.38, 0.89; p=0.005) and recurrence-free survival (HR 0.56; 95%CI 0.35, 0.89; p=0.01) in favour of those who switched to anastrozole, but no difference in overall survival (HR 0.56; 95%CI 0.28, 1.15; p=0.1). A meta-analysis of these trials concluded that there was an overall survival advantage to switching to anastrozole (OS HR 0.71; 95%CI 0.52,0.98; p=0.04) although the study groups differed in their entry criteria and also on both baseline data and design and only the two smaller trials contributed to this significant difference. [64] The study populations differed with those patients randomised in the ITA study being 90% node positive with many of these women having also received chemotherapy, whereas the ABCSG/ARNO study included mainly grade 1 and 2 tumours and no patients received chemotherapy. No difference in overall survival was seen in the ABCSG8 trial which was a true sequencing trial with 2579 patients randomised prior to starting tamoxifen in contrast to the other two trials which were switching trials.

Exemestane has been examined in the adjuvant setting with sequential use of exemestane therapy after 2-3 years of tamoxifen to complete 5 years of therapy, and results at 3 years showed a significant improvement of switching over 5 years tamoxifen alone with a reduction in events (HR 0.68; 95%CI 0.56, 0.82; p<0.001)

and an absolute disease free survival benefit of 4.7%. [65] These benefits in disease-free survival for switching over tamoxifen were sustained at 58 months follow-up. [66] The potential increased cardiovascular risk associated with aromatase inhibitor use was highlighted in this study with a small but non-significant excess of myocardial infarcts in the exemestane group.

In addition to use in the adjuvant setting, the role of using an aromatase inhibitor after 5 years of tamoxifen has been investigated in the MA.17 trial. This study randomised 5187 postmenopausal women who had completed 5 years tamoxifen within the previous three months to receive either letrozole or placebo for five years. Results from this study at a median follow-up of 30 months showed a significant disease-free survival benefit with letrozole (HR 0.58; 95%CI 0.45, 0.76; p=0.00004) and also a distant disease-free survival benefit (HR 0.60; 95%CI 0.43, 0.84; p=0.002). No difference in overall survival was found for the whole intention-to-treat population (ITT), but for patients with node-positive disease a significant improvement in overall survival was evident (HR 0.61, 95%CI 0.38, 0.98, p=0.04).[67, 68] Letrozole treatment did not result in a higher risk of hypercholesterolemia, cardiovascular events or clinical fractures, but there was a trend towards more patients having newly diagnosed osteoporosis with letrozole (p=0.07). Toxic effects were primarily of grade 1 or 2, and 4.5% of women in the letrozole group discontinued because of toxic effects, which overall was not significantly different from the 3.6% of women in the placebo group who withdrew for similar reasons (p=0.11).

An analysis of the benefit of prolonged treatment with letrozole compared with placebo over time compared hazard ratios at 12 month intervals to 48 months median

treatment, and found a significant improvement in disease-free survival that increased over time (HR 0.59 at 6 months to HR 0.19 at 48 months, $p<0.0001$) and a significant improvement in distant disease-free survival over time (HR 0.51 at 6 months to HR 0.21 at 48 months, $p=0.0013$), irrespective of nodal status. [69] No significant difference in overall survival was found. These results would indicate that prolonged treatment with letrozole (up to 4 years) improves disease-free and distant disease-free survival compared with shorter periods of treatment.

In a sub-group analysis of ER and PR status within the ITT population of the MA.17 trial, significant benefit for disease-free survival was found for letrozole in tumours that were ER+/PR+ (HR 0.49; 95%CI 0.36, 0.67) and ER-/PR+ (HR 0.56; 95% CI 0.15, 2.12), but not for those tumours that were ER+/PR- (HR 1.21; 95% CI 0.63, 2.34), with similar findings for distant disease-free survival. [70] For overall survival there was a 42% improvement in survival for patients on letrozole compared with placebo in the ER+/PR+ subgroup (HR 0.58; 95% CI 0.37, 0.90). The subgroup of ER+/PR- and ER-/PR+ patients on letrozole did not appear to benefit for overall survival when compared with the placebo group. While this subgroup analysis contained small numbers of ER+/PR- tumours (n= 636) compared to the whole group which may weaken the analysis, it has furthered discussion of the role of PR in determining response to aromatase inhibitors. A retrospective analysis from the ATAC trial showed that tumours with ER+/PR- status had a greater relative benefit with anastrozole for disease-free survival (HR 0.43; 95%CI 0.31, 0.61) than tumours that were ER+/PR+ (HR 0.84; 95%CI 0.69, 1.02). [71] However, results of central analysis of the study tumours for ER and PR did not shown any benefit of ER+/PR- tumours over ER+/PR+ for disease-free survival. [72] Similar to the MA.17 analysis,

the numbers in the subgroups that were ER+/PR- were smaller than the ER+/PR+ group (n= 1372 vs n=5709), and analysis was retrospective. A central review of ER and PR status was performed for patients in the BIG 1-98 trial of letrozole versus tamoxifen in the adjuvant setting resulting in a significant disease-free survival benefit for ER+/PR+ tumours treated with letrozole (HR 0.67; 95%CI 0.51, 0.88), and a disease-free survival for ER+/PR- with letrozole (HR 0.88; 95%CI 0.55, 1.41). This did not amount to a significant difference in disease-free survival between the two subgroups of ER+/PR+ and ER+/PR- (p=0.32). [73] The biological differences between these two subsets of tumours and the potential mechanisms underlying the different clinical responses to tamoxifen and aromatase inhibitors will be discussed later.

Following the results of these randomised studies, national recommendations have been revised. In September 2005, the Scottish Medicines Consortium (SMC) advised that anastrozole had been accepted for restricted use in women with early invasive breast cancer that is hormone sensitive, and in 2006 was granted restricted use for switching after 2-3 years of tamoxifen. [74, 75] Restricted use of adjuvant letrozole was advised in May 2006, while the SMC stated that another aromatase inhibitor [anastrozole] was available for the same indication at a lower cost. [76] In March 2005 the SMC advised that letrozole was accepted for use for treatment of invasive early breast cancer in postmenopausal women who have already received standard tamoxifen therapy, and that treatment should continue for three years. [77] The National Institute of Clinical Excellence has set out preliminary recommendations that the aromatase inhibitors anastrozole, exemestane and letrozole, within their licensed indications, are recommended as options for the adjuvant treatment of early

oestrogen-receptor-positive invasive breast cancer in post-menopausal women, and that the choice of treatment strategy (that is, primary adjuvant treatment with an aromatase inhibitor, switching from tamoxifen to an aromatase inhibitor or use of an aromatase inhibitor after completion of 5 years of tamoxifen treatment) should be made after discussion between the responsible clinician and the patient about the risks and benefits of the options available. [78] Consideration of the strategy to be adopted should include whether the patient has received tamoxifen as part of their treatment so far, the side-effect profiles of the individual drugs and, in particular, the assessed risk of recurrence.

Aromatase inhibitors and neoadjuvant use

Until recently neoadjuvant therapy of breast cancers consisted predominately of cytotoxic chemotherapy. [79-82] Studies comparing neoadjuvant versus adjuvant use of chemotherapy regimens have shown that while disease-free survival, progression-free survival and overall survival are not changed by the timing of treatment, there is an improvement in all of the above outcomes in patients who have had a good clinical response to treatment in the neoadjuvant setting. [83-85] These results highlight one of the key benefits of using neoadjuvant treatment as a way of predicting response and therefore potentially a survival benefit.

Endocrine treatment is now emerging as an attractive alternative in postmenopausal women with large operable or locally advanced hormone receptor positive breast cancers, many of whom cannot tolerate the toxicities of chemotherapy. A small study from Russia reported results of 121 postmenopausal women with T2-4 ER and/or PR positive breast cancer who were randomised to receive either neoadjuvant doxorubicin and paclitaxel chemotherapy (n=62) or neoadjuvant endocrine treatment

with anastrozole (n=30) or exemestane (n=29) for 3 months prior to surgery. [86] Clinical and mammographic objective response rates were similar for endocrine therapy and chemotherapy, and there was a trend for increasing rates of breast-conserving surgery in favour of endocrine therapy (p=0.054) with no significant differences in local recurrence rates at 34 months. Grade III/IV toxicity for alopecia, neutropaenia, cardiotoxicity and neuropathy were experienced by significant numbers of women in the chemotherapy group. Neoadjuvant endocrine therapy was better tolerated; the most common adverse events reported were hot flushes, fatigue, vaginal bleeding and arthralgia. These data confirm that endocrine therapy is a safe alternative to chemotherapy in postmenopausal women with ER+ cancers, with similar response rates but less toxicity. There is some evidence to suggest that neoadjuvant chemotherapy is less effective in ER+ breast cancer than in ER- breast cancer. A retrospective study of 1,731 patients who had primary chemotherapy showed a significantly lower rate of pathologic complete response to chemotherapy in patients with hormone receptor–positive tumours compared with those with hormone receptor–negative tumours (8% versus 24%; p< .0001). [87] These data are consistent with the observation that patients with hormone receptor–positive disease are less chemosensitive and respond better to endocrine therapy.

Early studies of neoadjuvant hormonal therapy used tamoxifen but did not select patients on the basis of ER or PR to identify those most likely to respond. Most studies with tamoxifen involved elderly patients and compared surgery with or without post-operative tamoxifen to the use of tamoxifen alone, and thus were not designed to assess tamoxifen in the true neoadjuvant setting. These studies do, however, show the effect of tamoxifen as a primary treatment. There have been three

large randomised trials of tamoxifen alone versus surgery alone. While one trial found no difference between the two treatments at six years, [88] a study from Nottingham found a significant increase in local progression in the group who received tamoxifen alone at a median follow-up of 145 months. [89] This was confirmed in a multi-centre European study that reported a shorter time to progression, a worse disease-free survival and worse loco-regional control in women who received tamoxifen alone. [90] A recent meta-analysis of the randomised studies of surgery versus primary endocrine therapy for operable breast cancer in elderly women found no difference in overall survival. [91]

Several randomised trials have compared tamoxifen alone with surgery followed by tamoxifen [92-96] and in all but one of these studies [96] there was a significant increase in local events in the tamoxifen alone group. In one study there was a significantly higher mortality in patients treated with tamoxifen alone, but this increase in mortality was only apparent after three years follow-up. [93] The authors of these studies concluded that long-term hormonal treatment without surgery should be reserved only for patients whose life expectancy is very limited. A meta-analysis of the above studies found a non-significant trend in favour of surgery with tamoxifen over tamoxifen alone for overall survival (HR 0.86, $p=0.06$), and a significant improvement in local disease control with surgery and tamoxifen compared with tamoxifen alone (HR 0.28, $p<0.00001$). [91]

Initial studies performed in Edinburgh suggested there may be benefits to using aromatase inhibitors rather than tamoxifen as neoadjuvant therapy in postmenopausal women with ER+ cancers. [97-99] These results led to a series of randomised studies.

The PO24 trial compared 4 months of neoadjuvant letrozole with tamoxifen in 337 postmenopausal women with large breast cancers that required mastectomy or were locally advanced and inoperable and were ER or PR positive. [100] The primary endpoint of P024 was the percentage of patients in each treatment arm with objective responses (complete or partial response) determined by clinical palpation of the breast cancer. Secondary endpoints were overall objective response rate (ORR) determined by mammogram and ultrasound at 4 months, and the percentage of patients in each treatment arm who became eligible for breast-conserving surgery. World Health Organization response criteria based on bidimensional measurements of area were applied. The results demonstrated that letrozole achieved a significantly higher clinical response rate by palpation than tamoxifen (55% versus 36%; $p< 0.001$) in the intention to treat population. The superiority of letrozole was observed irrespective of baseline tumour size (T2 vs >T2). Letrozole was significantly more effective than tamoxifen irrespective of the assessment method, although response rates assessed by ultrasound and mammography were lower than those assessed by clinical examination. Median time to response was 66 days in the letrozole group and 70 days in the tamoxifen group. The higher response rates assessed by clinical examination were reflected by significantly more letrozole-treated patients than tamoxifen-treated patients being suitable for and undergoing breast-conserving surgery after treatment (45% vs 35%; $p=0.022$). Even in patients with locally advanced breast cancer, significantly more patients from the letrozole arm than from the tamoxifen arm were eligible for breast-conserving surgery. At the end of therapy, 135 (88%) patients in the letrozole arm underwent some type of surgery, compared to 139 (82%) patients in the tamoxifen arm.

The only other factor besides treatment that influenced the likelihood of patients being suitable for breast-conserving surgery was tumour size at presentation, with patients with T2 tumours being more likely to be candidates for breast-conserving surgery than larger tumours (p=0.0001). In this randomised study, letrozole was at least as well tolerated as tamoxifen.

In the P024 study, tumour responses to letrozole versus tamoxifen were also evaluated according to biopsy-confirmed ER and/or PR status. [101] Both letrozole and tamoxifen achieved significantly more responses in patients with ER+ tumours than those with ER- tumours. Differences in response rates between these two agents were most marked for tumours that were both ER+ and/or PR+ and also positive for the markers HER-1 and/or HER-2 (response rate to letrozole 88% versus 21% for tamoxifen; p=0.0004). There was also some evidence of a direct correlation between the degree of ER expression and the incidence and extent of tumour response. In the P024 randomised trial, clinical responses were related to the level of ER expression as determined by immunohistochemistry (IHC) using the semi-quantitative Allred scoring system (0–8). There were no tamoxifen-induced responses at ER levels below a score of 6, in contrast to letrozole-induced responses of >30% at a score of 3.

Two large multicentre, double blind, double-dummy, randomised trials have been published, both comparing efficacy of preoperative use of anastrozole and tamoxifen for postmenopausal women with hormone receptor positive tumours.

The IMPACT study (*IM*mediate *P*reoperative *A*rimidex, Tamoxifen or *C*ombined with *T*amoxifen) recruited 330 patients from UK and Germany, of which 292 were analysable for response, who were randomised to receive anastrozole or tamoxifen or

both in combination for 3 months prior to surgery. [102] Patients had pre-treatment surgical assessment for mastectomy or breast-conserving surgery. The primary endpoint was objective clinical response measured by callipers. As a secondary endpoint, ultrasound response was measured. This study found no difference in objective response between the three treatments as measured by callipers and ultrasound, except for those patients who had HER-2+ tumours, where anastrozole had a numerically higher clinical response rate than tamoxifen (p=0.09) and the combination arm. It was also found that anastrozole enabled significantly more breast-conserving surgery in patients initially thought to require mastectomy than tamoxifen or combination (anastrozole 46% versus tamoxifen 22%; p=0.03).

The PROACT (*PR*e*O*perative *A*rimidex *C*ompared with *T*amoxifen) trial, performed in a number of centres in Europe and USA, recruited postmenopausal women with similar criteria to the IMPACT trial, but also included patients who were inoperable, and those on concurrent chemotherapy. [103] Patients were randomised to receive either 1mg anastrozole (n=202) or 20 mg tamoxifen (n=201), both with placebo, for 3 months. The primary endpoint was objective tumour response measured by ultrasound scan. Calliper measurement was included as a secondary endpoint, alongside surgical assessment at baseline and 3 months. No significant difference in objective response rate (ORR) was seen between treatment arms in all patients, although a trend was noted in favour of anastrozole in those patients who had hormonal therapy alone. There was, however, a significantly higher ORR in favour of anastrozole in those patients initially assessed as requiring mastectomy (36.6% vs 24.2% on ultrasound, p=0.03; and 48.6% vs 35.8% with callipers, p=0.04). This also translated into a significant surgical improvement for those initially requiring

mastectomy with 43% of those treated by anastrozole having breast-conserving surgery versus 30% with tamoxifen (p=0.04), and greater numbers of those with initially inoperable tumours having a surgical procedure performed with anastrozole compared with patients randomised to tamoxifen. No significant adverse events were noted.

A combined analysis of these two studies has been presented but not published, with a total combined population of 535 patients, and an analysis performed of ORR and improvement in surgery. [104] There was a trend in favour of anastrozole in ORR as measured by callipers and ultrasound in the whole group, but this was not statistically significant. There was however, a significant improvement in ORR in favour of anastrozole in the subgroup of patients who were thought to require mastectomy or to have tumours that were inoperable at initial diagnosis; response rate as measured by callipers being 47% vs 35%, (p=0.026) and on ultrasound 36% vs 26%, (p=0.048). For the purposes of the analysis feasible surgery was defined as the surgery required at 12 weeks and actual surgery as surgery that was actually performed at 12 weeks. There was a significant reduction in the extent of feasible (anastrozole 47% versus tamoxifen 35%, p=0.021) and actual surgery (anastrozole 43% versus tamoxifen 31%, p=0.019) in those patients whose tumours were thought to require a mastectomy or were inoperable at initial assessment.

Promising results were shown in an initial study of exemestane in Edinburgh, in which women with large operable or locally advanced primary breast cancer were treated with exemestane for 12 weeks prior to surgery. [98] Twelve patients were treated, of whom 10 had >50% reduction in tumour size, and 2 had stable disease defined as <50% reduction or <25% increase in size. No patients had an increase in

tumour size >25%. Ten patients initially required mastectomy, but only two required mastectomy after neoadjuvant exemestane (breast-conservation rate 80%).

In the "German Neoadjuvant Aromasin® Initiative" (GENARI) trial 27 post-menopausal women with hormone receptor positive T2-4 tumours were treated with 16 weeks exemestane. Of 27 patients, 10 had a partial response and 17 had stable disease, and 14 patients underwent breast-conserving surgery. [105]

In a French study 42 postmenopausal women with ER positive operable breast cancer were given 4-5 months of exemestane prior to surgery. [106] Clinical response as measured on ultrasound confirmed clinical objective response in 73.3%, with a pathological partial response rate of 16.7%. Breast conserving surgery was achieved in 57.1%.

A Japanese study has reported results of four months neoadjuvant exemestane in ER+ and/or PR+ large breast cancers. [107] A pathological response was observed in 13 (43%) of 30 patients who underwent surgery at 4 months, and a clinical response was seen in 27 (66%) of 41 evaluable patients. Breast conserving surgery was performed in 27 (90%) of 30 patients who underwent surgery at 4 months.

In another trial of neoadjuvant exemestane 55 women (age $\geq$65) with ER+ tumours $\geq$3cm not suitable for breast conservation were given treatment for 6 months, after which time 51% had partial response, 47% had stable disease and 2% had progressive disease on mammography, with a breast conservation rate of 55.8%. [108]

In the only reported randomised study of neoadjuvant exemestane, 151 women with hormone receptor positive breast cancer to be treated with either exemestane (E) or tamoxifen (T) for 3 months pre-operatively. [109] Clinical objective response rate assessed by palpation was the primary endpoint. Secondary endpoints were response

measured on mammogram and ultrasound, and number of patients undergoing breast-conserving surgery. Clinical objective response on palpation was superior in the exemestane group (76.3%) compared to tamoxifen (40.0%; p=0.05). There was no significant difference in objective response on ultrasound (E 60.5% vs T 37.3%; p=0.092) or on mammogram (E 64.0% vs T 37.3%; p=0.082). Exemestane treatment resulted in a higher rate of breast-conserving surgery than tamoxifen (36.8% versus 20.0%; p=0.05). Exemestane had a similar reported adverse events rate to tamoxifen.

Neoadjuvant hormone therapy is thus effective at downstaging tumours, particularly large tumours initially thought to be inoperable or requiring mastectomy. Randomised trials have shown that the newer aromatase inhibitors letrozole, anastrozole and exemestane increase the numbers of women who are suitable for breast-conservation compared with tamoxifen, and that letrozole is superior to tamoxifen in terms of clinical response.

A recent meta-analysis of aromatase inhibitors versus tamoxifen for neoadjuvant treatment included the PO24, IMPACT, PROACT and the Semiglazov exemestane trials and pooled results from these four trials. [110] Combined results showed superior rates for aromatase inhibitors compared with tamoxifen for clinical objective response rate (ORR) (RR; 1.29; 95% CI 1.14, 1.47; p< 0.001), ultrasound ORR (RR 1.29; 95% CI 1.10, 1.51; p=0.002) and breast-conserving surgery rate (RR 1.36; 95% CI 1.16, 1.59; p< 0.001).

When compared to neoadjuvant chemotherapy, aromatase inhibitors have similar objective response rates and rates of local recurrence after downstaging and breast conserving treatment, but are better tolerated due to their lower toxicity. Aromatase

inhibitors are now the neoadjuvant hormonal agents of choice in appropriately selected postmenopausal women with hormone sensitive breast cancers. Patients who are most likely to gain benefit from neoadjuvant therapy include: women with locally advanced breast cancer whose disease is not suitable for an initial surgical approach but following response may become operable either by mastectomy or breast conserving surgery; patients with single unifocal cancers of a size relative to the breast size that would require mastectomy, but that with response to neoadjuvant therapy would be suitable for breast conserving surgery; patients with large unifocal cancers that could be treated by breast conserving surgery but where breast conserving surgery would remove a significant volume of the breast and would be unlikely to produce a satisfactory cosmetic outcome. [111]

There are certain tumour types that have been shown to respond slowly or in which response is difficult to assess such as invasive lobular carcinomas (ILC) and mucinous carcinomas, and these cancer types may therefore be less suitable for this treatment. In one study of neoadjuvant chemotherapy invasive lobular carcinomas were found to have lower rates of pathological complete response and lower rates of breast conserving surgery than invasive ductal carcinomas, although this did not translate into poorer overall survival at a median 68 months follow-up. [112]

Randomised studies of neoadjuvant endocrine therapy have treated patients for 3-4 months, based upon previous experience with neoadjuvant chemotherapy. These studies have not addressed the issue of the optimum duration of treatment. One prospective study published recently from Edinburgh included 184 women with large operable or locally advanced ER+ breast cancers who were treated with neoadjuvant letrozole. [113] Tumour samples from this study were utilised for the study outlined

later on within this thesis. One hundred and twenty seven patients (69.8%) had a complete or partial response by 3 months and only four patients had disease progression (>25% increase in tumour volume). Of the 119 patients who had surgery at 3 months, 99 had breast conserving surgery and 19 had mastectomy. The 19 undergoing mastectomy included 15 patients who had locally advanced breast cancer not initially considered suitable for surgery but whose tumours became operable following 3 months of letrozole. Sixty–three patients continued on letrozole beyond 3 months. The mean reduction in clinical volume in the first 3 months in these 63 patients was 46.7%, with a median of 52%. There was a sustained response with continued treatment with further reductions in volume between 3 and 6 months (mean 46.6%, median 50%), between 6 and 12 months (mean 47.8%, median 37%) and 12–24 months (mean 35.8%, median 33%). Prolonged treatment with neoadjuvant letrozole treatment resulted in an increase in the number of patients who were converted from requiring a mastectomy or who were inoperable at diagnosis to be later suitable for breast conserving surgery from 81 of 134 (60%) at 3 months with 96 (72%) eventually being treated with breast conservation. At a median follow-up in this group of 3 years, the median time to treatment failure in women had not yet been reached with 70% of those on prolonged letrozole treatment having their disease controlled on letrozole alone. These data show that in selected patients prolonged treatment with letrozole can result in sustained response with tumour shrinkage. There are no randomised studies of the use of aromatase inhibitors as neoadjuvant endocrine therapy only versus neoadjuvant endocrine therapy followed by surgery, but studies for tamoxifen showed poorer locoregional control without surgery. [88-90, 114] The authors of this study opined that surgery should be offered to patients after a

satisfactory response to neoadjuvant letrozole has been achieved. In patients who are not suitable for or who refuse surgery, neoadjuvant letrozole given for prolonged periods appears safe and appears to provide this group of women with long-term disease control.

Resistance to therapy and predicting response

Results from long term studies with tamoxifen have shown that while some patients will have their disease well controlled by hormone therapy alone, many patients will develop resistance. Results from neoadjuvant studies with letrozole have shown similar findings, with some tumours being resistant from the start, others developing resistance over time. [113] In the neoadjuvant setting, the option is open for excision of a tumour that has ceased to respond to therapy. The therapeutic dilemma in the adjuvant setting is that the tumour has been excised and there is no predictive marker to say which cancers will respond and which are resistant or will develop resistance to endocrine treatment until a clinically evident recurrence is apparent. Clearly having a predictive marker for patients who are likely to respond and those who are likely to be resistant would be of great therapeutic benefit.

One marker that has been identified in neoadjuvant trials as a potential marker of response to treatment is proliferation as assessed by Ki67 (MIB-1).

In the PO24 study there was a significantly greater reduction in proliferation with letrozole, with a reduction in geometric mean Ki67 of 87% with letrozole compared with 75% for tamoxifen ($p = 0.0009$). [115] Results from analysis of tumours samples from the BIG 1-98 trial of letrozole versus tamoxifen have shown a significantly poorer DFS for those patients with tumours with high Ki67 at baseline (HR 1.4; 95%CI 1.1, 1.9; $p=0.02$), with a better DFS for highly proliferative tumours treated

with letrozole than with tamoxifen (HR [Let:Tam] 0.53; 95%CI 0.39, 0.72; p=0.09). [116]

As part of the IMPACT trial, immunohistochemical analysis was assessed after 2 weeks of treatment and at surgery (12 weeks after commencement of hormonal treatment). [117] Initial results revealed that there was a significantly greater reduction in Ki67 in the anastrozole group than tamoxifen or combination groups at 2 weeks (p=0.004) and 12 weeks (p<0.001) but found no significant relationship between Ki67 changes and clinical response. Analysis at a median 37 months follow-up have confirmed a statistically significant correlation between Ki67 after 2 weeks treatment and relapse-free survival (HR 1.96; 95%CI 1.23, 3.07; p=0.004). Ki67 at baseline was not significantly correlated to outcome on multivariate analysis, nor was the change in Ki67 (rather than absolute value for Ki67 at 2 weeks). The implication of these findings is that there is a predictive value in the treatment-determined Ki67 at 2 weeks, which could potentially be used as a prognostic tool for predicting response to neoadjuvant therapy.

Further data are reported from the Edinburgh neoadjuvant letrozole series mentioned previously for 63 patients who had tumour biopsies taken at 0, 2 and 12 weeks (2 week biopsies taken between 10-14 days of starting treatment) for Ki67, and for whom clinical and pathological response data were collected. [118] A clinical response was seen in 76.2%, and a pathological response in 75.8%, with some disconcordance between the two outcomes. While there was no difference in pre-treatment Ki67 counts for responders and non-responders, there was a significantly higher Ki67 at 2 weeks in those that had no pathological response compared to those that had a pathological response (9.35% versus 4.02% respectively, p=0.024), and the same was

seen at 3 months (8.11% versus 3.47%, p=0.009). A significant reduction in Ki67 was seen in responders (both clinical and pathological) between 2 weeks and 12 weeks, but not for non-responders.

A change in Ki67 of 40% between different paired biopsies was taken as being meaningful by the authors, and significantly more pathological responders (42 of 47) had a reduction of ≥40% compared with pathological non-responders (9 of 15, p=0.034). No difference was seen between clinical responders and non-responders. Different patterns of changes in Ki67 over the treatment period were detected. The largest cohort of tumours (n=47) showed substantial decreases at 10–14 days that were maintained or fell further at 3 months, but there was a cohort of five patients in which a decrease at 10–14 days was followed by a substantial rise in score at 3 months. There is therefore evidence for the predictive value of Ki67 at baseline and at 2 weeks for treatment response with aromatase inhibitors.

The development of resistance to endocrine therapy is a major clinical problem occurring either de novo or acquired during treatment. ER positivity is the strongest predictor of response to treatment but the development of resistance indicates the influence of other factors, as this resistance can occur without loss of ER expression in tamoxifen resistance. [119, 120] In the case of tamoxifen this may be because of the partial agonist effects to stimulate non-genomic activity of ER in cells with high EGFR and HER-2. [121] An intrinsic mechanism of tamoxifen resistance has also been identified in 8% of Caucasian women carrying inactive alleles of cytochrome P450 2D6 (*CYP2D6*) resulting in failure to convert tamoxifen to its active metabolite, endoxifen, and consequently less responsiveness to tamoxifen. [122]

Figure 1 shows the normal mechanism of ER signalling. [123] There are a number of co-activators in the oestrogen bound cell, as opposed to co-repressors present when not in oestrogen bound state, thus modulation of ER is likely to be a balance of co-activation and co-repressor activity. The co-activator complexes (histone acetyltransferase (HAT) complexes) stimulate ER activity following ligand stimulation through direct interaction with AF2. Drugs such as tamoxifen and raloxifene inhibit AF2 activation (active in breast epithelium) and thus ER activity, but do not prevent activation of AF1 (active in other tissues such as the uterus). Pure anti-oestrogens such as faslodex inhibit both AF1 and AF2 and are thus anti-oestrogenic in all tissues. In the case of aromatase inhibitors the oestrogen availability for binding is reduced, shutting off genomic and non-genomic signalling, further implicating factors other than ER expression influencing resistance.

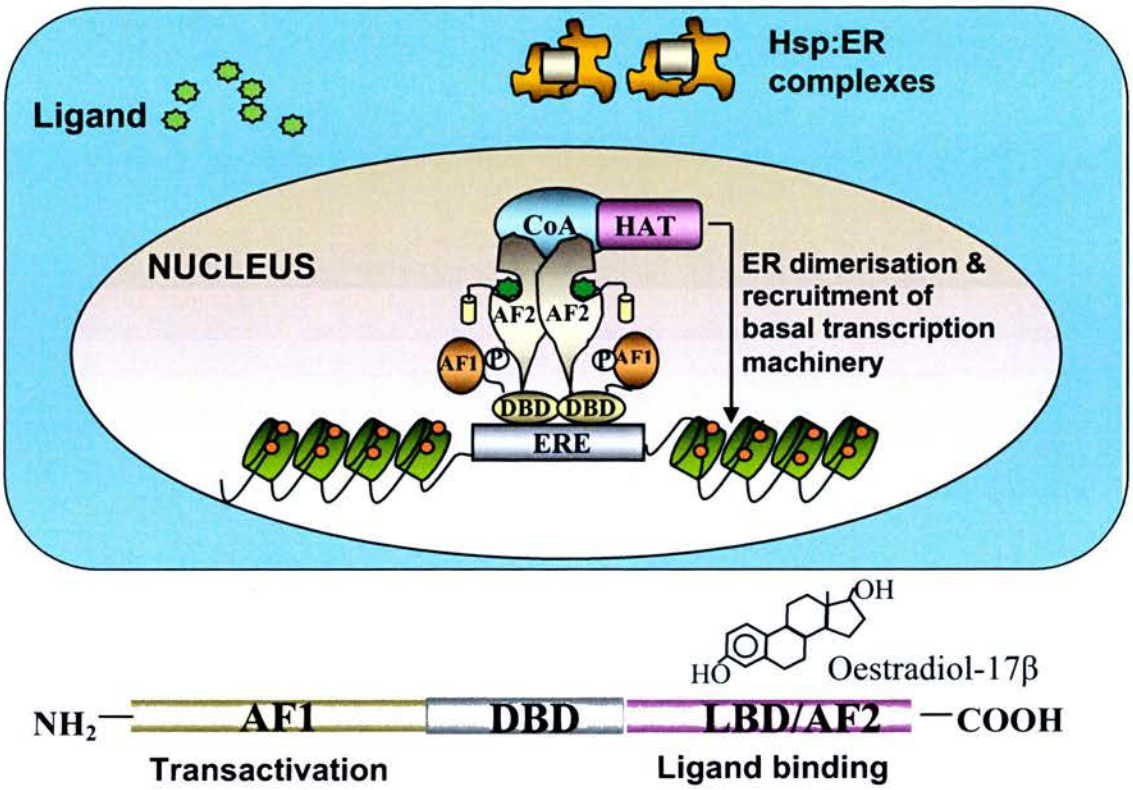


Figure 1
In the normal cell, oestrogen binds to ER resulting in a conformation change by binding as a dimer to oestrogen response elements (EREs) in the gene promoters at 2 distinct ER activation domains AF1 (regulated by phosphorylation) and AF2 (regulated by oestrogen binding), which in turn activate transcription of oestrogen regulated genes. (AF- activation domains, CoA- co-activators, DBD- DNA-binding domain, LBD- ligand-binding domain). Courtesy of Dr LA Martin, Institute of Cancer Research, Breakthrough Breast Cancer Centre, London.

A number of molecular mechanisms have been proposed to be involved in endocrine resistance. These include ER mutations resulting in increased sensitivity to ligand recruitment, ligand independent activation of ER (through growth factor signalling pathways), increased expression of co-activator proteins mediating ER activity and non-genomic effects of ER, such as direct interaction with signal transduction pathways, also known as post-translational modification. [123-125] **Figure 2**

ER activity classically is thought to require ligand binding but growth factors can stimulate ER in the absence of a ligand, such as epidermal growth factor (EGF), Protein Kinase A, steroid receptor co-activator (Src), HER-2 and PI3K/ Akt. Of particular significance in this thesis is the phosphorylation by Akt that occurs at ser 167 resulting in ligand-independent activation of ER. [126] A number of therapeutic agents are available or are under development as antagonists to these growth factors, such as HER-2 antagonist herceptin (discussed later), gefitinib and erlotinib (EGFR antagonists), and inhibitors of the mTOR/ PI3K/ Akt pathway RAD001 and CCI-779 (discussed later).

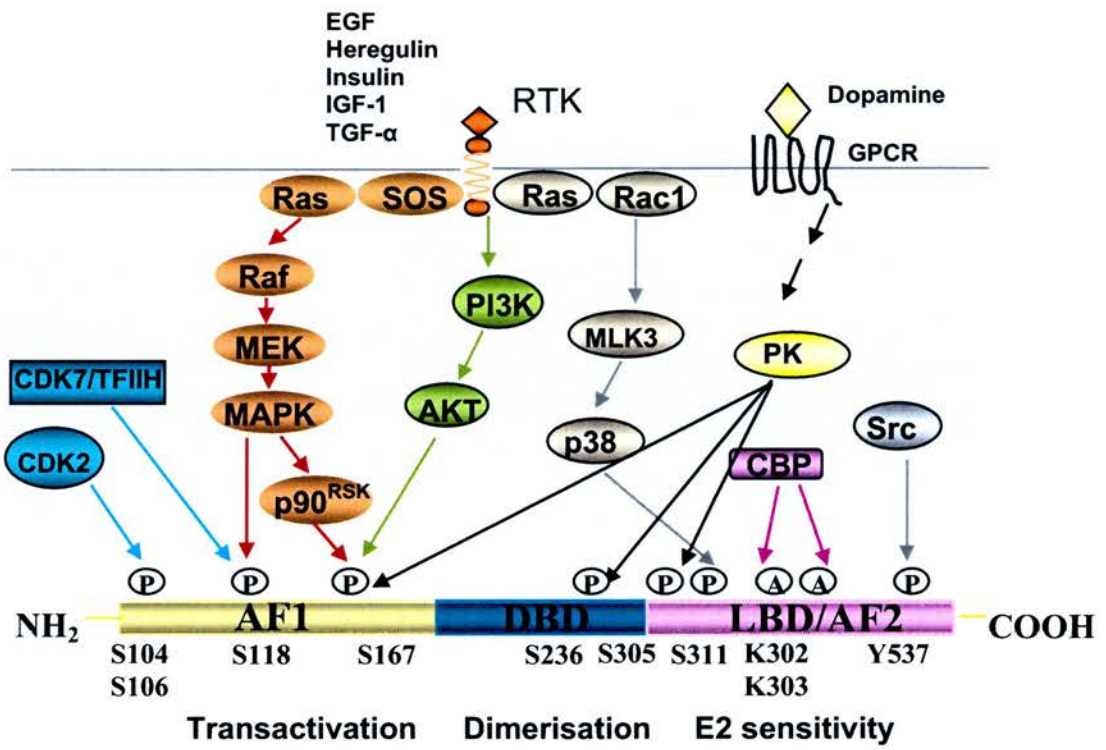


Figure 2
Post translational modifications of the oestrogen receptor-signalling pathway, demonstrating growth factor pathways that have been implicated as activating ER in the absence of oestrogen. Courtesy of Dr LA Martin, Institute of Cancer Research, Breakthrough Breast Cancer Centre, London.

As well as the structural and functional changes to the ER in endocrine resistance, it has been recognised that there is an influence of PR in response to treatment. As mentioned previously, the ATAC and BIG 1-98 studies both showed a better response to aromatase inhibitors over tamoxifen in ER+/PR- tumours, indicating resistance to SERMs in ER+/PR- tumours and less resistance to oestrogen withdrawal. [71, 73]

It had previously been thought that the PR was dependent upon ER and that absence of PR reflected a non functional ER and thus resistance to hormone therapy. However the results from the ATAC trial suggest that there is a response to oestrogen withdrawal in ER+/PR- tumours indicating simple loss of ER function via low levels of oestrogen or ER is not the whole explanation. Indeed, there is a suggestion that the benefit seen for anastrozole over tamoxifen in the ATAC trial was largely due to reduced efficacy in the ER+/PR- tumours. [127] In a study of tamoxifen treated tumours both ER and PR were independent predictors of overall survival, with a greater response to tamoxifen in ER+/PR+ compared to ER+/ PR- tumours. [128] There is good evidence to date that there are significant biological differences between ER+/PR- tumours to account for these different levels of responsiveness to endocrine therapy. ER+/PR- tumours have higher levels of HER-2 positivity and EGFR signalling than ER+/PR+ tumours, and loss of PTEN associated with upregulation of mTOR/Akt/PI3K pathway results in loss of PR, implicating other regulatory mechanisms. These proposed mehcanisms include loss of PR due to hypermethylation and repression of the PR promoter, or genetic loss at the PR gene locus in up to 40% of breast cancers. [127] Also implicated are growth factor mediated downregulation of PR independent of ER, as IGF-1, EGF and heregulin have been

shown to lower PR levels in cell lines, and/or growth factor influenced translational modification of ER resulting in alteration of oestrogen-regulated genes, of which PR may be an example.

All of this emerging evidence of the significance of PR in endocrine resistance and the biological differences between PR+ and PR- cancers is suggestive of inherent resistance of ER+/PR- tumours to tamoxifen, and that aromatase inhibitors should be used in preference in these tumours, or with combination treatment of tamoxifen and growth factor inhibitors.

Molecular targeted therapy

Receptor tyrosine kinases and growth factor receptors regulate numerous functions in normal cells and have a crucial role in oncogenesis. Epidermal growth factor (EGF) was one of the first to be identified in 1962, with identification of the EGF receptor in 1978 in epidermoid carcinoma cells. The development of cloning techniques has allowed characterisation of the molecular mechanisms by which EGFR and other tyrosine kinase receptors function. [129]

The targeted therapy that has been most investigated to date is that against HER-2/neu receptors. Amplification of the HER-2/neu oncogene is present in 20-25% of breast tumours, and is associated with reduced overall survival and shorter time to relapse in node-positive patients. [130, 131] When amplified the gene produces high expression of HER-2 cell surface receptors. Trastuzumab is a monoclonal antibody that was developed to recognise HER-2 cell surface receptors, and has been shown to be active against HER-2 positive disease in both metastatic and early breast cancer. Early studies of efficacy and safety in metastatic breast cancer yielded objective response rates of between 23-35% in those with over-expression of HER-2. [132, 133]

Several large clinical trials of trastuzumab in early breast cancer have reported results. The HERA study included 5081 women with histologically confirmed HER-2 amplified (IHC 3+ or FISH amplification positive) node-positive breast cancer or node-negative tumours >1cm who had received primary treatment including adjuvant chemotherapy and were randomised to observation or to one year of trastuzumab or two years of trastuzumab. [134] Interim results after one year showed that patients in the one-year trastuzumab arm had a significant improvement in disease-free survival

(HR 0.54, p<0.0001) and distant disease-free recurrence (HR 0.49, p<0.0001) and a trend towards better overall survival (HR 0.76, p=0.26) compared with the observation group. At 2 year follow-up an overall survival benefit was shown for trastuzumab over placebo. [135] The most recent data from the HERA trial presented at St Gallen 2009 at a median 4 year follow-up showed significantly better disease-free survival (HR 0.76; 95%CI 0.66, 0.87; p<0.0001), but not overall survival (HR 0.85; 95%CI 0.70, 1.04; p=0.1087) with trastuzumab. [136] These latest results are for the intention-to-treat (ITT) population, and this is thought to be influenced by the large number of patients who crossed over to the trastuzumab arm of the trial after the one year data were presented (885 patients of original 1689 in observation group crossed to trastuzumab arm).

Two studies that randomised patients either to receive trastuzumab concurrently with chemotherapy or chemotherapy alone reported a combined analysis. The NSABP trial B-31 randomised 2043 women to doxorubicin and cyclophosphamide followed by paclitaxel or the same chemotherapy with one year of trastuzumab commencing on the same day as paclitaxel. The NCCTG trial N9831 randomised 1633 women to the same arms as above but had a third study arm not included in the combined analysis in which patients were randomised to doxorubicin and cyclophosphamide followed by paclitaxel and then commencing trastuzumab after completion of paclitaxel therapy. In the combined analysis of 3351 patients (control arm n=1679, trastuzumab n=1672) there was a significantly longer disease-free survival (HR 0.48, p<0.0001), distant disease-free survival (HR 0.47, p<0.0001) and improved overall survival (HR 0.67, p=0.015) after a median follow-up of 2 years. [137] At median follow-up of 3 years, there continued to be benefit for trastuzumab for DFS (HR

0.49; 95%CI 0.41, 0.58; p<0.0001) and OS (HR 0.63; 95%CI 0.49, 0.81; p=0.0004). [138]

In the BCIRG 006 study 3222 women with node positive or high risk node negative HER-2 positive tumours were randomised to receive either doxorubicin and cyclophosphamide followed by docetaxel (AC → T) or doxorubicin and cyclophosphamide followed by docetaxel and trastuzumab (AC → TH) or docetaxel, carboplatin and trastuzumab (TCH). [139] Interim results at 36 months median follow-up showed an absolute disease free survival (DFS) benefit of AC → TH over AC → T of 6% (HR 0.61; 95%CI 0.48, 0.76; p<0.0001) and an overall survival benefit of AC → TH over AC → T (HR 0.59; 95%CI 0.42, 0.85; p=0.004). The combination of TCH also had overall survival (HR 0.66; 95%CI 0.47, 0.93; p=0.017) and disease free survival benefit (HR 0.67; 95%CI 0.54, 0.83; p=0.0003) over AC → T.

In the FinHer study 1010 women with axillary-node–positive or high-risk node-negative cancer to receive three cycles of docetaxel or vinorelbine, followed by (in both groups) three cycles of fluorouracil, epirubicin, and cyclophosphamide. [140] The 232 women whose tumours had an amplified HER-2 gene were further randomised to receive or not to receive nine weekly trastuzumab infusions. In this study, trastuzumab was administered before other cardiotoxic therapies and concomitantly with potentially synergistic chemotherapy for only nine weeks to test the hypothesis that such a schedule would limit cardiotoxicity and maintain efficacy. In those patients who were HER-2 positive there was a significant improvement in recurrence-free survival (HR 0.42, p=0.01) and a trend for better overall survival

(HR 0.41, p=0.07) after a median of three years follow-up. Numbers in this study were smaller than the other published studies of adjuvant trastuzumab.

The most significant adverse event emerging from trastuzumab studies has been cardiotoxicity, primarily congestive cardiac failure, with an incidence of 1.4% in women receiving trastuzumab as a single agent. [132, 133] In patients with metastatic disease receiving trastuzumab concomitantly with paclitaxel or anthracyclines higher incidences of cardiac dysfunction have been reported (13% and 27% respectively). [139, 141] From study results available it appears the risk of cardiac dysfunction varies with the type and timing of chemotherapy, in that concomitant use of trastuzumab with non-anthracyclines is low-risk, use of sequential anthracycline then trastuzumab is higher risk, and concomitant trastuzumab and taxane after anthracyclines is associated with a risk of severe congestive heart failure. [142]

These promising results with targeted therapy against HER-2 have sparked renewed enthusiasm for discovery of and development of therapies to combat specific molecular targets in oncogenesis.

Of particular relevance to this thesis is the potential use of agents that target specific molecular pathways for combination treatments. More specifically, there is a reasonable body of scientific evidence to suggest that these agents may be useful in endocrine resistant tumours, either endocrine-naïve or those that have become resistant over time. [143-145] One such potentially important target, the mammalian target of rapamycin (mTOR) will be discussed in this thesis.

Oestrogen deprived MCF7 cells have increased mTOR and Akt activity, which has led to the hypothesis that both proteins are an essential part of adaptation of breast cancer cells to low oestrogen levels. [146] It has previously been shown that patients

treated with tamoxifen were more likely to relapse if they had high expression of p-Akt, and that such patients have a decreased overall survival. [147] A study in breast cancer cell lines with aberrant Akt activity has shown that by inhibiting mTOR tamoxifen response can be restored. [148] Furthermore it has been shown in breast and ovarian cell lines that by combining tamoxifen and RAD001 there is an additive antitumoural effect, [149] also seen with the combination of letrozole and RAD001 in breast cancer cell lines. [143]

The mTOR pathway

The mTOR pathway: background

The mammalian target of rapamycin (mTOR) is a 289kDa serine/threonine protein kinase that belongs to the phosphatidylinsotide-3OH kinase related kinase (PIKK) family. The PIKKs are a subfamily of kinases related to phosphoinositide 3-kinase (PI3K) by a similarity on the catalytic domains. The first members of the PIKK family to be molecularly cloned from studies in budding yeast cells were TOR1 and TOR2, identified as the target of the drug rapamycin when bound with FK506 binding protein-12 (FKBP-12). The mammalian orthologue of TOR (mTOR) was isolated shortly thereafter by several groups of investigators, thus it is also known as FKBP-12 and rapamycin-associated target (FRAP), rapamycin and FKBP-12 target-1 (RAFT1), rapamycin target-1 (RAPT1) or sirolimus effector protein (SEP). [150-154] mTOR exists in two distinct complexes within the mammalian cell, one containing mTOR, G-protein beta-subunit-like (GβL) and raptor (regulatory associated protein of mTOR), and the other containing mTOR, GβL and rictor (rapamycin-insensitive companion of mTOR). [155-158] **Figure 3** The roles of these components will be discussed later, as most of what is understood about these complexes is as a direct result of drug studies with rapamycin. The mTOR protein consists of up to 20 tandemly repeated motifs at the amino-terminus called HEAT motifs, which are thought to form interfaces for protein-protein interactions. [159, 160] At the carboxyl terminus there is a mutated FRAP-ataxia-telangiectasia (FAT) domain, a transformation/ transcription domain, a FKBP-12-rapamycin binding (FRB) domain,

a catalytic kinase domain (similar to those found in the PI3K family), a probable autoinhibitory or repressor domain and a FAT-carboxyl (FATC) terminal domain.

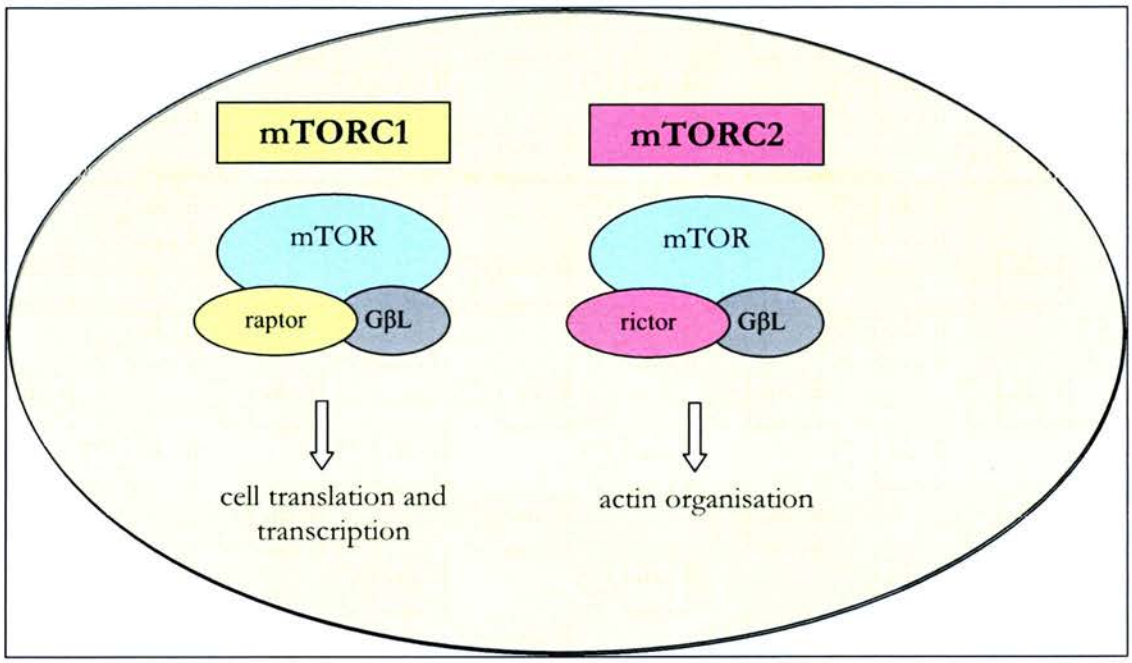


Figure 3
mTOR exists in two distinct complexes within the mammalian cell, one containing mTOR, GβL and raptor (mTORC1) and the other containing mTOR, GβL and rictor (mTORC2).

Downstream of mTOR

P70S6 kinase (S6K1) and eIF4E binding proteins (4E-BPs, also known as translational repressor protein PHAS-1) are the two best characterised downstream effector molecules of mTOR. [161] **Figure 4** It has become apparent that these actions of mTOR are through the mTOR1 complex with raptor. The actions of mTOR2 complex with rictor will be discussed separately.

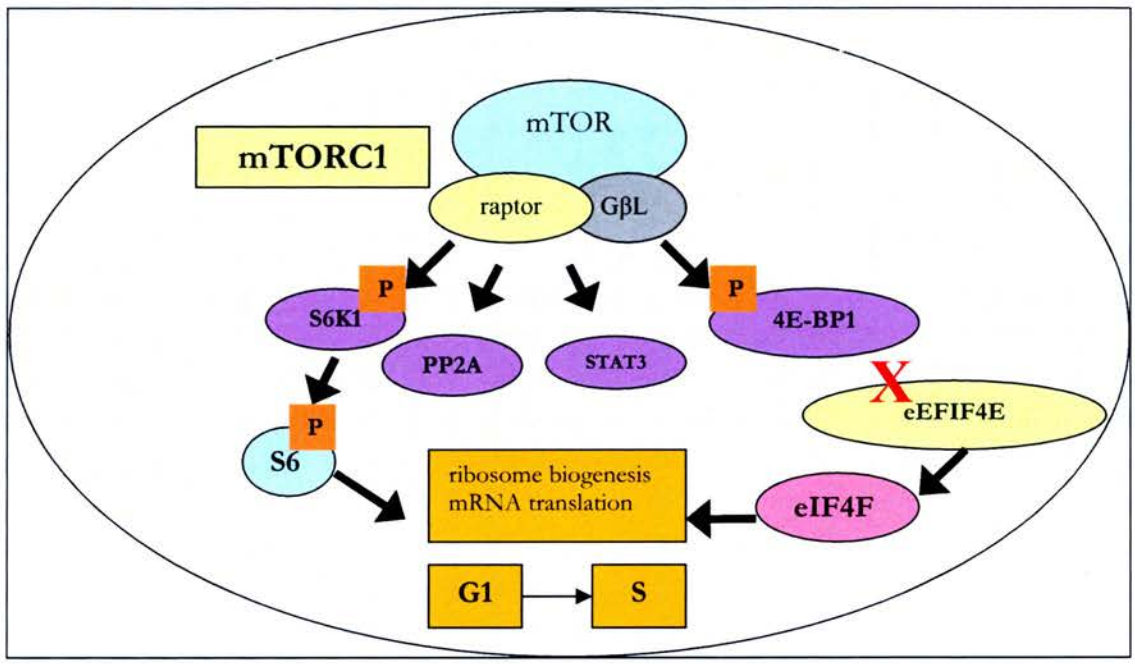


Figure 4
Actions of mTOR in complex with raptor (mTORC1) on S6 kinase 1 and 4E-BP1, thus promoting progression of the cell cycle from G1 to S phase (see text below).

S6K1 is a serine-threonine kinase, activation of which by mTOR enhances the translation of mRNA that bears a 5'-terminal oligopyrimidine tract (TOP). [159] S6K1 requires phosphorylation at two sites for its full activation: at Thr389 in its C-terminal hydrophobic motif and phosphorylation of its T loop. mTORC1 mediates phosphorylation of Thr389. [161] Activated S6K1 phosphorylates the 40S ribosomal protein S6 to actively translate mRNAs with TOP, including ribosomal proteins, elongation factors and insulin growth factor-II and is thought to regulate cell size. [160, 162-164] S6K1 has been shown to phosphorylate mTORC1 at ser 2448, [165, 166] with ser 2481 the main site of phosphorylation of mTORC2. [167] Studies comparing normal breast, benign and malignant breast tumours have shown immunohistochemical overexpression of S6K1 and S6K2 in breast cancer, [168] and in a study of 452

premenopausal breast cancer patients, p70S6 kinase overexpression was independently predictive for poor locoregional disease control (HR 2.5, p=0.006) and disease-free survival (HR 1.8, p=0.025) at median 10.8 year follow-up. [169]

The second signalling pathway promoted by mTOR involves phosphorylation of 4E-BP1 and inactivation of 4E-BP1 suppressor proteins. The phosphorylation of 4E-BP1 results in a dissociation from eIF4E, facilitating the subsequent formation of the eIF4F complex consisting of eIF4E, eIF4G, eIF4A and eIF3, thereby enhancing cap-dependent protein translation. [159, 170, 171] The eIF4F complex is necessary for the recruitment and positioning of ribosomes at mRNA sites, as eukaryotic ribosomes do not have the ability to locate and bind to the 5' end of mRNA, but need translation initiation factors to guide them. This is a crucial step toward activating translation of mRNAs with specific regulatory elements in the 5'-untranslated terminal region (5'UTR), especially c-myc, cyclin D1, vascular endothelial growth factor (VEGF) and ornithine decarboxylase. [162, 172]

Other proteins that have been shown to be directly affected by mTOR include PP2A and STAT3. PP2A is a serine /threonine phosphatase that is involved in a variety of cellular functions such as cellular metabolism, DNA replication, transcription, translation, cell proliferation and cell transformation. mTOR controls the binding of catalytic subunits and regulatory proteins within PP2A, thereby negatively regulating the function of these subunits, and affecting the translation by eukaryotic elongation factor 2 (eEF2). [162, 173] STAT3 is a transcriptional activator that is directly phosphorylated by mTORC1 resulting in regulation of transcription factors. [173]

Upstream of mTOR

The phosphoinositide 3-kinase (PI3K) family is deregulated in oncogenesis resulting in disruption to a cascade of signals that normally regulate basic cellular properties such as proliferation, survival and angiogenesis. [174] There are multiple mechanisms for this deregulation, including mutation or silencing of the PTEN tumour suppressor gene which, in the normal cell, acts to inhibit the activation of the PI3K-Akt cascade. **Figure 5** Without this suppressor mechanism, the hyperactivation of Akt, also known as protein kinase B (PKB), is associated with resistance to apoptosis, increased cell growth, cell proliferation and cellular energy metabolism. [175] All of the above enzymes are implicated in the regulation of the mTOR pathway, primarily through the interaction of Akt and mTOR. This interaction is complex and not well understood due to continued new developments in the discovery of how Akt is influenced by rictor-mTOR and raptor-mTOR in apparent antagonism.

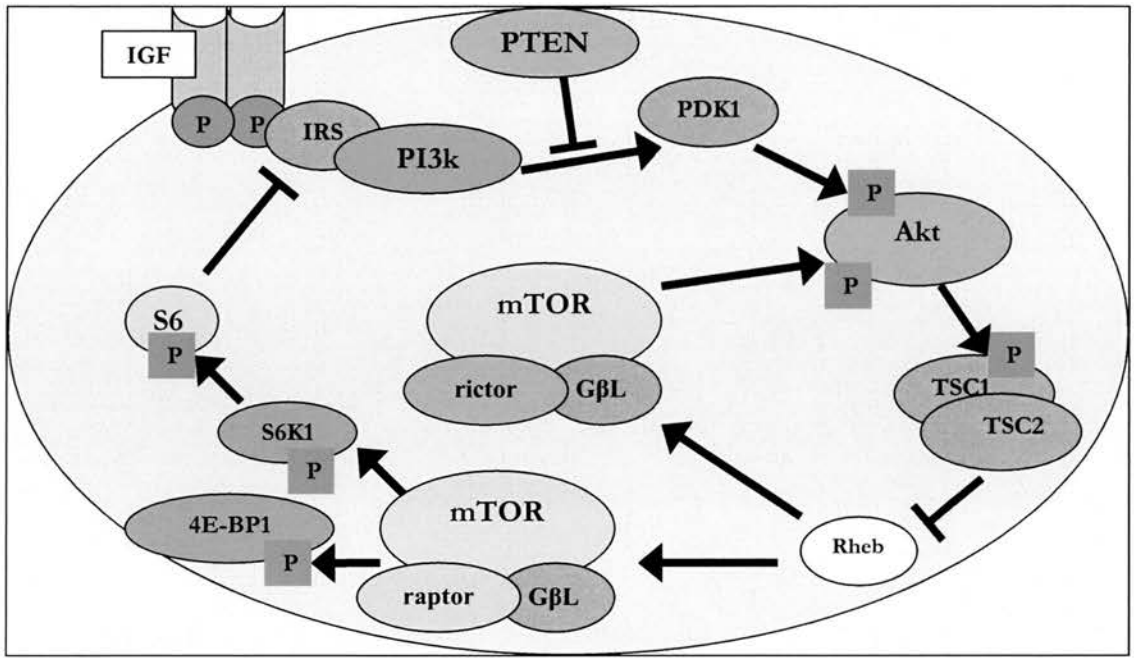


Figure 5
The interaction of mTOR as mTORC1 and mTORC2 with PI3K/ Akt pathway (see above text).

A study of RNA interference (RNAi) in Drosophila cells with knockdown of aspects of the mTOR pathway was performed to determine the role of mTOR upon Akt signalling. [176] What was found is described in **Figure 6**. In the normal Drosophila cell, Akt stimulated mTOR which stimulated S6kinase with a resulting negative feedback loop to control Akt activation. Direct inhibition of S6kinase resulted in upregulation of Akt, as did inhibition of mTOR raptor. If, however, mTOR was inhibited, there was little change in the activation of Akt, or a slight decrease in activation, indicating that mTOR is involved in activation of Akt.

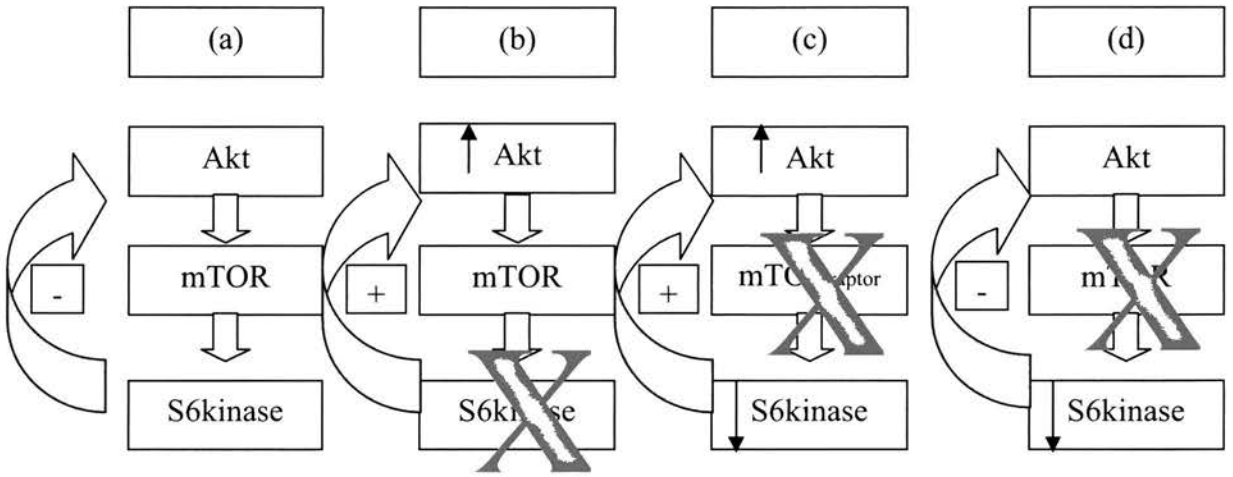


Figure 6
Findings from Sarbassov et al, Science 2005, [176] in Drosophila with direct inhibition of different parts of the mTOR pathway. (a) normal pathway with no inhibitors: Akt stimulates mTOR activation which activates S6 kinase with a negative feedback loop to maintain similar levels of Akt. (b) when S6 kinase is directly inhibited, there is loss of the negative feedback loop and upregulation of Akt. (c) if mTOR raptor complex is inhibited, there is subsequent reduction in p-S6 kinase and thus loss of negative feedback with upregulation of Akt. (d) If both complexes of mTOR are inhibited, however, there is downregulation of S6kinase but with no reduction in Akt, suggesting an action of mTOR that is necessary for phosphorylation of Akt that is not involving raptor.

mTOR as a therapeutic target in cancer

The majority of what is understood about the mTOR molecule is due to the discovery of rapamycin in the 1970s. After isolation from a strain of *Streptomyces hygroscopicus* in the soil of Easter Island (Rapa Nui), it was discovered that as well as being a potent fungicide, rapamycin's antiproliferative properties extended beyond fungi to have high potency as both an immunosuppressant and antineoplastic agent against mammalian cells. More specific details on how rapamycin achieved these effects remained elusive for 20 years until several groups of investigators identified the mTOR protein kinase in 1994. Since then derivatives of rapamycin have been developed, namely cell cycle inhibitor-779 (CCI-779, Wyeth-Ayerst, MA), AP23573 (Ariad Pharmaceuticals, Cambridge, MA) and RAD001 (Novartis Pharmaceuticals, Basel, Switzerland). All three drugs are currently undergoing clinical development as potential anticancer agents, but the focus of this thesis will be on RAD001 (everolimus, Certican©) and its effects in early breast cancer.

RAD001 in cancer studies

RAD001 is currently being assessed as an agent in a number of cancers. Studies in prostate cancer have shown that particularly in PTEN-deficient cell lines, RAD001 amplifies the autophagic pathway of cell death and is thus capable of radiosensitising these tumours. [177]

In ovarian cancer, a study has been performed in Tag-DR26 mice which develop ovarian tumours with strong immunoreactivity for Tag, phospho-Akt, phospho-mTOR, and phospho-p70S6K in early and advanced lesions. [178] These mice when treated with RAD001 from 5 weeks of age to 20 weeks of age at 5mg/kg nearly all

developed ovarian tumours but RAD001 treatment significantly delayed tumour onset and progression (median time for ovaries to reach a volume of 50 mm^3 was 90 days in the placebo group compared with >122 days in RAD001-treated mice; median tumour burden measured at time of euthanasia was 429.2 mm^3 in controls versus 26.2 mm^3 in RAD001-treated mice). RAD001 treatment resulted in a significant reduction in the number of mice with advanced disease and inhibited ascites formation and peritoneal dissemination.

In a cell line study of human pancreatic neuroendocrine tumour (NET), BON cells, known to have constitutive Akt/mTOR phosphorylation, were treated with RAD001 at different concentrations for 72 hours. [179] Lost expression of the PI3K antagonist PTEN occurs in 54% of poorly differentiated neuroendocrine carcinomas and 76% of all NETs display constitutive Akt phosphorylation. Results from this study showed inhibition of proliferation by 50% and induction of apoptosis with RAD001 treatment.

One clinical study has been reported of RAD001 in patients with advanced haematological malignancies. [180] Patients with histologically confirmed advanced, relapsed, or refractory acute myeloid leukaemia (AML) or myelodysplastic syndrome (MDS), chronic lymphocytic leukaemia (CLL), T-cell leukaemia, myelofibrosis, or mantle cell lymphoma (MCL) were treated with RAD001 5mg (first three patients) or 10 mg orally once daily (one cycle lasting 28 days). Toxicity data were available for 27 patients and included grade 1 or 2 hyperlipidemia (44%), elevation of transaminases and/or alkaline phosphatase (41%), anorexia (37%), mouth ulcers (37%), diarrhoea (29%), hyperglycaemia (26%), and hypomagnesaemia (22%). One patient with refractory anaemia with ringed sideroblasts developed a

biopsy-proven grade 3 cutaneous leucocytoclastic vasculitis (LCV) after 120 days of therapy, which was believed to be related to RAD001. Thirty-eight infectious episodes were noted in 21 (78%) patients at some time during the therapy. Fourteen (37%) of the 38 infectious episodes that occurred in 9 patients required hospitalisation and in some patients this was prolonged. One objective response was sustained of 26 evaluable for response. Although no patients with CLL patients achieved a complete or partial response, three patients had a 27% to 34% reduction in lymphadenopathy, documented radiologically, after two to three cycles of therapy. The most promising results to date with RAD001 are in advanced renal cell cancer. [181] Two hundred seventy-two patients with metastatic renal cell carcinoma with progressive disease were randomised (2:1) to RAD001 or placebo, with progression-free survival (PFS) the primary outcome. There were 191 PFS events (47% of 410 pts) reported by central review: 37% on RAD001 compared with 65% receiving placebo. As a consequence of these interim analysis results, the Independent Data Monitoring Committee stopped the study to allow remaining patients on placebo to receive RAD001. Most common adverse events (AEs; all grades/ grade 3–4) were mouth ulcers (RAD001 36/ 4%, placebo 7/ 0%), anaemia (RAD001 28/ 7%, placebo 15/ 5%), and fatigue (RAD001 28/ 2%, placebo 20/ 4%). 10% of pts had AEs leading to discontinuation with RAD001 versus 4% with placebo. 68 deaths were observed, and study follow-up is ongoing to assess the secondary endpoint of overall survival. Studies with other mTOR inhibitors are at a similar stage in development as oncological agents as RAD001, but lack the advantage of RAD001, it being licensed for a different purpose, namely that of multi-drug immunosuppression for transplant. Valuable insights into the function, safety and tolerability of RAD001 are thus

already available. CCI-779 (Wyeth Pharmaceuticals) has been shown in pre-clinical breast cancer cell lines to inhibit cell growth, particularly in PTEN wildtype cell lines, and those with elevated baseline Akt and mTOR signalling. [182] A delay in tumour growth with treatment with CCI-779 has also been seen in human pancreatic neuroendocrine tumours (PNET) and malignant glioma xenografts. [183] CCI-779 has been trialled as a single agent in 109 patients with locally advanced or metastatic breast cancer who had failed to respond to anthracycline or taxane based regimens. [184] Clinical benefit was seen in 36 patients (37%), including patients with liver metastases. Median duration of response was 5.4 months. Interestingly, no response was seen in the 33 HER-2-neu negative patients.

Pharmacology of RAD001

RAD001 is a novel macrolide that is similar to rapamycin but bears a hydroxyl-ethyl chain giving it increased polarity for oral formulation. RAD001 has a licence for use in multi-drug immunosuppression after transplantation, and is thus well investigated in terms of its pharmacology and toxicity in the transplant population. The pharmacological discussion herein will focus upon the oncological studies that have taken place, but evidence from the transplant setting will be drawn upon where appropriate.

RAD001 is formulated as 5mg tablets, and is metabolised mainly by CYP3A4 in the liver. Co-administration of other compounds that are known inhibitors or inducers of CYP3A should be avoided, which includes grapefruit, certain anticonvulsants and antibiotics. **Appendix 1** Taken orally, RAD001 is rapidly absorbed with peak levels after an hour and an elimination half-live of 30 hours.

The mechanism by which RAD001 inhibits mTOR is similar to the action of rapamycin, which has high affinity for the immunophilin FKBP-12 and when bound together interact with mTOR. It is thought that the main interaction with mTOR is within mTOR complex1 (in association with raptor), but there is evidence to suggest that with prolonged treatment mTOR complex 2 (in association with rictor) is also inhibited.

RAD001 has a licence for use in solid organ transplantation as part of multi-drug immunosuppression, and as a result has more toxicity data available than would be normal for a drug undergoing phase II testing. None of these studies have been published and details of results are owned by Novartis Pharma AG.

In healthy subjects and stable renal transplant patients undergoing single dosing up to 4mg there were few side effects, headache being most notable. A small reduction in platelet and neutrophil counts was seen but this was transient. Two multiple dose studies were carried out with placebo control of doses up to 10mg/day in 63 stable renal transplant patients who were also taking cyclosporin, which was expected to increase the exposure to RAD001. The dose limiting toxicity in transplantation was established as being thrombocytopenia ($<100 \times 10^9$/L) at 10mg/day. Longer term studies up to 24 months were carried out in 1404 kidney transplant patients, and a consistent increase in blood lipid levels and reduction in platelet and leucocyte counts occurred with longer durations of treatment.

In phase 1 oncology studies to date, the most frequent adverse events reported have been stomatitis, diarrhoea, mouth ulceration, gastroenteritis and hypercholesterolaemia.

In a phase 1 oncology study to identify an optimal dosage of RAD001 given weekly, 20-30 mg / weekly was tolerated satisfactorily and resulted in downregulation of p70S6kinase in peripheral blood mononuclear cells which were used as a marker of pharmacodynamic effects. This was in patients with advanced cancers (none were breast). Daily dosing of up to 10mg in the transplant setting resulted in no increase in adverse events and with a half-life of 30 hours, daily dosing of 5mg, a cumulative dose of 35mg per week was chosen as the initial phase 2 dose for this study.

Aims

Within this thesis the candidate has attempted to answer the questions defined below through a number of modalities. While these aims run throughout the thesis, each method of investigation has a separate section with individual aims specified at the beginning of each section. It is important to stress at the outset the common thread of these methods and the ultimate unifying aims of this thesis.

These aims are:

- To assess the safety and tolerability of RAD001 in postmenopausal women with early breast cancer
- To assess the effects of 5mg RAD001 upon tumour proliferation
- To investigate potential biological markers within the mTOR pathway as predictive for response to RAD001
- To clarify the role of any biological marker identified above in breast cancer cell lines treated with RAD001
- To assess the role of any biological marker identified above in the resistance and sensitivity of breast cancer to endocrine agents, and thus the potential for RAD001 as a combination agent

Clinical Study

Aims

The principal aim of the clinical study was to investigate the safety and tolerability of RAD001 at a dose of 5mg in postmenopausal women with early breast cancer in the pre-operative setting. The collection of breast cancer biopsies from each patient was a prerequisite for entry into the RAD001 study, in order to meet the primary aims of the biological study.

Patients and methods

The clinical study was a pre-operative 2 week study of patients with operable early breast cancer. The initial target accrual was 30 patients, with 25 evaluable patients with adequate tissue samples at diagnosis and at surgery who had also completed the course of treatment with RAD001 and had surgery within 14-16 days of starting RAD001. If after 30 patients less than 25 were evaluable, more patients would be recruited until 25 evaluable patients were obtained. The non-evaluable patients would be replaced with 25 evaluable patients at the final analysis. The patients were all postmenopausal females, identified at the staging meeting at the Edinburgh Breast Unit. Inclusion and exclusion criteria are shown below.

Inclusion Criteria

- Postmenopausal women defined as >12 months since last menses. In patients whose last menses was ≤12 months before start of treatment, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels must be in postmenopausal range: FSH ≥35IU/L and LH ≥40 IU/L
- Fresh tissue available from core biopsies taken at diagnosis.
- No evidence of metastatic disease
- Operable breast cancer
- Written informed consent obtained from all subjects
- Performance status ≤2 (ECOG/WHO scale)
- Life expectancy ≥4 months.
- At least 2 weeks since last major surgery. A shorter interval was permissible if the patient had recovered with no continuing complications.
- Screening laboratory values at entry:
    - Haemoglobin ≥10.0g/dL
    - WBC ≥3,500/mm^3
    - Neutrophils ≥1,500/mm^3
    - Platelets ≥150,000/mm^3
    - Creatinine ≤1.5 x upper limit of normal (ULN) for the testing laboratory, or creatinine clearance ≥60mL/min
    - Adequate liver function as defined by bilirubin ≤1.5 of upper limit of normal (ULN), albumin ≥30g/l, serum ALT ≤2.5 x ULN and Alkaline Phosphatase ≤2.5 x ULN.

Exclusion Criteria

- Prior therapy for breast cancer
- Inflammatory breast cancer
- Concurrent medical or psychiatric problems unrelated to breast cancer, which would significantly limit full compliance with the study or expose the patient to extreme risk or decreased life expectancy.
- Treatment with another investigational drug within 30 days or five half-lives (whichever is longer) prior to entry into the study.
- Presently receiving or expected to require concurrent chemotherapy, immunotherapy, radiotherapy, chronic corticosteroid therapy or other hormonal therapy for breast cancer.
- Presently receiving warfarin anticoagulant therapy.
- Current treatment with high dose oral corticosteroid therapy.
- Patients being treated with drugs recognized as being strong inhibitors of the isoenzyme CYP3A within the last 7 days. **Appendix 1**

Patients were identified as eligible for the study at the staging meeting at which the initial management plan was discussed by the multidisciplinary team (MDT). The date of surgery was allocated at the MDT meeting, and if the date of surgery was within 14 days of the MDT meeting, the patient was considered ineligible for the study, ensuring that no patient had surgery delayed as a result of entering the study. The named consultant saw the patient that day and discussed the proposed management plan with the patient and family, and introduced the potential for entering the RAD001 study. Interested patients then discussed the study with the

research fellow or research nurse and were given a patient information sheet (**Appendix 2**), and the patient was allowed at least 24 hours to decide upon entry. If the patient did not wish to re-attend for randomisation on another day but wanted to enter the study on the day of discussion, the study documentation was completed and the drug dispensed with the patient aware that she should further consider the study and withdraw from the study before or after commencing study medication if she so decided. Once the patient had decided upon entry into the study, a consent form was signed (**Appendix 3**). Safety bloods were taken on the day of randomisation in addition to the routine bloods taken within the previous 10 days for screening. Also performed were assessments of general health including pulse, blood pressure, physical examination, electrocardiogram (ECG) if not already performed, height and weight. World Health Organisation performance scale was assessed for each patient. Patients were dispensed 14 days of RAD001 5mg once daily, and advised to contact the research team in the event of any questions or side effects. A patient diary was completed each day by the patient to allow assessment on Day 13 (pre-operative admission day) of compliance with medication and monitoring of side effects experienced by patients if not notified to the research team during the study period. **Appendix 4** Patients were strongly advised to contact the study team in the event of any side effect that was unexpected in its nature or severity. A study information sheet was sent to the patients' registered general practitioner in case of contact by the patient regarding the study. **Appendix 5**

At the end of the study period, patients were admitted on the day prior to surgery (study Day 13) or the morning of surgery (study Day 14), and were seen by a

member of the research team for assessment of side effects, compliance and repeat of safety bloods.

Safety data was collected at time of consent to enter the trial (Day 0) and on Day 13 when the patient was admitted to the ward for surgery the next day. For the few patients who were admitted on the morning of surgery, blood samples were taken on admission on Day 14. On both visits blood pressure, pulse, temperature, weight and height (latter only on Day 0) were recorded.

Blood samples were taken on Day 0 and Day 13/ 14 for full blood count, renal function, liver function and cholesterol. It was apparent to the researchers that in the first three patients there had been a drop in neutrophil counts after 14 days treatment with RAD001. In the next nine patients, full blood count samples were taken at Day 7 to determine any significant reduction in neutrophil counts prior to admission to surgery. Interpretation of the neutrophil counts in these patients and data made available from Novartis Pharmaceuticals regarding the nadir of Day 14 neutrophils led to the cessation of the Day 7 blood tests in the remainder of the study patients as it was clear there was no predictive value therein.

Statistical Analysis

The number of patients required for this study was 30 evaluable patients to power an 80% detection of reduction in Ki67 in 50% of patients. Analysis of variance (ANOVA) was performed to determine the significance of changes between pre and post treatment scores for all measurements. In the cases of data that were highly skew, log transformation was performed. In order to examine the linear relationship between the blood results and Ki67 counts, Pearson's Correlation (r) was used.

Ethical Approval

Ethical approval for this study was sought and obtained through the UK national application system (COREC). Approval was met through the Northern and Yorkshire MREC for the clinical and biological studies. **Appendix 6** Further application was made and approved by the Lothian MREC for retrospective use of tissue collected from patients who had been treated with endocrine agents. These patients had consented for the tissue to be collected and further ethical approval was obtained to utilise this tissue for the purposes of the analysis of endocrine resistant and sensitive breast tumour samples.

Approval was sought and obtained from the Lothian Hospital Research and Development department, and the Medicine and Health Regulatory Authority (MHRA) for this study. **Appendix 7** & **Appendix 8**

Results of clinical study

Patient recruitment

Recruitment for this study commenced in March 2005 and was completed in February 2006, during which time 91 patients were screened. Of those screened, 48 were excluded from the study for the following reasons: planned treatment with neoadjuvant letrozole (n=18), peri-menopausal (n=8), ongoing medical problems (n=8), operation date too early (n=7), medications in exclusion criteria (n=2), out of UK during study period (n=2). Forty-three patients were approached by the research team to discuss the study, of which 11 declined to enter. Four gave no reason, 4 did not wish to take medication prior to surgery, 2 did not want any extra visits to hospital or any extra intervention and one felt she was too old to participate. 32 patients were recruited to this study. One patient did not take any study medication due to a flu-like illness prior to commencing RAD001 and was thus excluded and returned all study medication. Patient 32 was recruited to reach the total of 25 evaluable patients required at the outset of the study who had completed the full 14 days of the trial medication.

Six patients withdrew from the study prior to completing 14 days of RAD001 5mg, in all but one case due to side effects, discussed in full below. One patient completed 11 days of medication prior to reaching the expiry date of the drug batch. While this had been anticipated, it was hoped that more trial drug would be available in time for the patient to complete 14 days but this was unfortunately not the case. Biopsy samples from all patients who withdrew early from the study were analysed for the same outcomes as those samples from patients who had completed 14 days.

Patient baseline characteristics

All patients were postmenopausal women with newly diagnosed early breast cancer. The mean age was 67.5 years (range 51-89), and all had a WHO performance scale of 0-2. Eleven patients had been on hormone replacement therapy in the past, and six had had a hysterectomy. Only four patients had no past medical history, and five were on no medication at the time of entry into the study. For the other patients, past medical history and concomitant medication are shown in **Tables 1 and 2**. Patients were staged pre-operatively as T1-2, N0, M0, with the exception of 3 patients who had palpable nodes at diagnosis, and were staged as T2 N1 M0. All patients proceeded to surgery after the 14 day study period. Eight patients had a mastectomy and axillary clearance and 23 had wide local excision of tumour with either axillary clearance (n=8) or sentinel node biopsy (n=15). Three patients required further surgery for complete excision of tumour, although initial excision samples were used for the purposes of this study.

Pt No	Age	Hysterectomy	HRT	PMH
1	79	No	No	CABG, IHD
2	73	No	No	Cholecystectomy, NIDDM
3	76	No	No	Hypertension, NIDDM
4	59	No	Yes	None
5	79	No	No	Thyroid adenoma
6	89	No	No	Cholecystectomy, Hypertension, Macular degeneration, Osteoporosis
7	70	No	No	Hypertension, RTA- knee fusion
8	54	No	Yes	Angina - coronary stent, CVA, OA spine
9	76	No	No	Rheumatoid arthritis
10	51	No	Yes	Dental abscess, Depression
11	67	No	No	Hypothyroid
12	78	No	No	CVA, Hypertension, Hypercholesterolaemia
13	52	Yes	Yes	Irritable bowel, OA
14	64	No	Yes	Appendicectomy, Basal Cell Carc, Ovarian cyst, TB lung
15	55	No	Yes	Bunionectomy
16	65	Yes	Yes	Bells palsy, Hypercholesterolaemia, Hypertension, Shoulder fusion, Tuberculosis
17	57	No	No	None
18	68	No	No	Hypercholesterolaemia, Hypertension
19	74	No	No	Hypertension, Osteoarthritis
20	62	No	No	Meningitis
21	77	No	Yes	COPD, Hypertension
22	67	Yes	Yes	None
23	52	No	No	None
24	74	Yes	No	Hypercholesterolaemia, Hypertension
25	60	No	Yes	Aortic stenosis, Hypercholesterolaemia, Hypothyroid, Lap chole, OA (knee), Sterilisation
26	75	No	No	Anaemia, Anal fissure, GORD, NIDDM (diet), Scarlet fever
28	76	No	No	Hypercholesterolaemia, Hypertension, IDDM
29	77	Yes	No	Arthritis, Cervical spondylosis, Hypertension, NIDDM
30	59	No	No	Endometrial polyps, Joint pain (knee)
31	53	Yes	Yes	Depression, Eczema, Goitre, Hypertension, OA, Psoriatic arthropathy, Spondylosis
32	75	No	No	Asthma, Hypercholesterolaemia, Hypertension, IHD, Migraines

Table 1
Age, past and current medical history at time of entry into RAD001 clinical study (coronary artery bypass graft (CABG), ischaemic heart disease (IHD), non-insulin dependent diabetes mellitus (NIDDM), road traffic accident (RTA), cerebrovascular accident (CVA), osteoarthritis (OA), tuberculosis (TB), chornic obstructive airways disease (COPD), gastro-oesophageal reflux disease (GORD)).

Pt No	Age	Concurrent Medications
1	79	Amlodipine, Aspirin, Atenolol, GTN, ISMN, Lactulose, Nicorandil, Simvastatin
2	73	Bendrofluazide, Lisinopril, Metformin, Nifedipine, Simvastatin
3	76	Amlodipine, Aspirin, Lisinopril, Metformin, Simvastatin, Ventolin
4	59	Omeprazole
5	79	Nitrazepam
6	89	Adcal, Bendrofluazide, Celluvisc eye drops, Fosamax, Paracetamol
7	70	Atenolol, Dihydrocodeine
8	54	Atenolol, Atorvastatin, Chlordiazepoxide, Frusemide, GTN, Methocarbamol, Omeprazole, Paracetamol
9	76	Sulfasalazine
10	51	Amoxicillin, Paroxetine
11	67	Amitriptyline, Thyroxine
12	78	Allopurinol, Atenolol, Frusemide, Simvastatin
13	52	Celebrex, Codydramol, Mebeverine
14	64	None
15	55	None
16	65	Bendrofluazide, Nifedipine, Simvastatin
17	57	None
18	68	Atenolol, Bendrofluazide, Lipitor, Lisinopril
19	74	Aspirin, Atenolol, Bendrofluazide, Celecoxib
20	62	None
21	77	Amitriptyline, Aspirin, Bendrofluazide, CoQ10, Propanolol
22	67	Co-codamol, Zimovane
23	52	None
24	74	Aspirin, Co-tenidone, Enalapril, Nifedipine, Simvastatin
25	60	Atenolol, Atorvastatin, Bendrofluazide, Thyroxine
26	75	Amlodipine, Atenolol, Bendrofluazide, Cocodamol, Ferrous sulphate, Ranitidine
28	76	Aspirin, Frusemide, Insulin, Lisinopril, Simvastatin
29	77	Atorvastatin, Bendrofluazide, Cocodamol, Enalapril, Gliclazide
30	59	Echinacea, Ibuprofen, Glucosamine
31	53	Ibuprofen, Paracetamol, Propanolol, Venlafaxine
32	75	Antihistamine, Aspirin, GTN, Simvastatin

Table 2
Age and concurrent medications for patients participating in the RAD001 clinical study.
(GTN: glyceryl trinitrate, ISMN: isosorbide mononitrate)

Tumour characteristics

Of the 31 tumours from which biopsies were analysed for RAD001 outcomes, 27 were invasive ductal carcinoma of no special type (IDCNST), although two of these displayed mixed mucinous features. Of the others, one was lobular, one was tubular, one was papillary and one was neuroendocrine. The latter two were initially thought to be IDCNST on diagnostic core biopsy. One tumour was thought to be tubular or cribriform on core biopsy but was IDCNST on excision pathology. The mean size of tumour was 23.9mm (range 11-41mm). On the excision specimen following 14 days of therapy, three of the tumours were grade 1, 16 were grade 2 and 12 were grade 3. Tumour grades from core biopsy and excision specimens as scored by the consultant pathologist correlated in 24 cases; in 5 cases the tumour was upgraded to grade 2 from 1, in two cases from grade 2 to 3, and in one case the tumour was downgraded from grade 3 to 2. Eleven patients had evidence of lymphovascular invasion (LVI). On pathological assessment of the axilla, 13 patients had at least one positive node, including 5 patients who had more than 4 positive nodes on histology. The mean number of nodes removed at axillary surgery was 12 (range 4-25). The Nottingham Prognostic Index was calculated for each patient using the formula NPI= size (cm) x 0.2 + stage (lymph node stage 1-3) + grade (1-3). [185] Seven patients had a score in the good prognosis category (NPI <3.4), 16 were in the intermediate prognosis group (NPI 3.4-5.4) and 8 were in the poor prognostic category (NPI>5.4). Pathology results for each tumour by patient number are shown in **Table 3**.

Pt No	Path size (mm)	Path type	Path Grade	LVI	Nodes Positive (n)	Total nodes excised (n)	NPI
1	17	NST	2	Y	0	5	3.34
2	26	NST	2	N	0	6	3.34
3	24	NST	2	Y	20	20	5.48
4	30	Lobular	2	N	4	18	5.6
5	30	NST	3	N	1	16	5.6
6	25	NST	2	N	0	6	3.5
7	41	NST	3	N	0	17	4.82
8	36	NST/MUC	3	Y	1	15	5.72
9	22	NST	2	Y	1	20	4.44
10	17	NST	2	Y	2	22	4.34
11	21	NST	2	N	0	17	3.42
12	30	NST/MUC	2	N	0	9	3.6
13	16	Tubular	1	N	0	9	2.32
14	13	NST	3	Y	2	9	5.26
15	27	NST	3	Y	0	13	4.54
16	14	NST	3	N	0	4	4.28
17	35	NST	3	Y	15	21	6.7
18	29	NST	2	N	0	4	3.58
19	35	NST	3	N	0	18	4.7
20	35	NST	2	Y	10	15	5.7
21	18	NST	3	N	0	18	4.36
22	11	Neuroendocrine	3	N	0	5	4.22
23	27	NST	3	N	7	12	6.54
24	35	Papillary	2	N	0	14	3.5
25	15	NST	2	N	0	4	3.3
26	22	NST	2	N	1	5	4.44
28	14	NST	2	N	0	6	3.28
29	20	NST	2	Y	8	25	5.4
30	18	NST	1	N	0	5	2.36
31	23	NST	3	Y	1	19	5.46
32	16	NST	1	N	0	5	2.32

Table 3
Pathology of tumours for each patient participating in RAD001 clinical study indicating size; type (invasive ductal no special type (NST), mucinous (MUC)); grade; lymphovascular invasion (LVI) present yes (Y) or no (N); number of positive nodes of total excised; Nottingham Prognostic Index (NPI).

Results for proliferation before and after treatment with RAD001 will be presented later, but correlations with the above tumour characteristics will be discussed here (Spearmans rank correlation (r)). There was a significant correlation between pre-treatment proliferation (Ki67%) and tumour grade for both core biopsy (r=0.59; 95%CI 0.29, 0.78; p=0.0013) and excision specimens (r=0.61; 95%CI 0.32, 0.79; p=0.0009)). No correlations were found between pre-treatment Ki67 and either tumour size, type, presence of LVI, node positivity, NPI, or HER-2 positivity. There was a trend toward highly proliferative tumours having lower ER pre-treatment, but this did not reach significance (r=0.35; 95%CI 0.01, 0.63; p=0.0554).

Tumours that were of higher grade had a significantly greater reduction in proliferation after RAD001 treatment (r=0.41; 95%CI 0.07, 0.67; p=0.024). **Figure 7** Tumour size, type, presence of LVI, node positivity, NPI, and HER-2 positivity were not found to correlate with the change in proliferation with treatment. There was a trend toward tumours lower in ER having a greater change in Ki67 with RAD001 treatment (r=0.35; 95%CI 0.01, 0.62; p=0.0588).

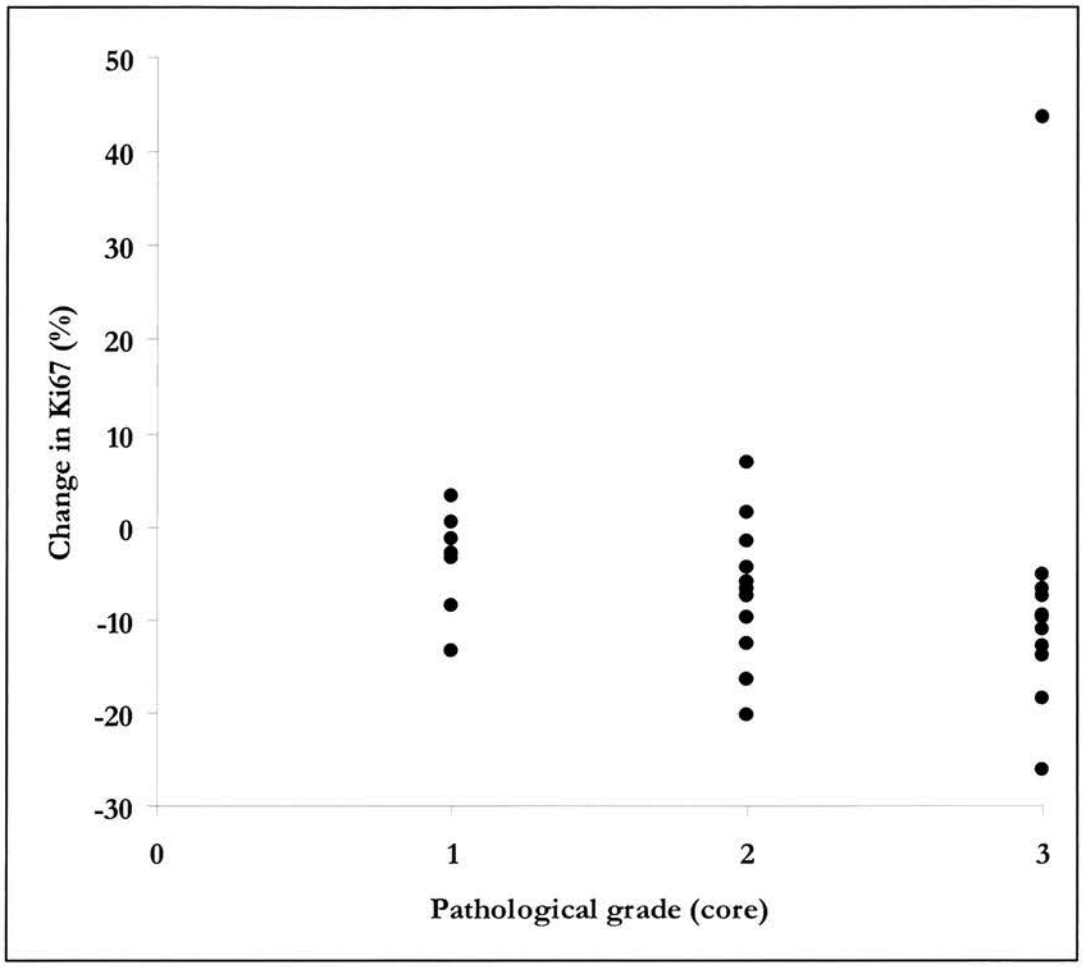


Figure 7
Tumour core biopsy grade correlated significantly with the change in Ki67 after RAD001 treatment, with high grade tumours having a greater reduction with treatment (r=0.41; 95%CI 0.07, 0.67; p=0.024).

Tolerability

Twelve of 31 patients reported no symptoms while taking RAD001 5mg for the two week treatment period. All adverse events reported are shown in **Table 4**. Each symptom reported during the two weeks, either reported by direct contact with the research team, or on submission of the diary at the end of the study period, is shown in the table. As can be seen, all adverse events were grade 1 or 2 on the NCIC-CTC scale. As stated above, five patients withdrew during the two week treatment period due to adverse events, of which two patients had more than one symptom that contributed to their withdrawal. Median duration of treatment in those that withdrew was 9.5 days (range 4-13 days). Four patients had mouth ulcers which were of sufficient impact with pain on swallowing or chewing to result in the patient withdrawing from the study, although one patient continued her medication until planned admission to hospital for surgery on Day 13. One patient who withdrew due to mouth ulcers had mouth ulcers prior to commencing treatment. Another patient who withdrew with mouth ulcers also experienced nausea and fatigue, and felt these symptoms contributed equally to her decision to withdraw from the study. A further four patients had mouth ulcers but did not feel these to be a significant problem as the ulcers were few in number or lasted for less than 2 days. The mouth ulcers reported appeared from study Day 3 to Day 12. Patients were advised to use antiseptic mouthwashes or throat lozenges, and a number of patients tried home remedies such as salt mouthwashes. The other patient who withdrew from the study experienced tiredness, nausea and anorexia for the first three days of the study period and withdrew on Day 4.

Two patients reported rashes; one was a small area of macular rash over the dorsum of her right hand during study Day 12-14, with no itch or scaling; the other patient had two acneiform spots over her anterior chest wall on study Day 7 that resolved spontaneously, as well as a flushing redness and heat on the left side of her neck on Day 11 that resolved spontaneously.

Two patients developed infections during the study period. One patient had a dental abscess requiring treatment with ciprofloxacin, and the other had a urinary tract infection and was treated with trimethoprim. A further patient experienced urinary frequency with a normal urinalysis, and her symptoms resolved spontaneously.

Toxicity Reported in Study	Patients Reporting Symptom, n (%)	Patients Withdrawing Because of Symptom, n (%)	NCIC-CTC Grade
Mouth ulcers	8 (25.8)	4 (12.9)	1–2
Fatigue	6 (19.4)	2 (6.5)	1
Nausea	3 (9.7)	2 (6.5)	1–2
Rash	2 (6.5)	0	1
Dry mouth	2 (6.5)	0	1
Infection	2 (6.5)	0	2
Headache	2 (6.5)	0	1
Indigestion	1 (3.2)	0	1
Light-headed/ dizziness	1 (3.2)	0	1
Itch	1 (3.2)	0	1
Easy bruising	1 (3.2)	0	1
Joint pains	1 (3.2)	0	1

Table 4
All toxicities reported in two week study period while taking RAD001 showing numbers (%) of patients reporting specific symptoms, and numbers (%) withdrawing from the study because of the specific symptoms. Some patients experienced and reported more than one symptom.

Safety

Safety assessments of general health were performed before and on Day 13/14 of treatment. Body mass index was calculated for each patient with a mean BMI of 27 both pre and post treatment (range pre-treatment 20-52). There was no significant difference in systolic blood pressure pre- and post-treatment (mean pre 137 versus mean post 136mmHg, p=0.71). There was a significant rise in pulse rate on the Day 13/14 measurements (71 b/m Day 0 versus 78 b/m Day 13/14, p=0.019). This difference may be accounted for by the level of anxiety experienced by patients on the second measurement due to admission to hospital. Similarly, a fall in peripheral temperature from 36.7 C to 36.5 C (p=0.0053) can be accounted for by the change of location from a warm clinic room to the relative cool of the admissions bay. There was no change in the Performance Status score for any patient during the treatment period of RAD001. All patients had a Performance Score of either 0 or 1.

Pre- and post-treatment mean values for the intention to treat (ITT) population for the blood samples collected pre- and post- treatment are summarised in **Table 5**.

	Pre-treatment (mean)	Post-treatment (mean)	p-value
Haemoglobin (g/dL)	13.7	13.6	0.7098
White blood cells ($x10^9$/L)	7.9	5.8	<0.0001
Neutrophils (mm^3)	5.01	2.95	<0.0001
Platelets ($x10^9$/L)	290*	208*	<0.001
Creatinine (µmol/L)	71.5*	73.9*	=0.3104
Sodium (mmol/L)	142*	142*	=0.8396
Potassium (mmol/L)	4.3	4.0	=0.0001
Albumin (mg/L)	42.8	42.5	=0.5599
Calcium (corrected) (mmol/L)	2.39	2.33	=0.0005
Bilirubin (µmol/L)	6.9*	5.6*	=0.0029
Alkaline phosphatase (mmol/L)	80.1*	86.0*	=0.0311
Alanine amino-transferase (U/L)	31.0*	34.8*	=0.0253
Cholesterol (mmol/L)	5.3	6.0	=0.0001
Triglycerides (mmol/L)	1.51*	1.81*	=0.0186
HDL (mmol/L)	1.64	1.66	=0.6885
LDL (mmol/L)	2.85	3.26	<0.0001
Glucose (random) (mmol/L)	5.6*	6.2*	=0.0542

Table 5
Pre-and post-treatment mean values for safety bloods taken in all patients participating in RAD001 clinical study.
***denotes non-normal distribution of data where logarithmic transformation was back-transformed therefore means shown are geometric.**

Haematology

There was no significant difference in haemoglobin levels with RAD001 treatment. **Figure 8** There was a significant fall in white blood cell count (WBC) (mean fall 2.11 $x10^9$/L, 95% CI 1.59 to 2.62, $p<0.0001$). **Figure 9** The change in WBC highly correlated with the pre-treatment WBC ($r=0.71$, $p<0.0001$), in that those with high WBC had the greatest fall with RAD001 treatment. There was no evidence to suggest a relationship between pre-treatment WBC and Ki67 pre-treatment or change in Ki67 (Ki67 results to follow), nor did the change in WBC correlate with the Ki67 pre-treatment or change in Ki67 with treatment.

There was a significant reduction in the neutrophil differential of the WBC (mean fall 2.06mm^3, 95% CI 1.55 to 2.57, $p<0.0001$). **Figure 10** The fall in neutrophils correlated to the pre-treatment neutrophil count ($r=0.78$, $p<0.0001$). There was no relationship between either pre-treatment neutrophils or change in neutrophils and pre-treatment Ki67 or change in Ki67 with treatment.

There was a significant fall in platelet count with RAD001 treatment (geometric mean fall 1.39 $x10^9$/L, 95% CI 1.26 to 1.53, $p<0.001$), such that the post-treatment count was 71% that of pre-treatment. **Figure 11** The change in platelets correlated with the pre-treatment platelet counts ($r=0.45$, $p=0.015$), in that those with high platelets pre-treatment had the greatest fall with RAD001 treatment. There was no relationship between either pre-treatment or change in platelet count and Ki67 pre-treatment or change in Ki67.

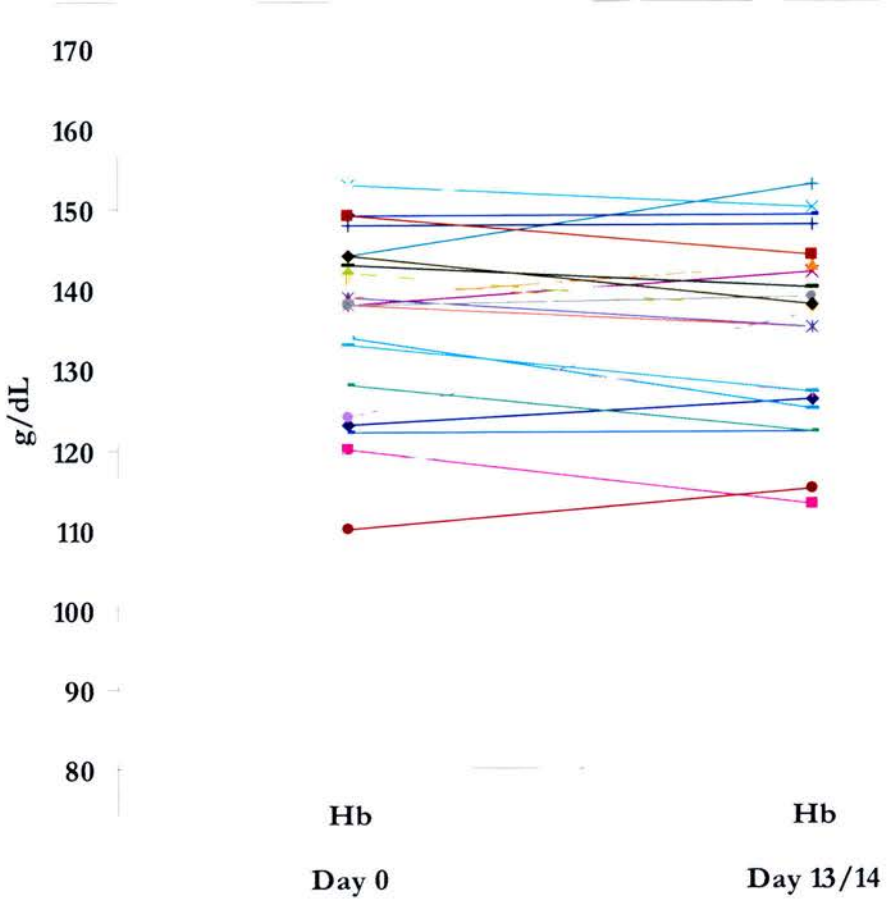


Figure 8
Individual values for haemoglobin pre- and post-treatment with RAD001 showing no significant change with RAD001 treatment (p=NS)

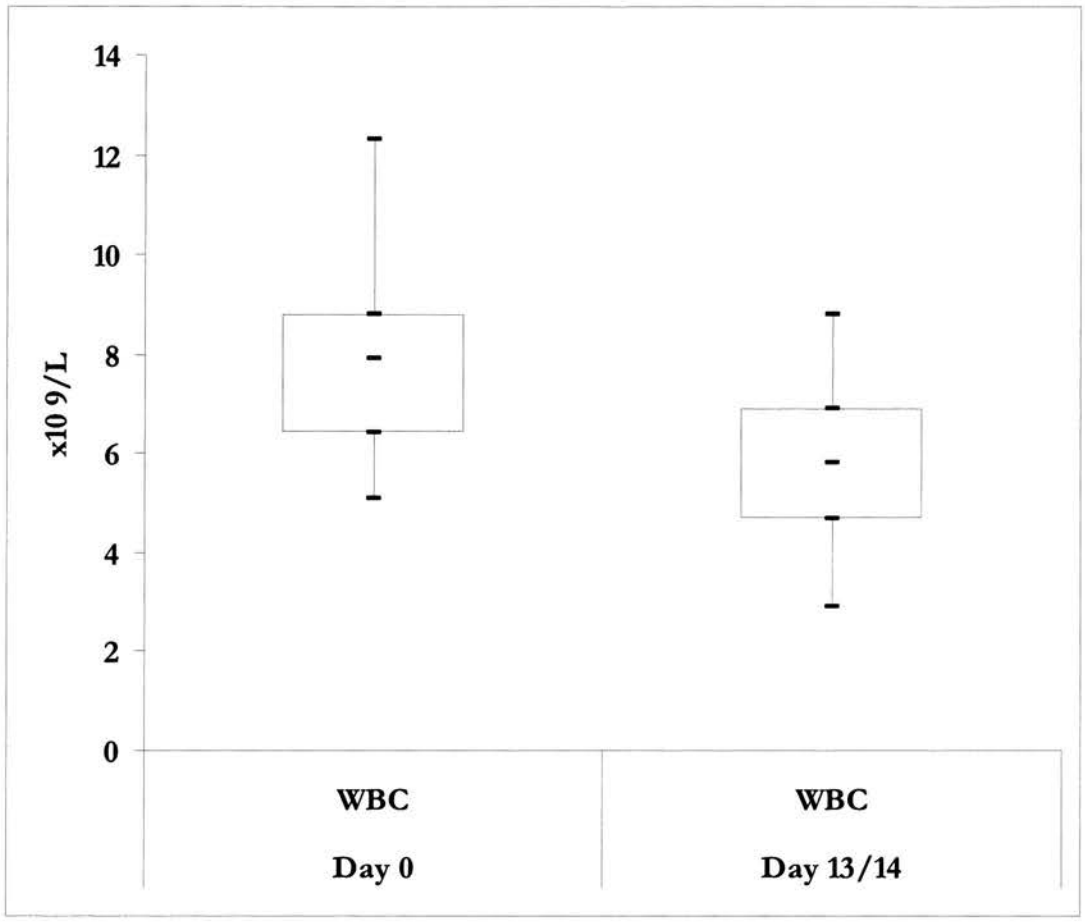


Figure 9
Results for white blood cell counts (WBC) before and after treatment with RAD001 demonstrating a significant fall with RAD001 treatment ($p<0.0001$) (min, 25th percentile, median, 75th percentile and max values shown as box plot)

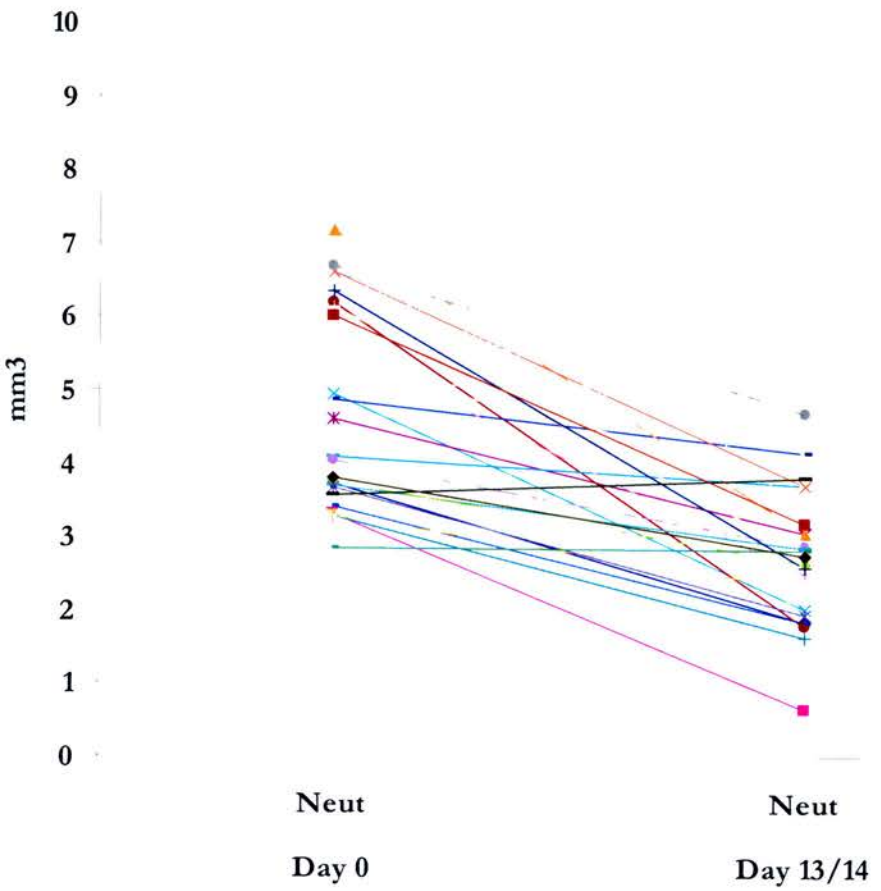


Figure 10
Individual values for neutrophil differential counts pre- and post-treatment with RAD001 ($p<0.0001$), demonstrating a significant fall with RAD001 treatment

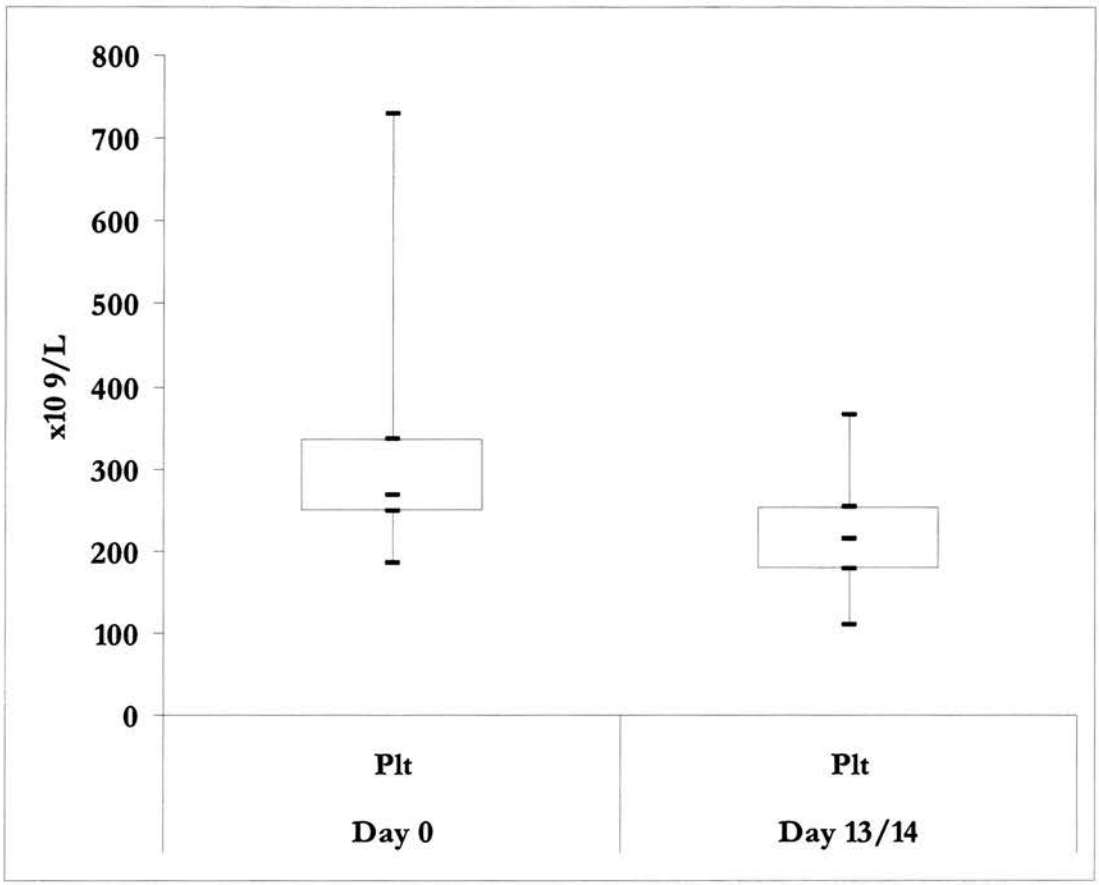


Figure 11
Results for platelet (Plt) counts pre- and post-treatment with RAD001 demonstrating a significant fall with RAD001 treatment ($p<0.0001$) (min, 25th percentile, median, 75th percentile and max values shown as box plot)

Biochemistry

There was no significant change in renal function as measured by creatinine. **Figure 12** There was no significant change in sodium (**Figure 13**) with RAD001 treatment but there was a significant fall in potassium (mean fall 0.3mmol/L, 95% CI 0.17 to 4.78, p=0.0001). **Figure 14** It is possible that this may be due to varying hydration status such as the fasting status of patients admitted on the same day for theatre. There was no difference in serum albumin levels with RAD001 treatment (**Figure 15**) but there was a significant reduction in corrected calcium levels with RAD001 (mean fall 0.07 mmol/L, 95% CI 0.03 to 0.10, p=0.0005). **Figure 16**

Liver function tests were all affected by RAD001 treatment, but with no consistent trend to suggest hepatotoxicity, as there was a significant reduction in bilirubin (geometric mean fall 1.23 mmol/L, 95% CI 1.08 to 1.41, p=0.0029) (**Figure 17**), but a rise in liver transaminase (ALT) (geometric mean rise 0.89 U/L, 95% CI 0.81 to 0.98, p=0.0253) (**Figure 18**) and alkaline phosphatase (geometric rise 0.93 mmol/L, 95% CI 0.87 to 0.99, p=0.0311) (**Figure 19**). There was no evidence to suggest any relationship between change in bilirubin with Ki67 pre-treatment or change in Ki67. There was a rise in random total cholesterol (mean increase 0.73 mmol/L, 95% CI -0.99 to 5.75, p=0.0001) (**Figure 20**) and random triglycerides (mean rise 0.08 mmol/L, 95% CI 0.73 to 0.97, p=0.0186). **Figure 21** Pre-treatment cholesterol was not associated with Ki67 pre-treatment or change in Ki67. There was, however, an association between Ki67 pre-treatment and the change in cholesterol (r=0.576, p=0.0017), in that those with high Ki67 pre-treatment had the greatest rise in cholesterol with RAD001 treatment. For the analysis of the two main lipoproteins (high density lipoprotein (HDL) and low density lipoprotein (LDL)) there was no

change in HDL (**Figure 22**) but a significant increase in LDL (mean increase 0.41 mmol/L, 95% CI -0.56 to 5.68, p<0.0001) with RAD001 treatment. **Figure 23** There was a trend towards a rise in random serum glucose (mean increase 0.90 mmol/L, 95% CI 0.81 to 1.00, p=0.0542). **Figure 24**

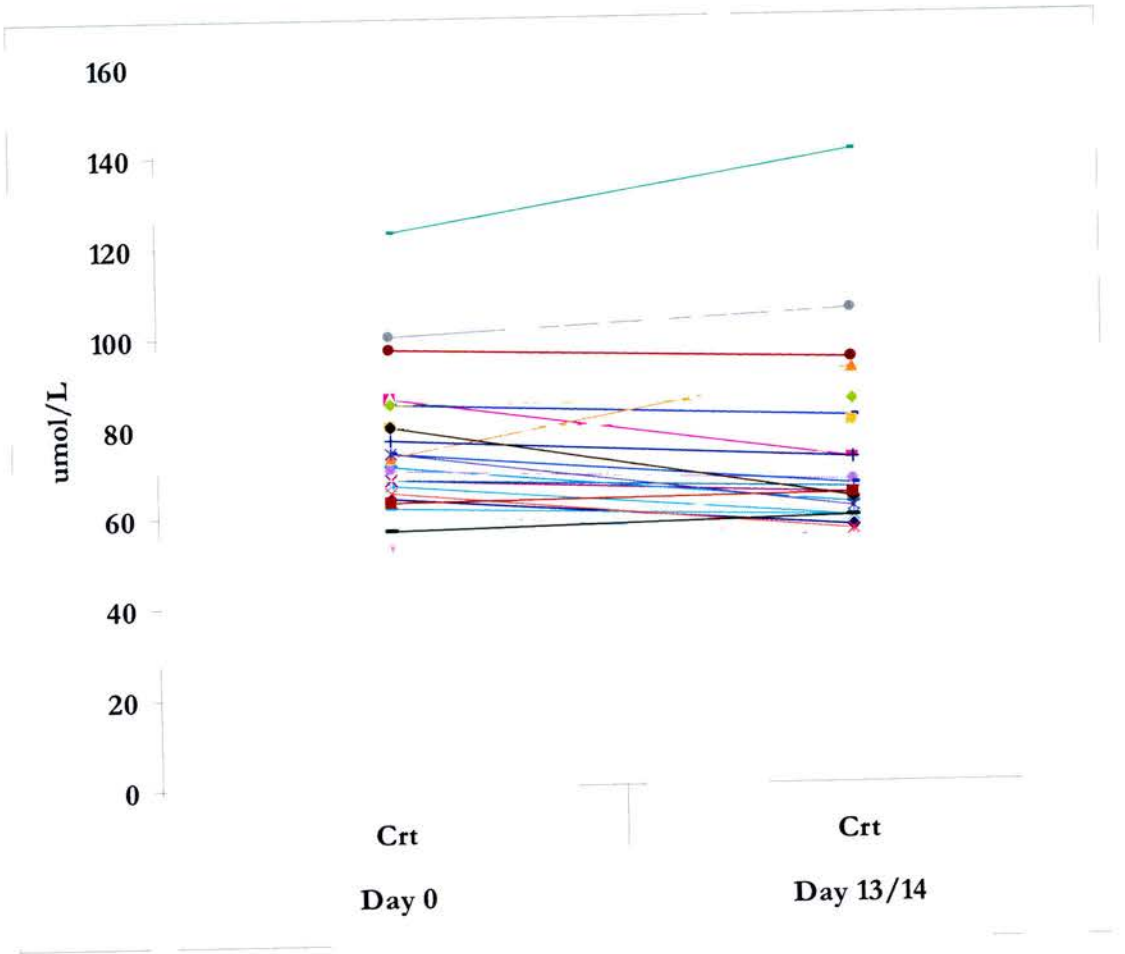


Figure 12
Individual values for creatinine (Crt) pre-and post-treatment showing no significant change with RAD001 (p=NS)

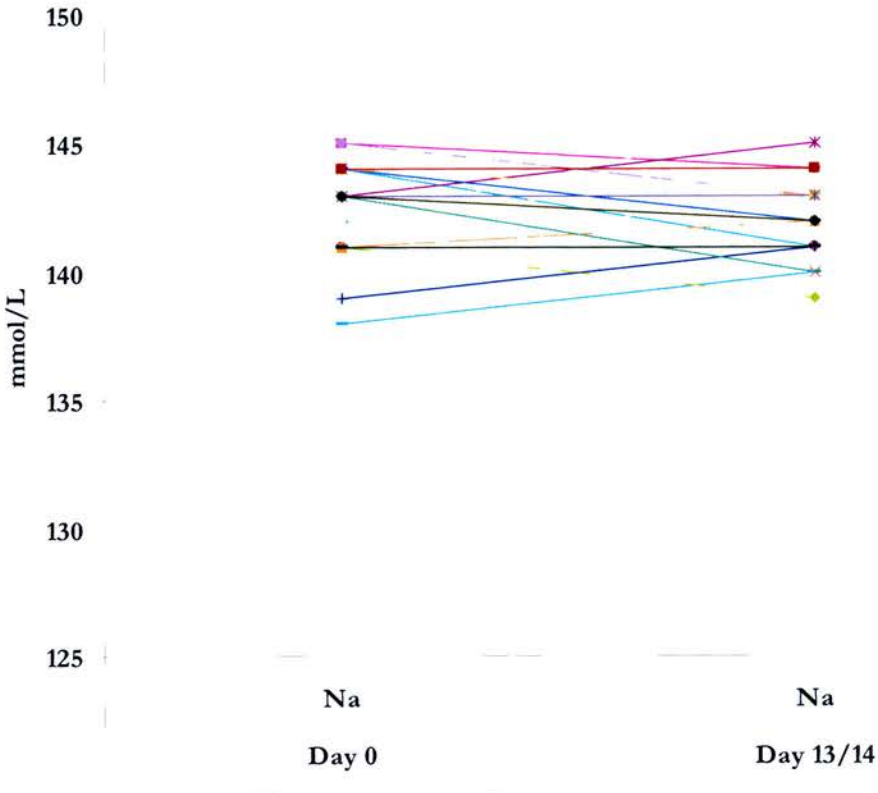


Figure 13
Individual values for sodium (Na) pre- and post-treatment with RAD001 showing no significant change with RAD001 treatment (p= NS)

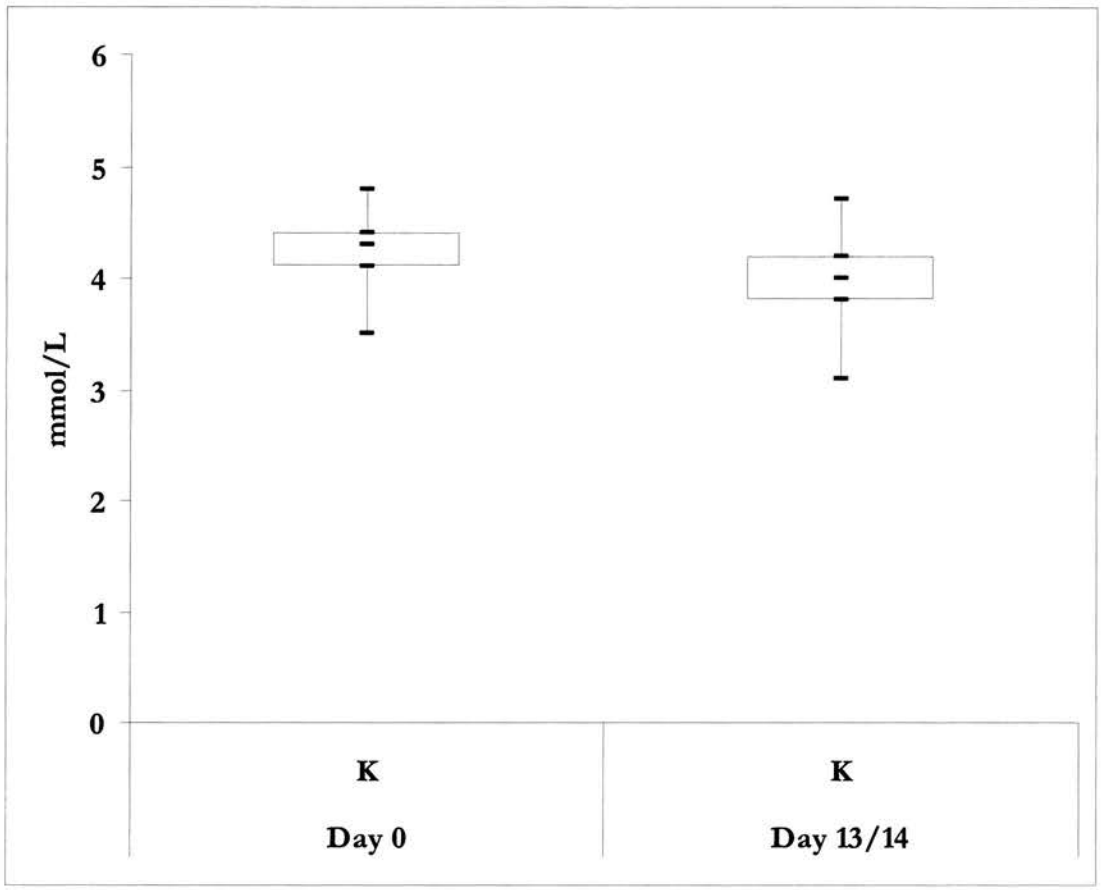


Figure 14
Values for potassium (K) pre- and post-treatment with RAD001 demonstrating a fall with RAD001 treatment (p=0.0001) (min, 25th percentile, median, 75th percentile and max values shown as box plot)

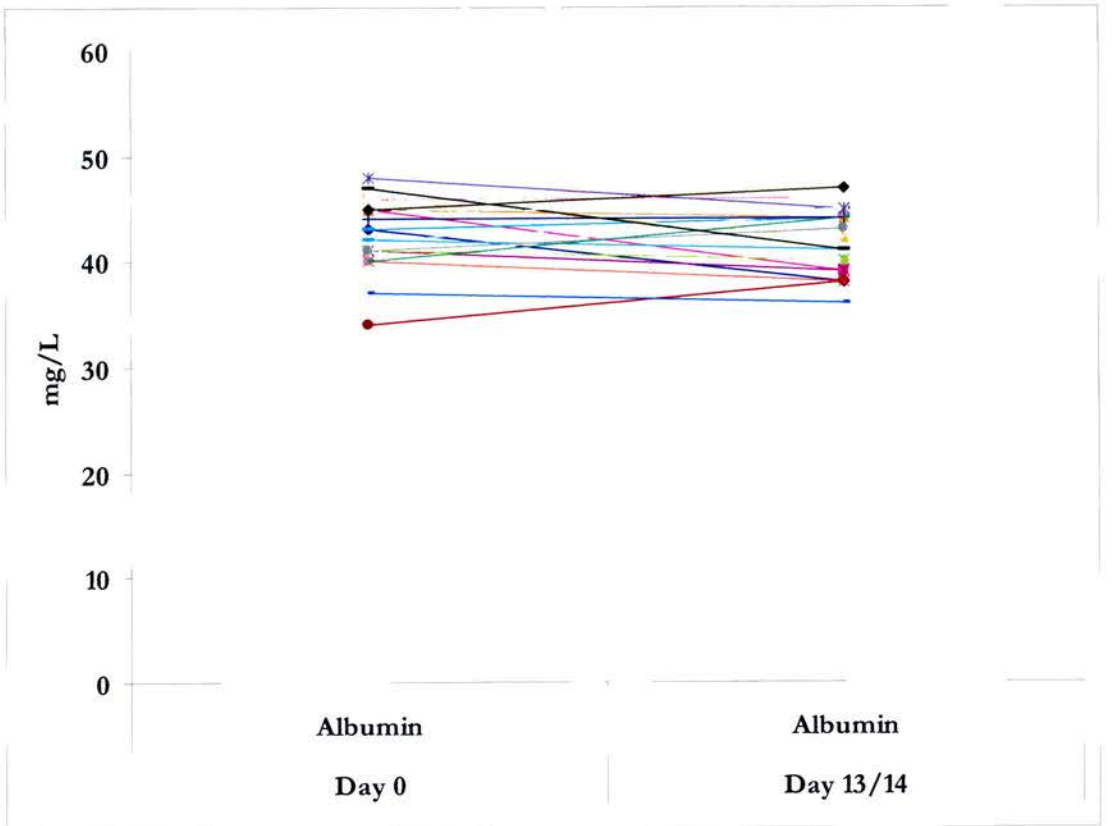


Figure 15
Individual values for serum albumin pre- and post-treatment showing no significant difference with RAD001 treatment (p= NS)

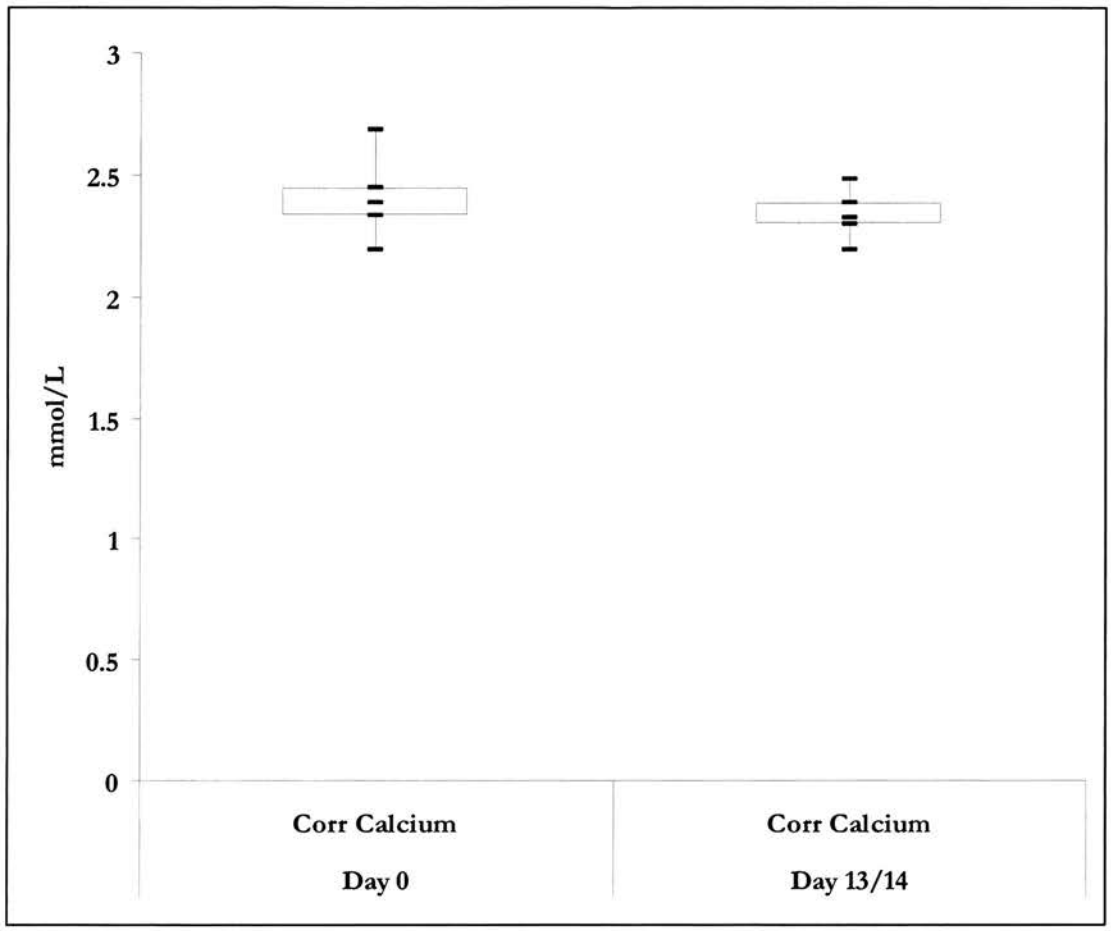


Figure 16
Values for corrected calcium pre- and post-treatment demonstrating a significant fall with RAD001 treatment (p=0.0005) (min, 25th percentile, median, 75th percentile and max values shown as box plot)

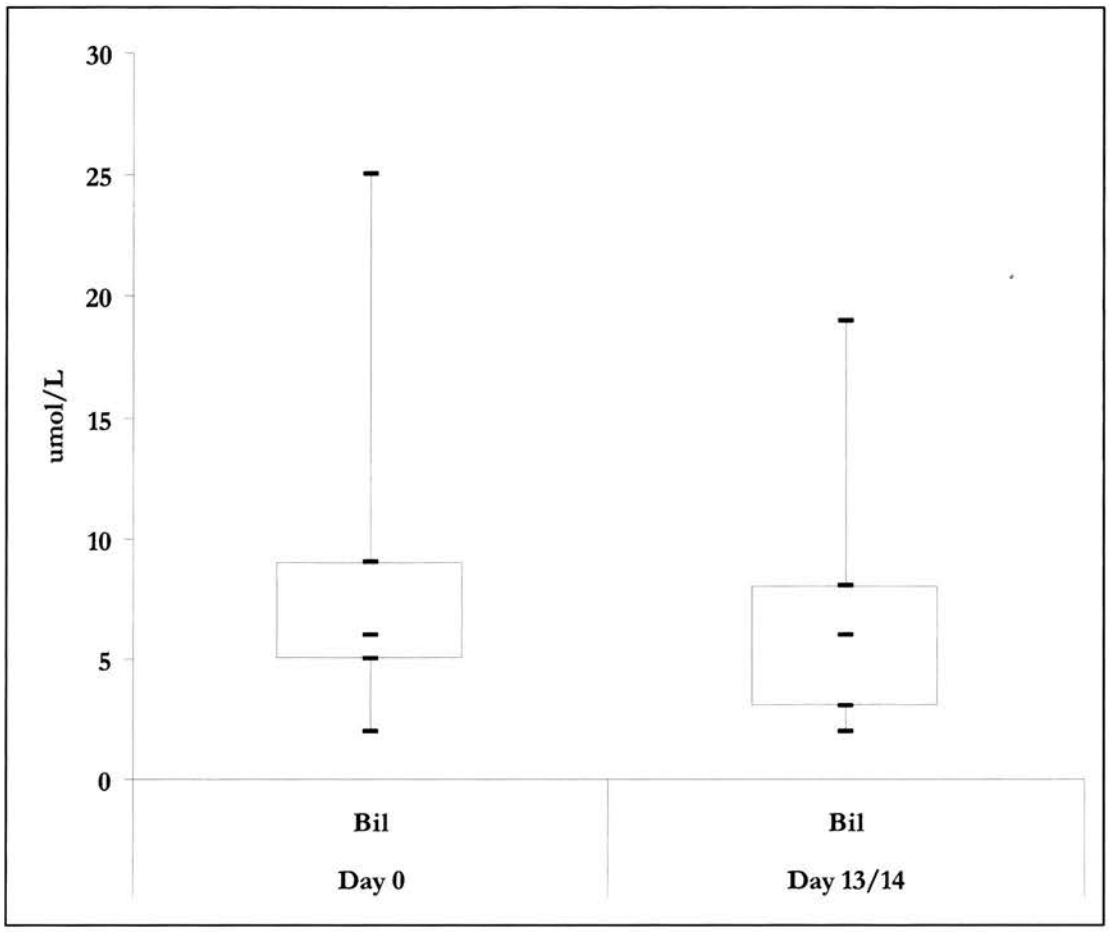


Figure 17
Individual values for serum bilirubin (Bil) pre- and post-treatment with RAD001 demonstrating a significant fall with treatment (p=0.0029) (min, 25th percentile, median, 75th percentile and max values shown as box plot)

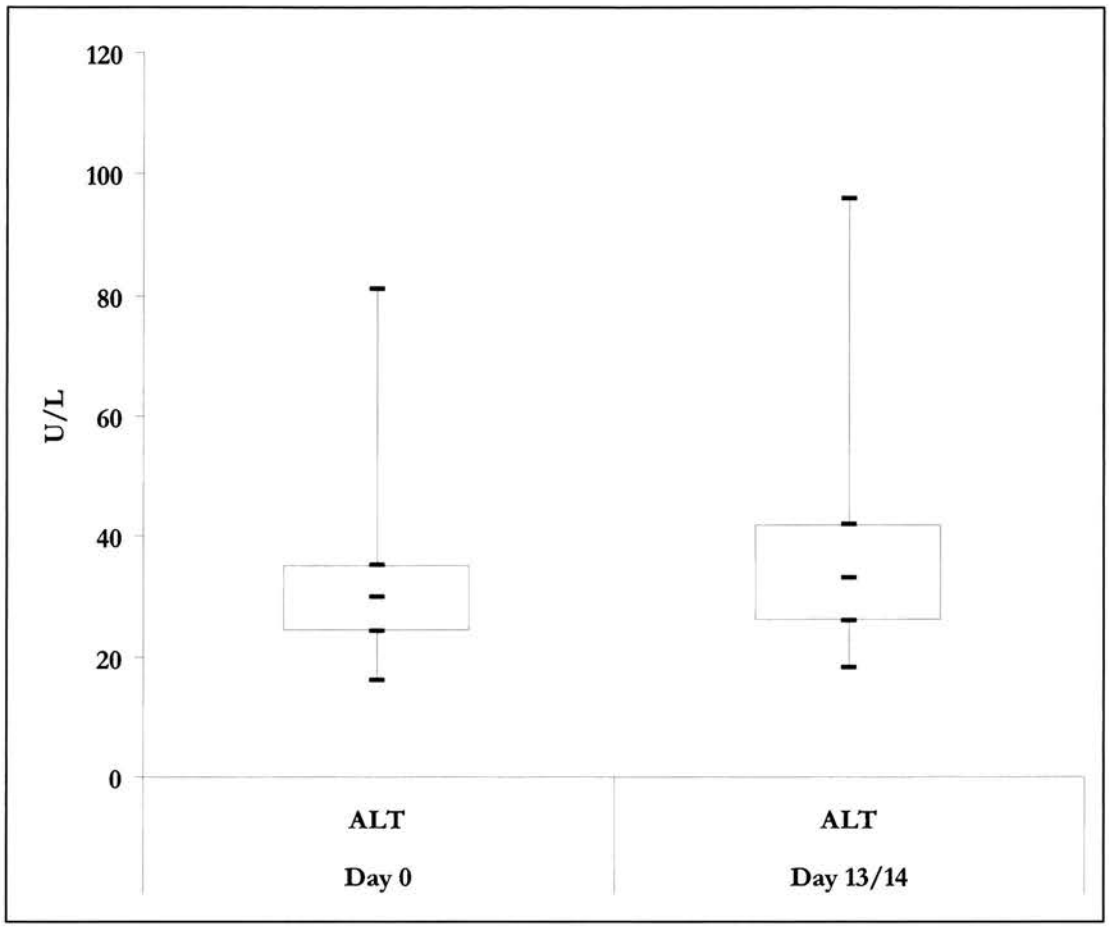


Figure 18
Results for alanine amino-transferase (ALT) pre- and post-treatment demonstrating a significant rise with RAD001 treatment (p=0.0253) (min, 25th percentile, median, 75th percentile and max values shown as box plot)

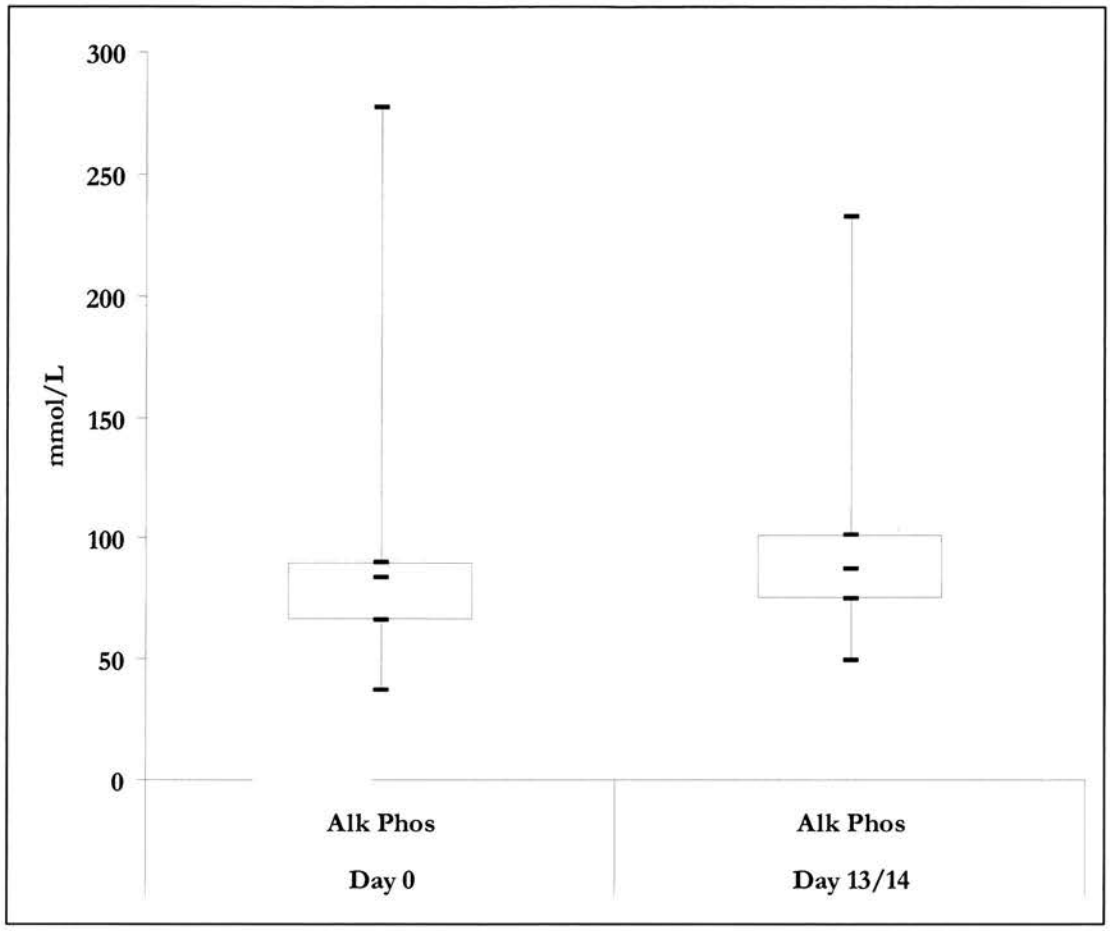


Figure 19
Results for alkaline phosphatase (Alk Phos) pre- and post-treatment demonstrating a significant rise with RAD001 (p=0.0311) (min, 25th percentile, median, 75th percentile and max values shown as box plot)

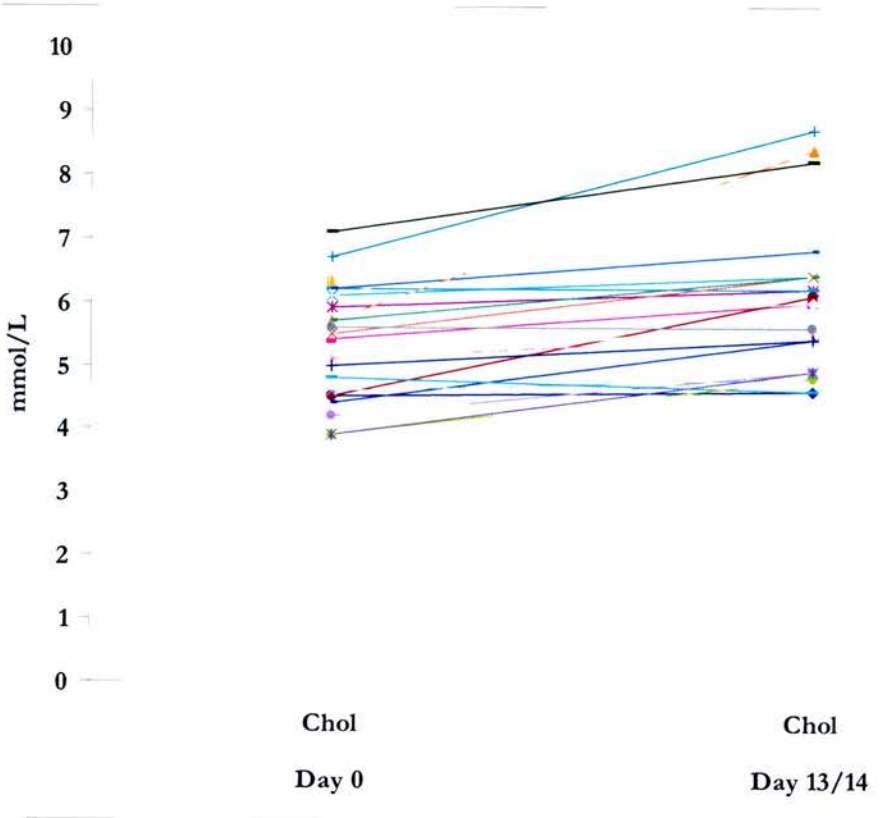


Figure 20
Results for random serum cholesterol pre- and post-treatment demonstrating a significant rise in cholesterol with RAD001 (p=0.0001) (min, 25th percentile, median, 75th percentile and max values shown as box plot)

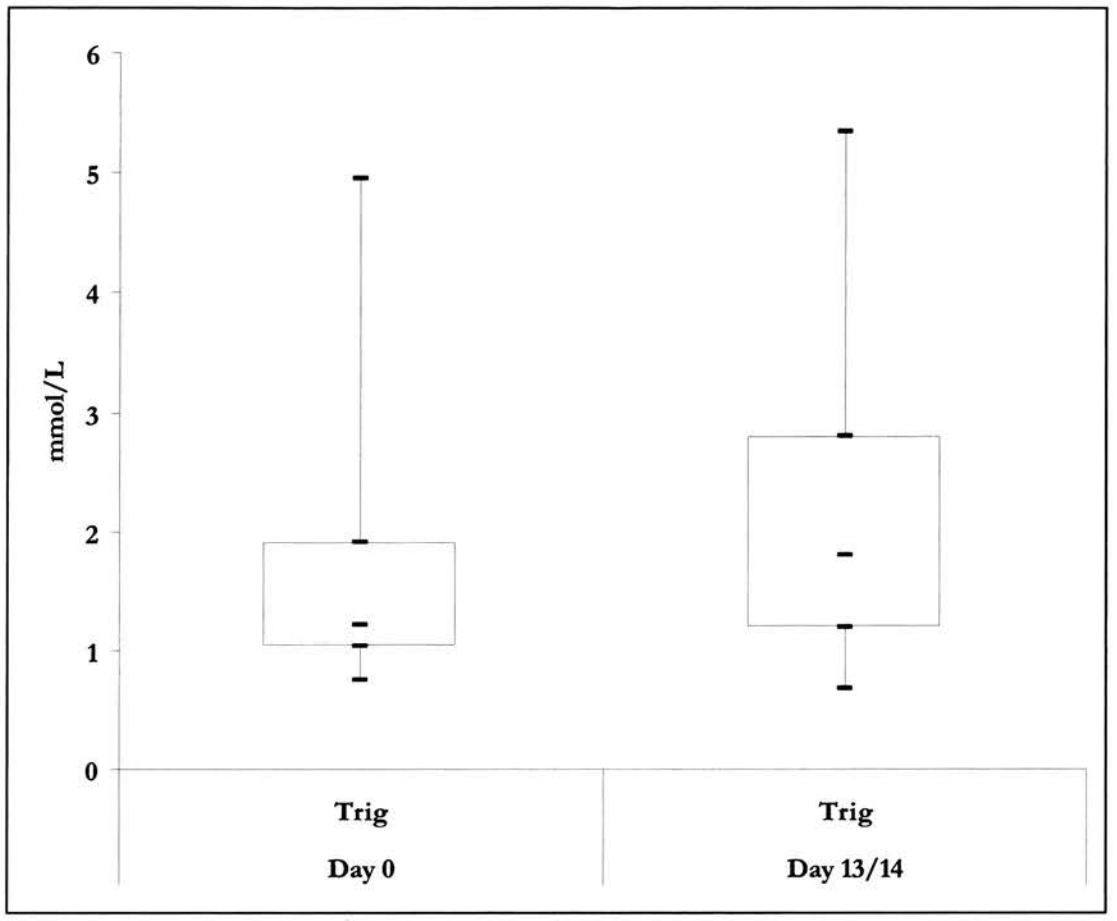


Figure 21
Results for serum triglycerides (Trig) pre- and post-treatment demonstrating a rise after treatment with RAD001 (p=0.0186) (min, 25th percentile, median, 75th percentile and max values shown as box plot)

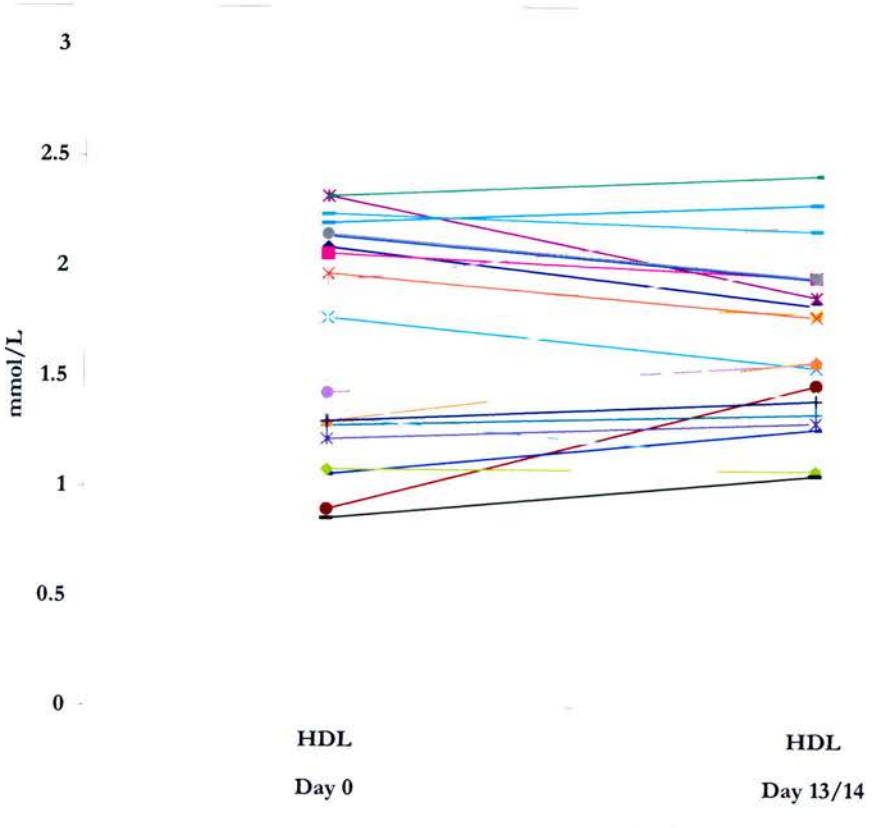


Figure 22
Individual values for high density lipoprotein (HDL) pre- and post-treatment with RAD001, showing no significant change with treatment (p= NS)

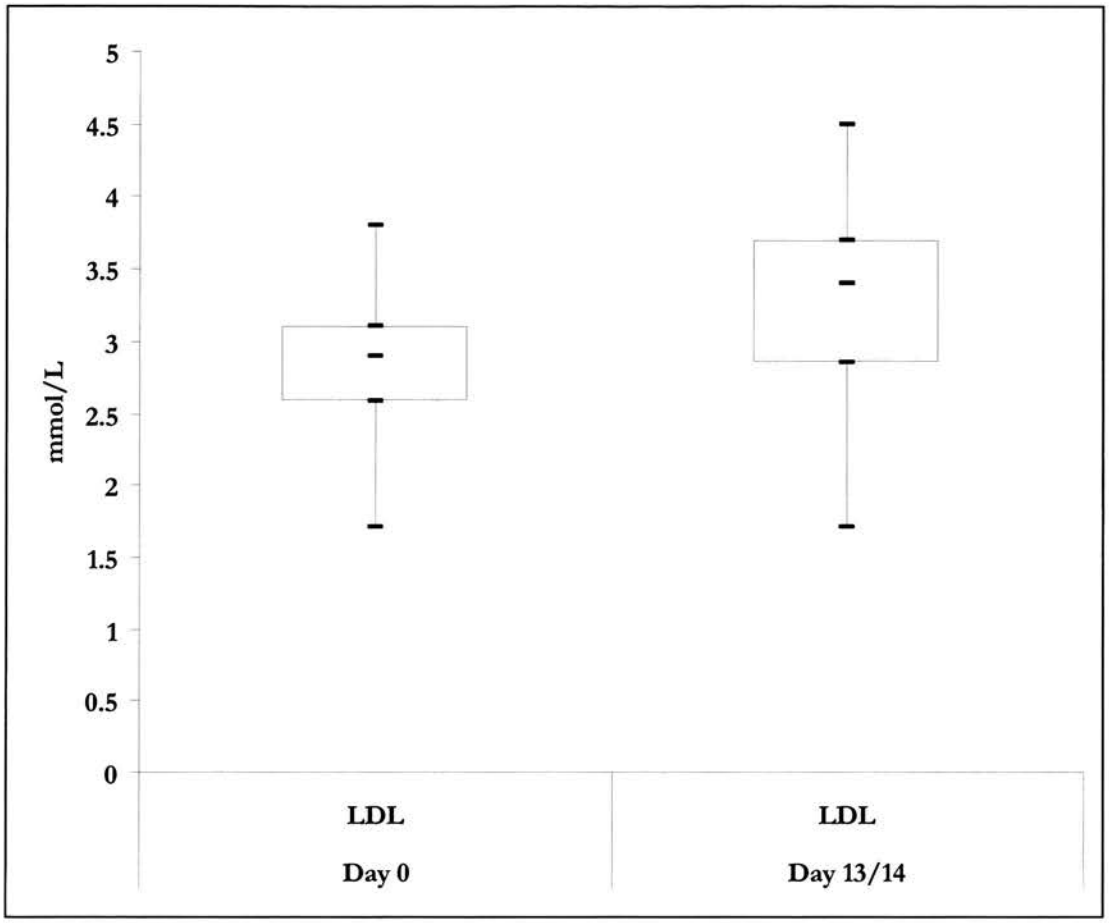


Figure 23
Results for low density lipoprotein (LDL) pre- and post-treatment demonstrating a significant rise with RAD001 ($p<0.0001$) (min, 25th percentile, median, 75th percentile and max values shown as box plot)

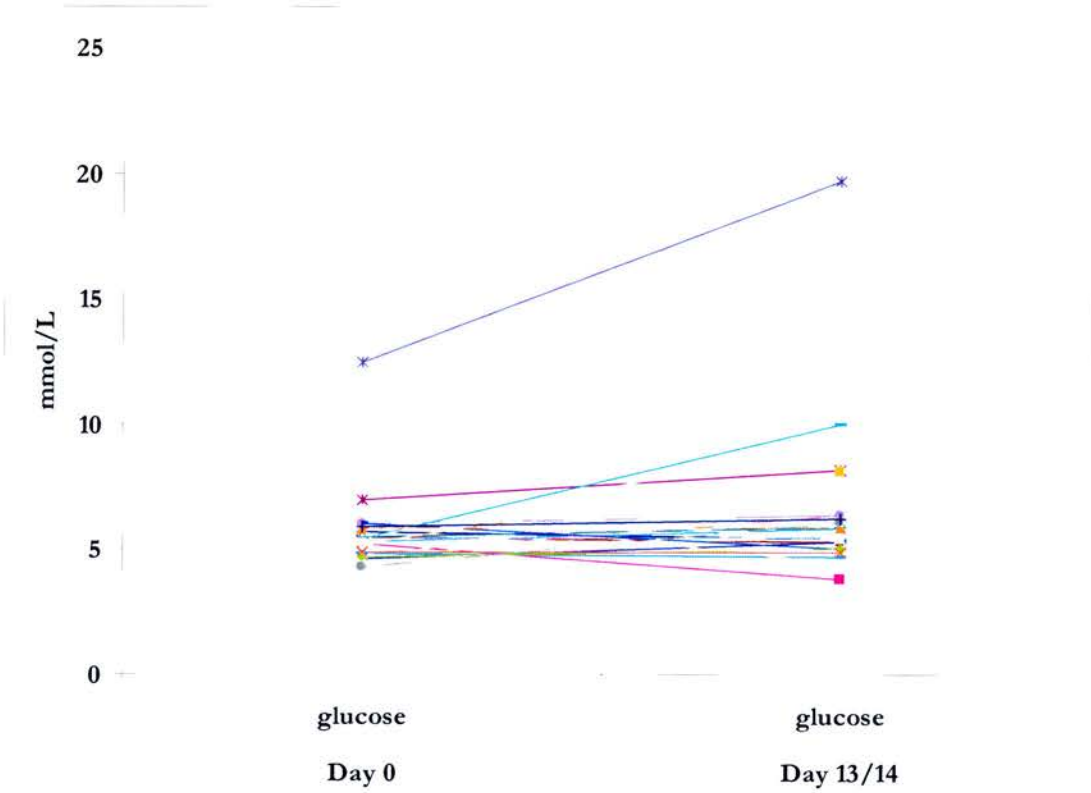


Figure 24
Individual values for random glucose pre- and post-treatment with RAD001, showing a trend toward a rise with RAD001 treatment (p=0.0542)

Peri- and post-operative complications

Data were collected on any complications occurring before, during or up to six weeks after surgery.

Eight patients had either wound infection or infected seroma during the study period. Three of these patients developed a suspected wound infection on the first post-operative day, all but one of which resolved with one course of antibiotics. None of the patients who developed immediate post-operative wound infection had a reduction in neutrophil count prior to surgery. One patient who developed an multiple resistant staphylococcus aureus (MRSA) wound infection had more than one course of antibiotics. This particular patient's husband was an inpatient in another hospital, and was known to be a MRSA carrier. Three further patients developed wound infections within four weeks of stopping RAD001 (4 weeks post-operatively). One patient had a re-excision prior to developing wound infection. Two of these patients had reduced neutrophils at the time of surgery, despite one of them having stopped the drug on Day 10 of the drug treatment. Two patients who had seromas drained in the post-operative period developed local infection 4-6 weeks after surgery.

Bleeding problems occurred in 4 patients of the total study group. Three patients had haematomas that required return to theatre for evacuation and haemostasis, two of whom had stopped the drug on Day 9 and Day 11. The other patient had reduced platelets (110 $x10^9$ /L), but an obvious single bleeding point was seen on return to theatre. One patient had bleeding into her drain post-operatively despite normal platelets and coagulation factors pre-operatively. This patient required iron replacement for anaemia post-operatively.

During the study period it was local policy to give patients unfractionated low molecular weight heparin prior to surgery, but due to an increase in bleeding problems experienced outwith this study, this was changed and a different form of prophylaxis introduced. [186]

The numbers of patients experiencing post-operative problems with bleeding or infection were greater than would have been expected within a normal population of patients undergoing breast surgery within the Edinburgh Breast Unit at that time. All the patients who bled had large tumours and underwent extensive mobilisation of breast tissue to close the breast defect. All apparent post-operative infections settled with antibiotics. Those who developed late infections were related to seromas. There was no correlation between reduction in white cells or neutrophils and infections, nor with reduction in platelets and bleeding problems.

Discussion

As can be seen from the high recruitment uptake of patients into the clinical study, the option of entering into a pre-operative study is an attractive one for patients. One reason for this may be the active participation and ability to take some form of potential treatment while awaiting surgery. Obviously in regions where there is only a short waiting time for surgery this benefit is diminished. The clear benefits of the design of this study for the investigator are the direct comparison of the same tumour before and after treatment, and the short study period, thereby not suffering the disadvantage of adjuvant studies where a long follow-up period is required in order to determine any treatment benefit. The challenge in the pre-operative setting is to find a biological marker that predicts for response in the long term. As previously discussed, there is increasing evidence for the role of Ki67 as a predictor of clinical response. [117, 118] A further discussion of these markers will take place after presentation of the results of the immunohistochemical studies carried out as part of this thesis.

From the above data, it can be concluded that RAD001 at a dose of 5mg is tolerable to patients with the exception of the most commonly reported symptom of mouth ulcers. Of the eight patients who experienced this side effect, four withdrew from the study with Grade I or II (mild or moderate) level symptom, defined as patients having pain on swallowing but being able to tolerate an adequate oral intake of liquid and solid. One of these patients had mouth ulcers at the time of entry into the study and withdrew on day 9 of treatment. Four other patients experienced mouth ulcers but remained in the study as they felt that their symptoms were not interfering with their quality of life. In general, those who withdrew had more mouth ulcers and they

were present for a longer period of time than those who remained in the study. The mouth ulcers resolved within 2 days in all patients after stopping RAD001. It is reasonable to expect that the level of tolerability of a drug in the early stage of treatment in a pre-operative setting will be different than that in an adjuvant or advanced study due to different levels of expectation and previous experience of patients. It is therefore encouraging that RAD001 was well tolerated in this pre-operative study.

The safety data are also reassuring from a group of patients who were typical of the medical fitness of women who experience breast cancer. It has become apparent from data now available from further studies carried out by Novartis Pharmaceuticals that the reduction in neutrophils found at 14 days in our study is the nadir and that this normalises with continued treatment. Significantly, there was no obvious increase in infective complications in the patients participating in this study, and in particular among those who had reduction in neutrophils counts. Reported rates of infective complications after breast surgery vary in the literature from 2% in retrospective studies to 18% in prospective studies with infective complications as primary outcome. [187-189] All those who had reduced neutrophils in this study had the expected response to surgery with a raise in neutrophil counts post-operatively. Similarly, although there were more bleeding complications than might be expected these did not occur in those who had a reduction in platelets. As mentioned above this RAD001 study period coincided with a local change in policy for deep vein thrombosis prophylaxis, with the resulting increase in bleeding complications, [186] and this may compound the results seen with RAD001.

One other published study has reported safety and tolerability data from a clinical study of RAD001 in cancer. [180] In that study patients with histologically confirmed advanced, relapsed, or refractory acute myeloid leukaemia or myelodysplastic syndrome, chronic lymphocytic leukemia, T-cell leukemia, myelofibrosis, or mantle cell lymphoma were treated with 5mg (first three patients) or 10 mg orally once daily for 28 days. From 27 patients for whom toxicity data was available, the following were reported as grade 1 or 2 toxicity: hyperlipidaemia (n=12, 44%), elevation of transaminases and/or alkaline phosphatase (n=11, 41%), anorexia (n=10, 37%), mouth ulcers (n=10, 37%), diarrhoea (n=8, 29%), hyperglycaemia (n=7, 26%), and hypomagnesaemia (n=6, 22%). One patient with refractory anaemia with ringed sideroblasts developed a biopsy-proven grade 3 cutaneous leukocytoclastic vasculitis (LCV) after 120 days of therapy, which was believed to be related to RAD001. Thirty-eight infectious episodes were noted in 21 (78%) patients at some time during the therapy. Fourteen (37%) of the 38 infectious episodes that occurred in 9 patients required either hospitalisation or prolongation of hospital stay. The frequency of infectious episodes did not seem to be increased in the study patients, in keeping with the results of the above results from this study of RAD001 in early cancer. The findings of hyperlipidaemia and elevation of liver transaminase and alkaline phosphatase with 14 days of RAD001 would correlate with these findings in the metastatic setting. One study in renal transplant patients treated with rapamycin found a trend towards correlation between higher triglyceride levels and lower levels of Akt phosphorylation, not found in this current study of RAD001. [190]

Two patients in this study were taking metformin for diabetes mellitus control. Metformin has been shown in cell line studies to activate AMPK in the presence of

TSC2, leading to phosphorylation of TSC1/2 complex, with a resulting reduction in mTOR signalling and dephosphorylation of S6 and 4E-BP1. [191] It is therefore unclear what effect concomitant treatment with metformin will have on tumours in combination with an mTOR inhibitor. There were not sufficient numbers of patients on metformin in this study to allow analysis of the effects of this drug on RAD001 efficacy. One of the patients on metformin developed mouth ulcers and stopped RAD001 after 10 days, while the other patient completed 14 days treatment with no side effects. In the study considered above in advanced haematological malignancy grade 3 hyperglycaemia occurred in six patients requiring insulin therapy, of which five had a history of diabetes mellitus and grade 2 hyperglycaemia was noted at baseline in four patients. [180] The hyperglycaemia resolved to baseline or better with cessation of RAD001. It has previously been shown that in patients with renal transplant rapamycin therapy was associated with impaired glucose tolerance and development of new-onset diabetes mellitus, [192] and that chronic exposure to rapamycin inhibits basal and insulin-stimulated activation of Akt, and leads to an increase in total insulin receptor substrate (IRS-1 and IRS-2) proteins, similar to the pattern seen in naturally occurring type 2 diabetes mellitus. [190] There was a trend towards a rise in serum glucose during our study of two week pre-operative treatment with RAD001, but this did not reach significance. Random glucose levels can be classified as normal <5.6mol/L, impaired glucose levels 5.6-11.1 mmol/L, diagnostic of diabetes >11.1mmol/L. [193, 194] One patient who had known diabetes mellitus had high levels both before at the start and finish of treatment with RAD001. Eight patients had normal random glucose before treatment but had glucose > 5.6 mmol/L after RAD001. Four patients had glucose >5.6 mmol/L before treatment but these

levels were normal after RAD001. In four patients both pre and post-treatment glucose levels were over 5.6 mmol/L. There was no dominant pattern seen in the results, probably because the samples were taken two weeks apart at different times of the day, and with differing fasting status of the patients. Further investigation of fasting blood glucose levels in patients with RAD001 would clarify any adverse effect, as it has been demonstrated that hyperglycaemia preoperatively is associated with increased cardiovascular mortality with surgery. [195]

The other mTOR inhibitor that has been trialled in the clinical setting in breast cancer is CCI-779 (Wyeth Pharmaceuticals). Tolerability from a study of two different doses of CCI-779 in patients with locally advanced or metastatic breast cancer revealed that all patients (n=106) had a drug-related treatment-emergent adverse event (TEAE) at some point in the treatment period (median 11 weeks treatment). [184] The most common of these was mouth ulcers (70%), with no difference between the two treatment groups dosing of 75mg or 250mg by weekly intravenous infusion. Similar rates of mouth ulcers have been reported with CCI-779 in advanced renal cancer and mantle cell lymphoma. [196, 197] The very high rate of mouth ulcers reported in these studies compared to our findings in the pre-operative setting may be due to the patient population included in these metastatic studies as these patients have been heavily pre-treated. However, the reported rate of mouth ulcers from the RAD001 study in metastatic haematological malignancies was 37%, and so there may be differences between CCI-779 and RAD001 safety and tolerability, possibly due to the methods of administration being oral for RAD001 and intravenous for CCI-779.

Biological Study

Materials and Methods

Collection of biopsy samples

All patients entering this study had diagnostic core biopsies taken at time of presentation with a symptomatic breast lump. The tissue was processed in accordance with local pathology department policy. The surgical excision specimen was processed by the pathology laboratory and once all diagnostic procedures completed, the tumour blocks were retrieved and sectioned for study purposes.
All patients entering this study had fresh tissue stored at the time of diagnostic core biopsy. This was in line with current policy in the Edinburgh Breast Unit approved by the local Ethics Committee. Patients who attended the symptomatic breast clinic with a clinically malignant palpable lump were approached by a member of the research team to discuss taking of additional core biopsies for research purposes. The uptake for this was high as most patients had up to 10 core biopsies taken for diagnostic purposes and the addition of a further 3-4 core biopsies for research caused no additional distress due to the infiltration of local anaesthetic and the additional time taken. The tissue samples were stored immediately in a liquid nitrogen canister within 2-3 minutes, and transferred to full storage in liquid nitrogen at the end of the clinic. For the purposes of this study during surgery core biopsies were taken for liquid nitrogen storage after the lump (in the case of wide local excision) or breast (in the case of mastectomy) had been removed from the patient. The diagnostic tissue was processed in accordance with local policy.

Analysis of biopsy samples

Immunohistochemical analysis was used to assess the effects of RAD001 on the paired samples from the patients. Staining was performed on all samples collected including those from patients who did not complete 14 days of treatment. Pre- and post-treatment sections were stained within the same staining run and anonymised prior to scoring.

Proliferation

Immunohistochemistry for Ki67 (MIB-1, diluted 1:50; Dako, Cambridge, UK) was performed as follows:

epitope retrieval was performed in citrate buffer pH6.0 in a microwave for 6 minutes, slides were then treated with 3% hydrogen peroxide (H_20_2) for 5 minutes to block endogenous peroxidase before washing and incubating with primary antibody as above at room temperature (RT) for 30 minutes before incubation with secondary antibody for 30 minutes (RT) (Dako EnVision+, K5007), then visualised with DAB chromogen for 10 minutes (RT) and counterstained with haematoxylin.

Hormone receptors

Immunohistochemistry for oestrogen receptor (ER, NCL-L-ER-6F11/2 diluted 1:25; Novocastra Laboratories, UK) and progesterone receptor (PR, PR636 diluted 1:100; Dako) were performed as follows:

for both ER and PR epitope retrieval was performed in citrate buffer pH6.0 in a microwave for 6 minutes, slides were then treated with 3% H_20_2 for 5 minutes to block endogenous peroxidase before washing and incubating with primary antibody as above (RT) for 1 hour for ER and 30 minutes for PR before incubation with

secondary antibody for 30 minutes (RT) (Dako EnVision+, K5007), then visualised with DAB chromogen and counterstained with haematoxylin.

mTOR pathway effects

In order to determine that RAD001 was downregulating mTOR, phospho-mTOR staining was carried out as described below. To show that the downregulation of mTOR resulted in reduction in activity of downstream effects, phospho-S6 staining was performed for ser 235/236 and ser 240/244 using a protocol worked up by Michael Stumm, Novartis as described below.

Immunohistochemistry for phospho-mTOR (serine 2448) and phospho-S6 (serine 235/236 and 240/244) were performed as follows:
for both phospho-S6 and phospho-mTOR epitope retrieval was performed in citrate buffer pH6.0 at 96°C for 10 and 20 minutes respectively, slides were treated with 3% H_20_2 for 10 minutes and 30 minutes respectively to block endogenous peroxidase and with casein (p-mTOR) or 10% normal goat serum (p-S6) before washing and incubating overnight at 4°C with the appropriate primary antibody (p-mTOR2448, 49F9, diluted 1:50; p-S6235/236 clone 2211, diluted 1:400; p-S6240/244 2215, diluted 1:100, all Cell Signaling Technology) before incubation with secondary antibody (Dako EnVision+, K5007) for 30 minutes at RT, visualized with DAB chromogen for 10 minutes at RT and counterstained with haematoxylin.

Immunohistochemical staining for phospho-Akt (serine 473) was performed as described below to determine the levels of expression and changes in expression with treatment with RAD001.

Epitope retrieval was performed in TRIS/ EDTA buffer pH9.0 at 97°C for 20 minutes, slides were then treated with 3% H_20_2 for 5 minutes before washing and incubating with primary antibody (p-Akt s473, clone 14-5, diluted 1:6; Dako, Denmark) for 30 minutes (RT) followed by incubation with secondary antibody and DAB and counterstaining as described above.

Several attempts were made to optimise immunohistochemical staining for phospho-PTEN (ser 380) by the candidate and members of the laboratory group of John Bartlett. No optimum method was reached and it was not possible to obtain satisfactory results for this within the time and financial restraints of this study.

HER-2

HER-2 assessment was performed routinely on the excision specimens of patients with poor prognostic scores at the Pathology Laboratory. Testing for HER-2 was performed by the same laboratory staff on those patients who did not routinely have this done for research purposes. Immunohistochemical testing was performed and scored by a pathologist. All samples scored as 2+ for HER-2 status by immunohistochemistry were tested by fluorescent in situ hybridisation (FISH). Overexpression of HER-2 was defined as tumours staining 3+ or 2+ and FISH positive, while all patients with tumours scored as 0 or 1+ were considered not to overexpress HER-2.

Apoptosis

Apoptosis was measured by assessment of active caspase-3 by the method described as follows:

Epitope retrieval was performed in TRIS/ EDTA buffer pH8.0 at 97°C, slides were then treated with 3% H_2O_2 for 10 minutes before washing and incubation in serum-free block (DAKO) for 1 hour. Primary antibody detecting cleaved caspase-3 (Abcam ab 2302, UK) was applied overnight at 4°C followed by incubation with secondary antibody (EnVision, DAKO) and DAB and counterstaining. [198]

Scoring and intra-observer and inter-observer correlations

All sections were checked by a pathologist and appropriate areas of cancer for scoring were ascertained. All samples except Ki67 and apoptosis were scored blind using the histoscore method [199] performed by two scorers, one of whom was the candidate. Ki67 was counted using a previously described and validated method of scoring the percentage of stained cells as a proportion of the total cancer cells. [200] A semi-automated method of calculating apoptosis using Ariol (Applied Imaging) was employed where sections were scanned at high resolution (20x) using Ariol and tumour areas were manually identified. Tumour cells were scored using a previously validated algorithm. [201] Inter-observer error was assessed for Ki67 count and for histoscores. **Figures 25 and 26** A significant correlation for both pre-treatment ($r=0.753$, $p<0.0001$) and post-treatment samples ($r=0.889$, $p<0.0001$) was achieved. The first count by the candidate had correlated significantly with the scores reported by the second scorer, and the mean of the two scores was used for results analysis. One patient's (pt 16) score was considerably different on first and second assessment by the candidate and that closest to the score of the second scorer was used in the results analysis.

Intra-observer error for the candidate was assessed for Ki67 counts and for histoscore on p-S6 (ser 235/236) by double scoring of both pre and post treatment samples with a four week interval between scores by the candidate.

The intra-observer correlations for histoscores for p-S6 (ser 240/244), and for Ki67 were highly significant. **Figures 27 and 28**

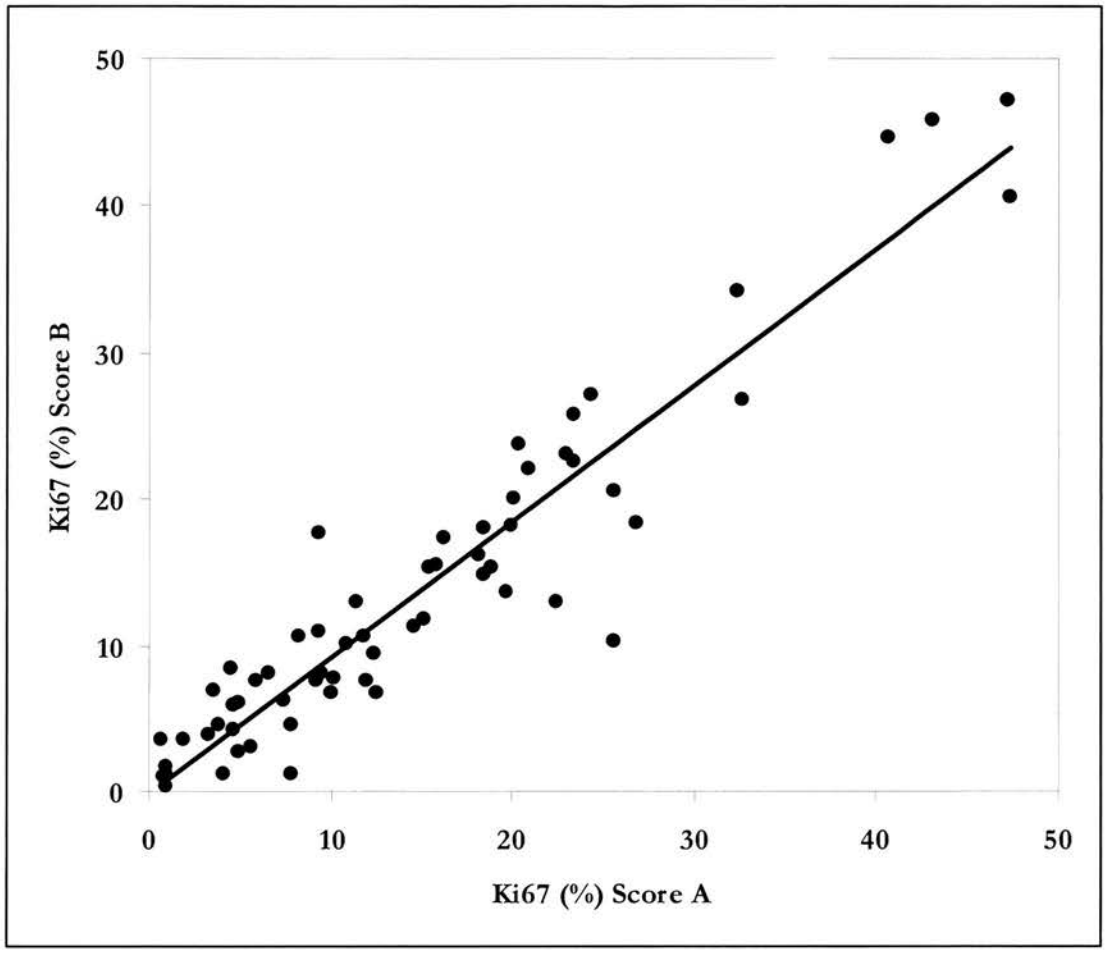


Figure 25
Inter-observer correlation for Ki67 (%) counts for pre and post treatment tumour samples (r=0.8846 (95% CI 0.8148-0.291, p<0.001)).

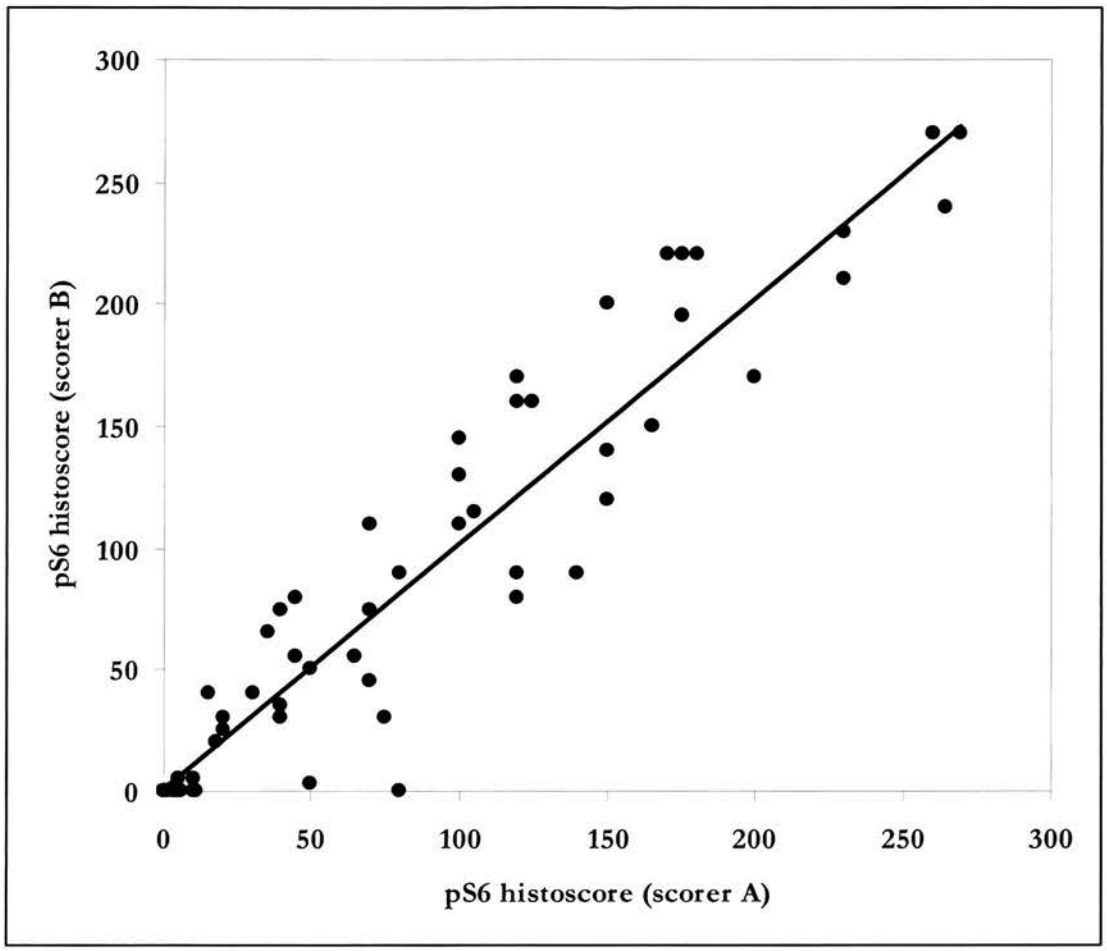


Figure 26
Inter-observer correlation for histoscore for p-S6 (ser 240/244) for pre and post treatment tumour samples (r=0.9485 (95% CI 0.9157-0.9688, p<0.0001)).

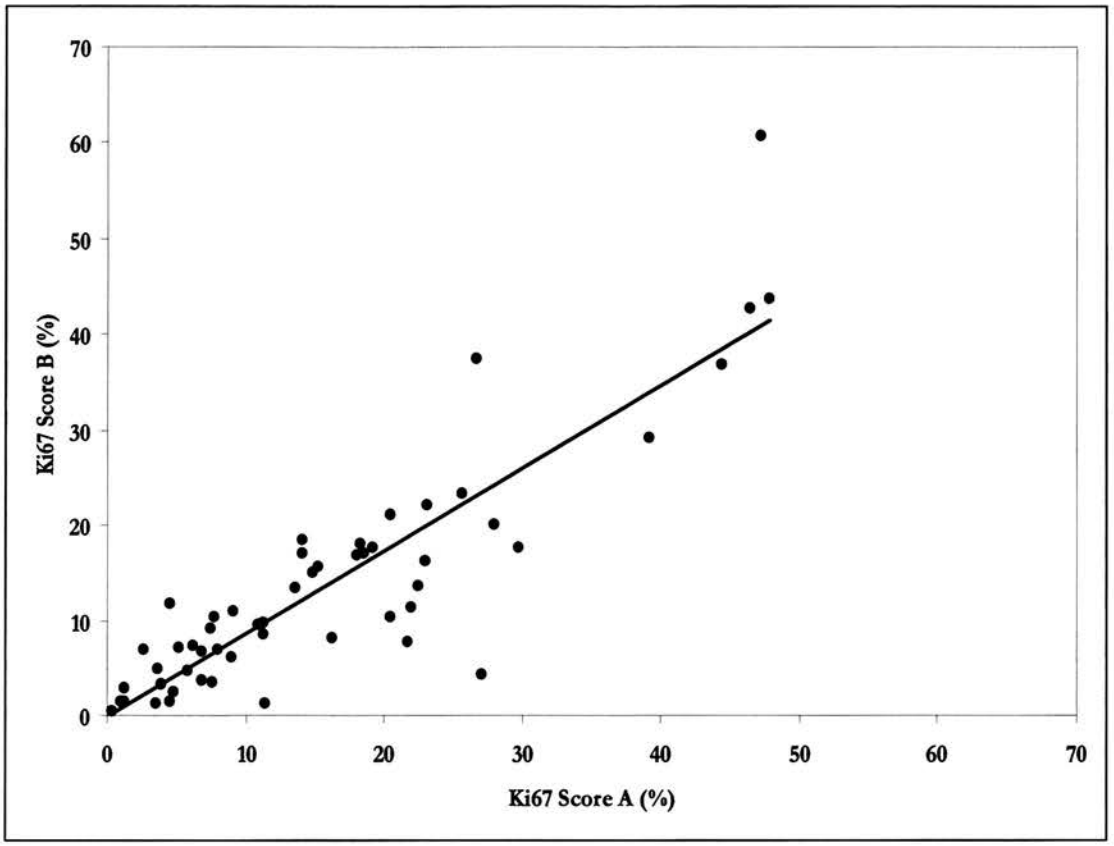


Figure 27
Intra-observer correlation for Ki67 (%) for pre and post treatment counts (r=0.843 (95%CI 0.7431 to 0.9072, p<0.0001)).

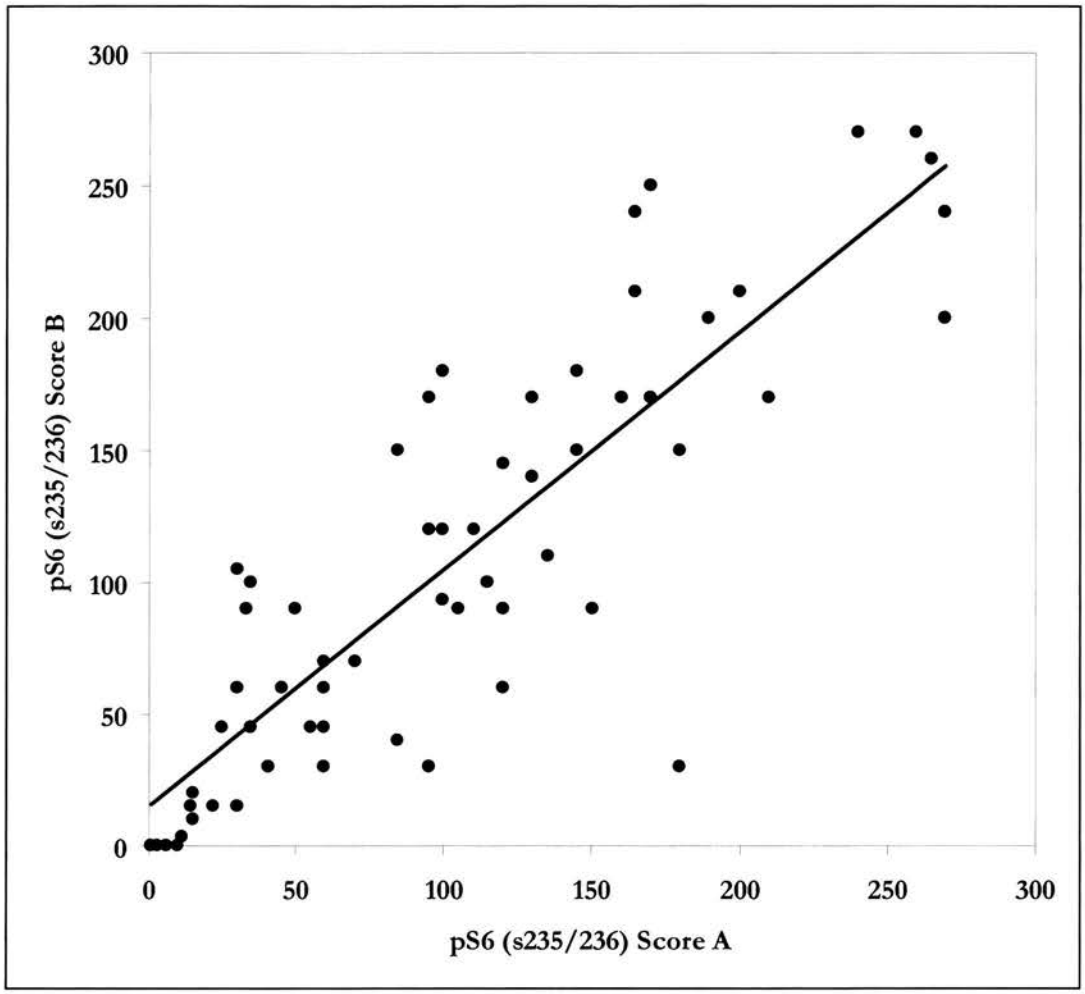


Figure 28
Intra-observer correlation for p-S6 (ser 235/236) histoscores for pre and post-treatment (r=0.8573 (95%CI 0.7730 to 0.9118, p<0.0001)).

Statistical Analysis

Results were analysed on an intention to treat (ITT) basis, after initial ANOVA showed no difference between those completing 14 days and those who took RAD001 for less than 14 days for change in proliferation as measured by Ki67, this being the primary outcome; neither was there any difference between those completing the full course or stopping early for the other markers of the mTOR pathway assessed.

ANOVA was also employed to determine differences between those that had a marked reduction in Ki67, "responders" (R), and those who did not, "non-responders" (NR). Estimations of "responders" and "non-responders" were based upon dual observer analysis of Ki67 staining; the 95% confidence interval for the mean difference in Ki67 counts between two observers was taken as the minimum difference which could be regarded as a change in Ki67 status between pre- and post-treatment samples. [202] Due to the non-normal distribution of the data, Ki67 scores were log transformed prior to analysis. The change between pre and post treatment scores for each patient was calculated as the difference in the logged scores.

In order to examine the linear relationship between the immunohistochemical and blood results with the Ki67 scores, Pearson's Correlation (r) was used. ANOVA was used to examine the change between pre and post treatment scores for all the measurements for each patient.

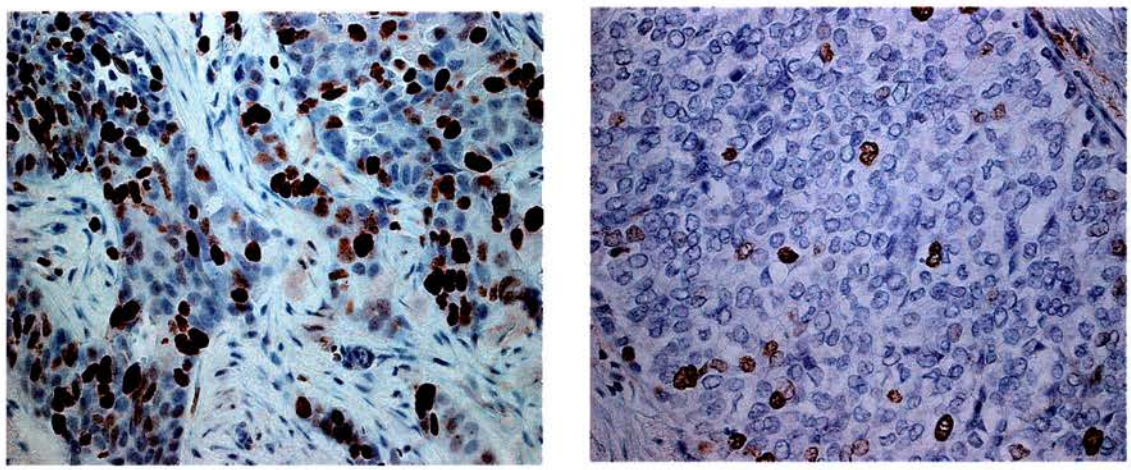
For the purpose of sub-group analysis data were split about the median value to differentiate between those with high and low scores for each variable.

Results of biological study

The results discussed below are for the intention-to-treat population of 31 patients who received up to 14 days RAD001 treatment. There was no difference between those who completed 14 days and those who had less than 14 days treatment for any of the outcomes discussed below. Using the 95% CI of the absolute value for change in Ki67%, 18 tumours were classified as "responders" and 13 as "non-responders".

Proliferation

Proliferation as measured by Ki67 was the primary outcome of the biological RAD001 study (staining before and after treatment shown in **Figure 29**). There was a significant reduction in proliferation as assessed by Ki67 (absolute median fall 8.5%, p=0.019). **Figures 30 and 31; Table 6** Using log transformed data, the post-treatment levels of Ki67 were 26% of those before treatment (95% CI 5, 56%). Those that had a high proliferation at diagnosis had the greatest absolute decrease in Ki67 (based on log transformed data r=0.36, p=0.046) with RAD001 treatment. The relationships between pre-treatment Ki67 and the change in Ki67 with treatment with the markers of the mTOR pathway examined will be discussed below with the results for each marker assessed.



(a) (b)

Figure 29
Example of proliferation by Ki67 showing a reduction in nuclear staining pre-(a) and post-(b) treatment with RAD001.

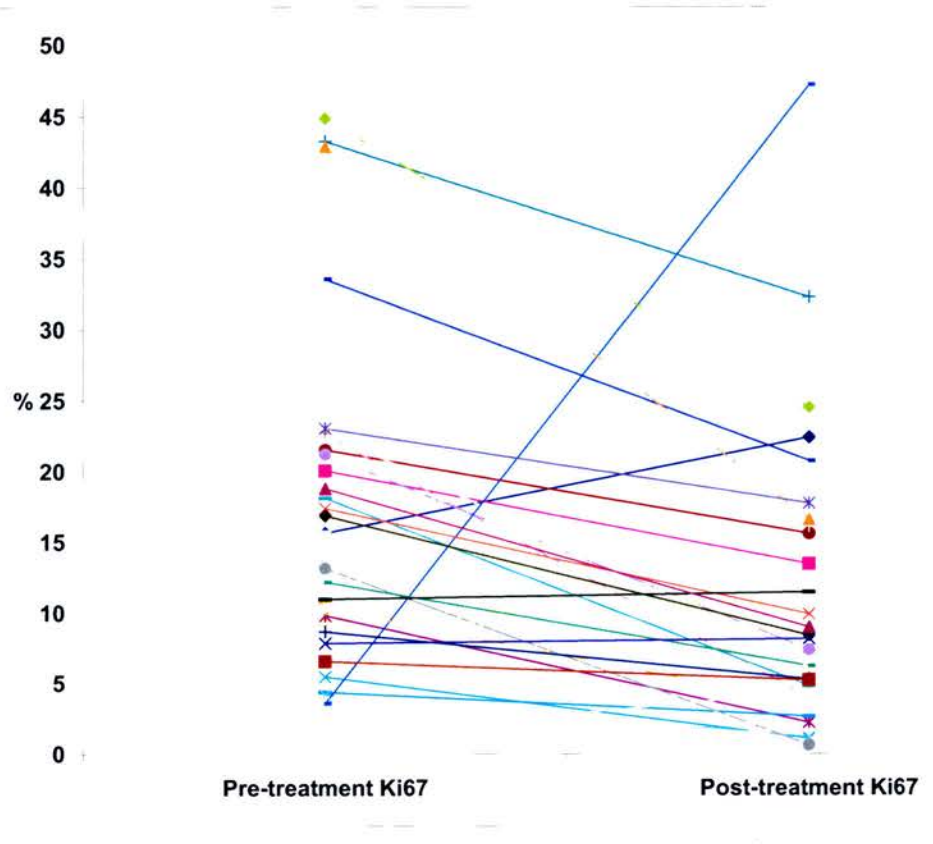


Figure 30
Individual values for Ki67 before and after treatment with RAD001 (p=0.019). One tumour (from Patient 17) is noted to have rise in proliferation after treatment.

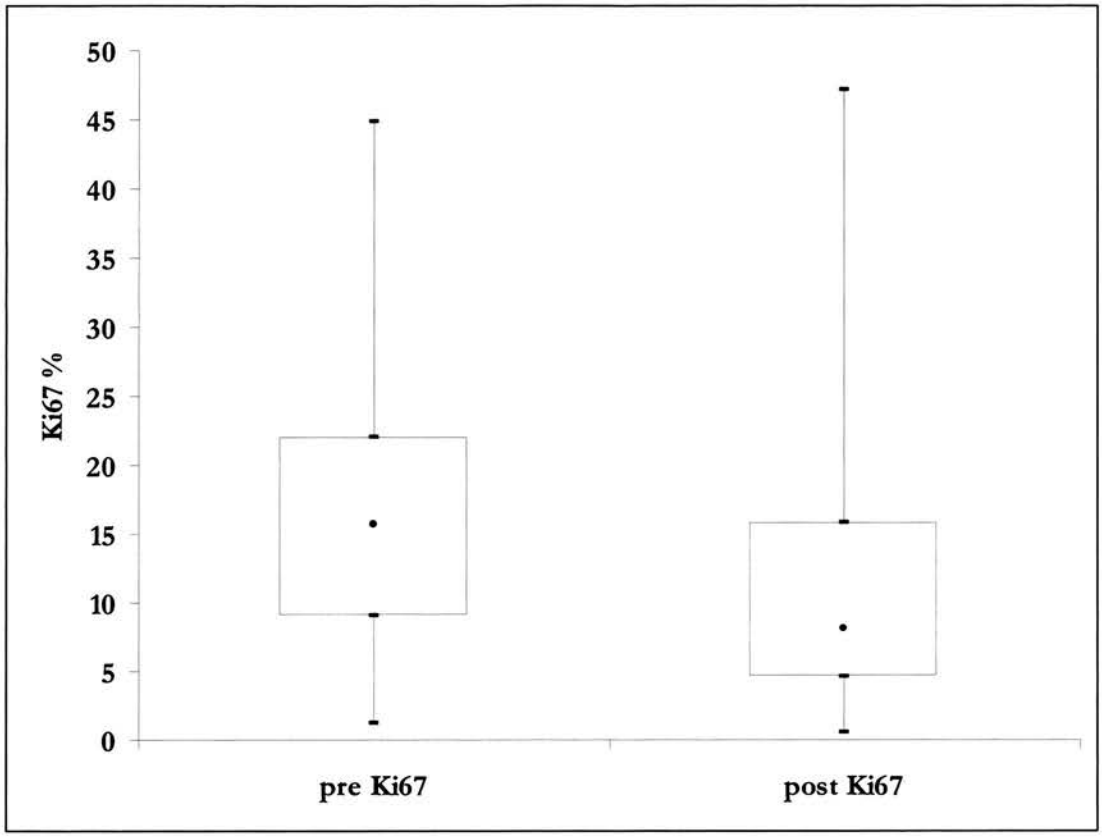


Figure 31
Proliferation (Ki67%) before and after RAD001 shown as box plot indicating min, 25th percentile, median, 75th percentile and maximum. There was a significant reduction in median Ki67 with RAD001 (p=0.019).

Patient number	Days of treatment completed	Ki67 pre-treatment (%)	Ki67 post-treatment (%)	%Ki67 difference
1	14	15.64464054	22.37932062	6.73468
2	10	20.07644202	13.43729499	-6.63915
3	14	9.49	10.89715232	1.402831
4	14	5.481474736	1.075595461	-4.40588
5	14	9.823652319	2.223224354	-7.60043
6	14	21.52219033	15.58552629	-5.93666
7	14	43.28024569	32.2784848	-11.0018
8	14	33.57424721	20.74116394	-12.8331
9	14	4.396040326	2.662083981	-1.73396
10	14	1.27	4.382147737	3.107816
11	14	15.39655555	5.668893311	-9.72766
12	14	22.8634227	6.522736121	-16.3407
13	9	4.23	1.208029547	-3.02269
14	14	22.59140438	3.982225303	-18.6092
15	14	21.21863803	7.365469905	-13.8532
16	14	25.72022868	16.0953047	-9.62492
17	14	3.59	47.20248447	43.60966
18	13	18.14672924	4.800763944	-13.346
19	14	44.9045913	24.52508402	-20.3795
20	14	10.33850024	3.576862616	-6.76164
21	14	42.8795435	16.59442075	-26.2851
22	14	17.40172389	9.906312325	-7.49541
23	14	23.04	17.74421659	-5.29804
24	14	13.14334833	0.593160477	-12.5502
25	11	8.674283646	5.274745273	-3.39954
26	4	12.20	6.229246176	-5.96992
28	14	10.97713215	11.45599451	0.478862
29	14	16.88578809	8.370806833	-8.51498
30	14	6.573965217	5.219653324	-1.35431
31	14	18.7829559	8.961546814	-9.82141
32	7	7.86	8.151975205	0.288313

Table 6
Individual numeric values for Ki67% before and after treatment with RAD001 (classified as responders (highlighted) and non-responders)

Oestrogen and Progesterone receptors

There was a significant reduction in ER scores with RAD001 treatment over 14 days (mean fall 40.5, 95%CI 16.9, 64.2, p=0.001). **Table 7** Data were split about the median (240) into high and low ER groups, demonstrating that those with lower ER pre-treatment (n=12) had a significantly greater fall in ER (mean fall 77.5, 95%CI 43.26, 111.74) compared with those with high ER pre-treatment (n=19; mean fall 17.21, 95%CI -10.00, 44.42, p=0.009). **Figure 32** This implies that tumours with high ER were more likely to preserve ER after treatment that than those with lower ER, who lost ER with treatment. There was however no correlation between Ki67 pre-treatment and ER scores before or after treatment, nor between ER score and change in Ki67 with treatment. There was no difference in pre-treatment ER or change in ER score with RAD001 between those that responded to treatment (as assessed by Ki67) and those that did not respond.

There was no significant change in PR with treatment, and no correlation between PR and Ki67 for either pre-treatment values or change in value with treatment. **Table 8** There was, however, a significantly different change in PR score with treatment between those that were classified on the basis of Ki67 change to have responded and those that did not respond, in that responders had an increase in PR after 14 days RAD001 (mean increase 14 (95%CI -1.5, 30.3) and non-responders had a decrease in PR score (mean decrease 14 (95%CI -32, 4; p=0.025). **Figure 33**

Patient number	Days of treatment completed	ER pre-treatment	ER post-treatment
1	14	285	270
2	10	170	15
3	14	285	270
4	14	200	160
5	14	260	190
6	14	285	260
7	14	160	15
8	14	170	10
9	14	295	280
10	14	210	165
11	14	240	240
12	14	255	260
13	9	210	180
14	14	210	210
15	14	155	10
16	14	240	180
17	14	230	15
18	13	300	300
19	14	160	90
20	14	297	230
21	14	240	240
22	14	240	300
23	14	285	285
24	14	285	300
25	11	270	210
26	4	275	240
28	14	240	240
29	14	225	240
30	14	180	240
31	14	240	225
32	7	240	210

Table 7
Individual numeric values for ER histoscores before and after treatment with RAD001 (classified as responders (highlighted) and non-responders by Ki67).

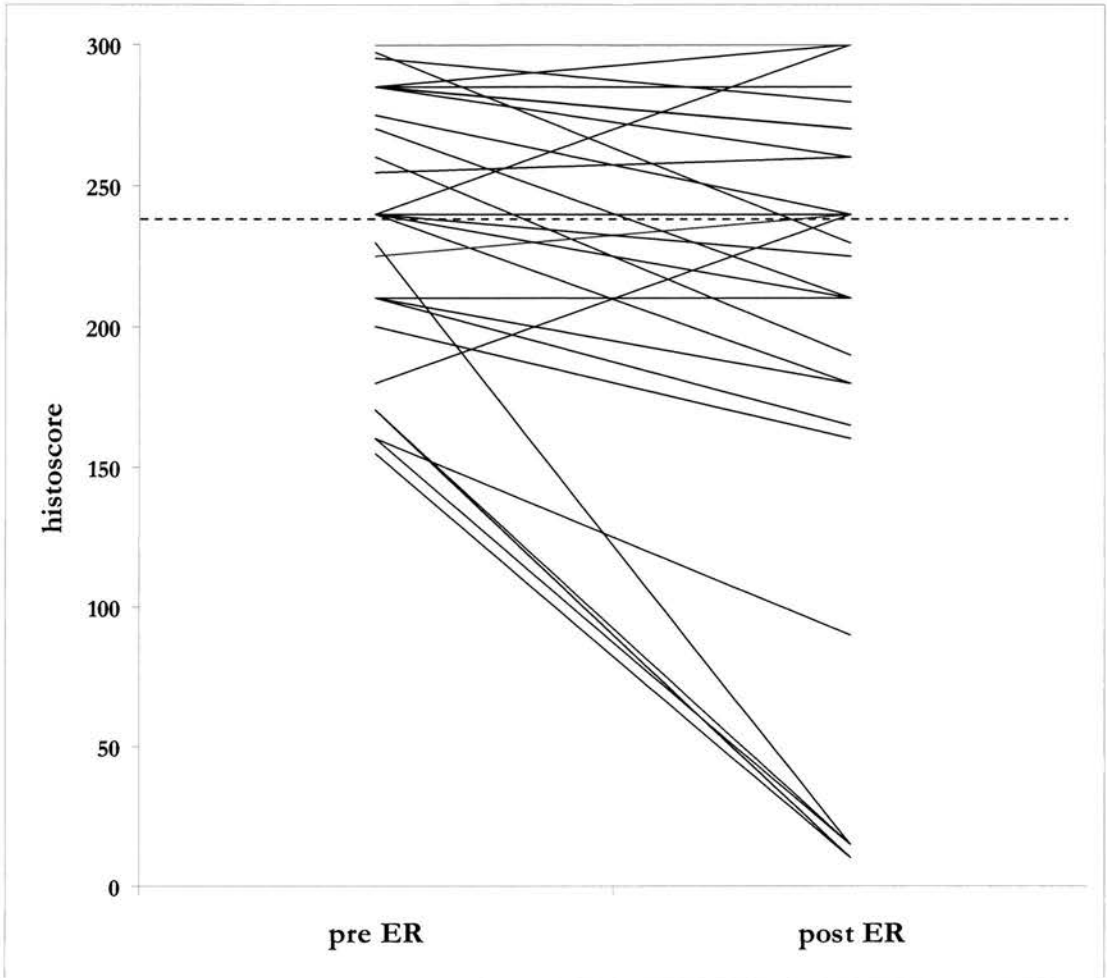


Figure 32
Individual values for ER score before and after treatment with RAD001, demonstrating a significant reduction in ER with RAD001 treatment (p=0.001). Those with high pre-treatment ER (above median 240, dashed line shown) showed no significant change in ER, but those with initially lower ER score (less than median 240) had a reduction with RAD001 treatment (p=0.009).

Patient number	Days of treatment completed	PR pre-treatment	PR post-treatment
1	14	70	70
2	10	0	0
3	14	18	15
4	14	0	2
5	14	0	0
6	14	250	220
7	14	0	3
8	14	18	0
9	14	155	150
10	14	140	130
11	14	90	120
12	14	180	255
13	9	0	0
14	14	140	210
15	14	0	0
16	14	140	160
17	14	6	2
18	13	3	3
19	14	1	5
20	14	200	190
21	14	100	30
22	14	120	145
23	14	5	3
24	14	180	200
25	11	170	90
26	4	150	180
28	14	230	170
29	14	70	150
30	14	240	270
31	14	140	140
32	7	260	240

Table 8
Individual numeric values for PR histoscores before and after treatment with RAD001 (classified as responders (highlighted) and non-responders by Ki67)

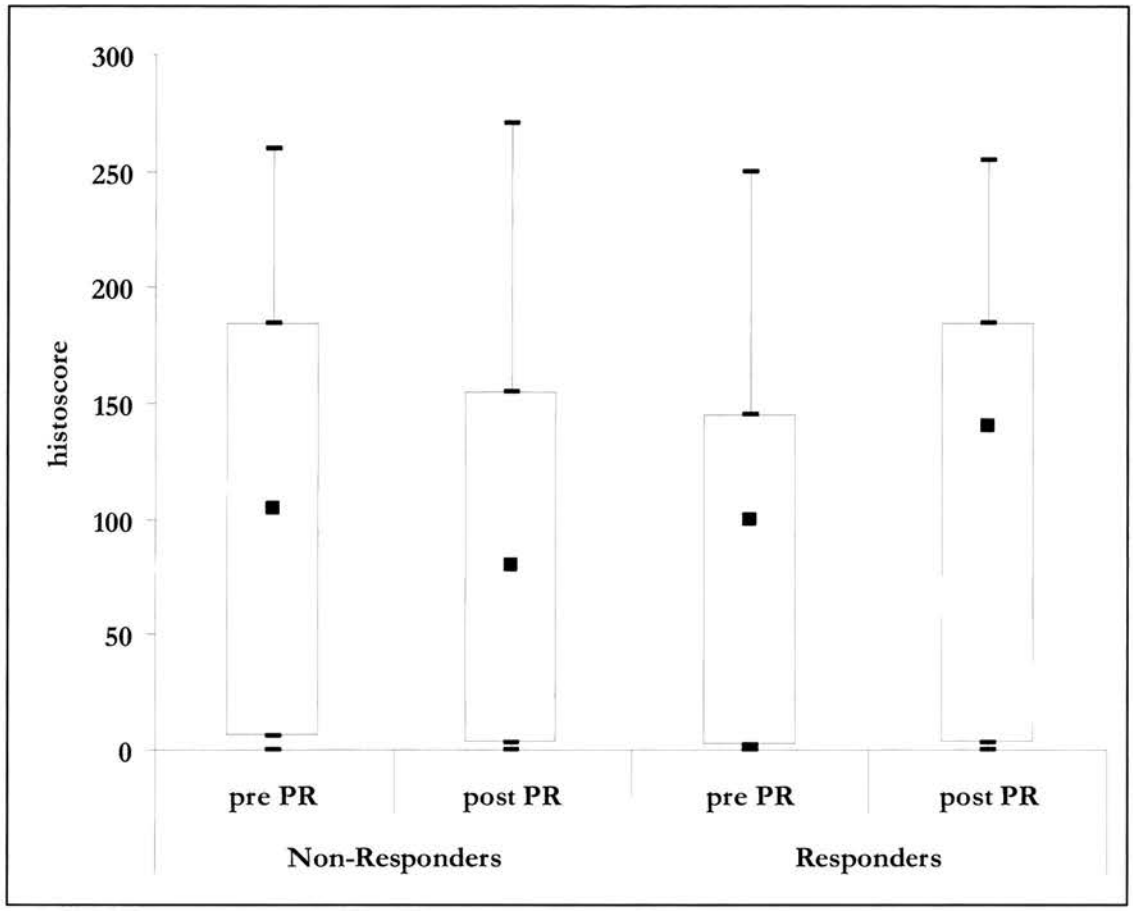


Figure 33
Results for PR scores before and after RAD001 shown as box plot indicating min, 25th percentile, median, 75th percentile and maximum. In those that did not show a response to RAD001 (defined by change in Ki67%) (non-responders) there is a small reduction in median PR with treatment and those that responded to RAD001 (responders) there was a small increase in PR with treatment. The difference between the two groups was significant (p=0.025).

HER-2

HER-2 scoring was performed by a trained pathologist, and scored as 0, 1, 2 or 3 on immunohistochemistry. Fluorescent in-situ hybridisation (FISH) testing was performed on those samples that were intermediate (score 2) on immunohistochemistry. Eight patients had tumours that were positive on IHC with a score of 3+. **Table 9** All those who had FISH testing for score 2+ were negative. Of those that were HER-2 positive, four were ER poor, and all were PR poor or negative. **Table 10** Response by Ki67 was seen in both HER-2 positive and HER-2 negative tumours. Of the 8 HER-2 positive tumours, 7 responded with a reduction in Ki67 (87.5%), but of 23 HER-2 negative patients, only 11 had a response (47.8%). This numerical difference in response between HER-2 positive and HER-2 negative did not reach statistical significance (p=0.10).

Patient number	Days of treatment completed	HER-2 IHC	HER-2 FISH
1	14	0	
2	10	3+	
3	14	3+	
4	14	1+	
5	14	3+	
6	14	1+	
7	14	3+	
8	14	3+	
9	14	1+	
10	14	1+; 2+	Negative (on 2+ tumour)
11	14	1+	
12	14	0	
13	9	1+	
14	14	2+	Negative
15	14	3+	
16	14	1+	
17	14	2+	Negative
18	13	0	
19	14	0	
20	14	2+	Negative
21	14	3+	
22	14	3+	
23	14	2+	Negative
24	14	0	
25	11	2+	Negative
26	4	1+	
28	14	2+	Negative
29	14	0	
30	14	1+	
31	14	2+	Negative
32	7	1+	

Table 9
HER-2 positivity on immunohistochemistry (FISH on ++): individual values (responders (highlighted) and non-responders by Ki67).

Patient number	ER (histoscore)	PR (histoscore)	HER-2 IHC
1	285	70	0
2	170	0	3+
3	285	18	3+
4	200	0	1+
5	260	0	3+
6	285	250	1+
7	160	0	3+
8	170	18	3+
9	295	155	1+
10	210	140	1+; 2+; FISH neg
11	240	90	1+
12	255	180	0
13	210	0	1+
14	210	140	2+; FISH neg
15	155	0	3+
16	240	140	1+
17	230	6	2+; FISH neg
18	300	3	0
19	160	1	0
20	297	200	2+; FISH neg
21	240	100	3+
22	240	120	3+
23	285	5	2+; FISH neg
24	285	180	0
25	270	170	2+; FISH neg
26	275	150	1+
28	240	230	2+; FISH neg
29	225	70	0
30	180	240	1+
31	240	140	2+; FISH neg
32	240	260	1+

Table 10
Combined results for ER, PR and HER-2 for each patient pre-treatment (responders (highlighted) and non-responders by Ki67).

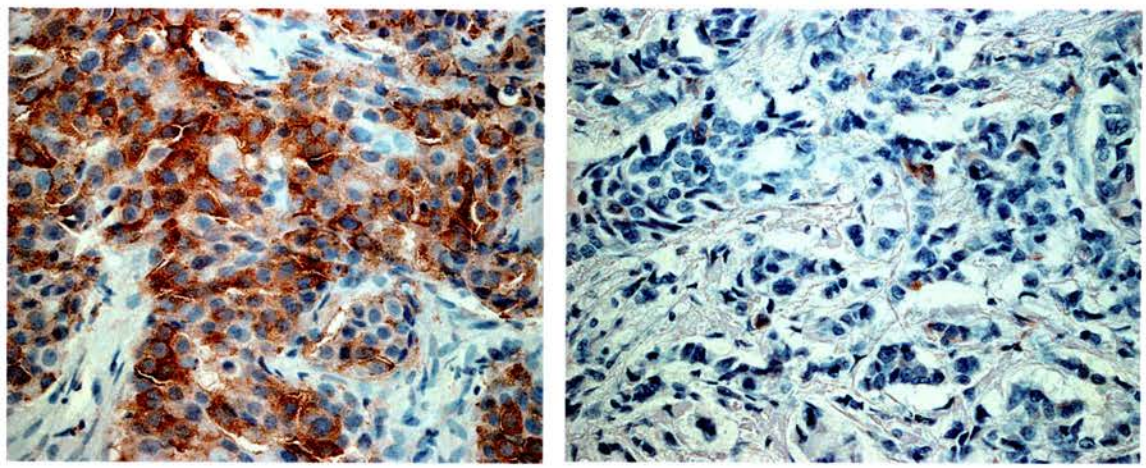
phospho-S6 kinase

Cytoplasmic staining for phospho-S6 was seen. **Figure 34** For both phosphorylation sites assessed (ser 235/236 and ser 240/244) there was a significant reduction in phosphorylation. For p-S6 s235/236 the mean histoscore pre-treatment was 129.4 and this fell to 58.3 post treatment (mean fall 71.2; 95%CI 41.9, 100.4; $p<0.001$). **Figure 35 and 37; Table 11** For p-S6 s240/244 the mean histoscore pre-treatment with RAD001 was 142.0 and 31.5 post treatment (mean fall 110.5; 95%CI 82.9-138.2; $p<0.001$). **Figure 36 and 37; Table 12**

There was no evidence for a relationship between pre-treatment scores for either site of phosphorylation of S6 and Ki67 before or after treatment, nor between the change in S6 and Ki67 before or after treatment. Reduction in phosphorylation of S6 by the mTOR inhibitor RAD001 would appear to be independent of proliferation.

There was a trend toward greater reduction in p-S6 in those tumours that responded to RAD001 but this did not reach statistical significance for either s235/236 (responders (R) mean fall 89.3; 95%CI 51.7, 127.0; non-responders (NR) mean fall 46.0; 95%CI 1.7, 90.3; p=NS) or s240/244 (R mean fall 124.5, 95%CI 88.5, 160.6; NR mean fall 91.2; 95%CI 48.8, 133.6; p=NS) sites of phosphorylation.

There was no difference in p-S6 expression at baseline between responders and non-responders.



(a) **(b)**

Figure 34
Example of cytoplasmic staining of p-S6 (ser 235/236) pre-(a) and post-(b) treatment with RAD001.

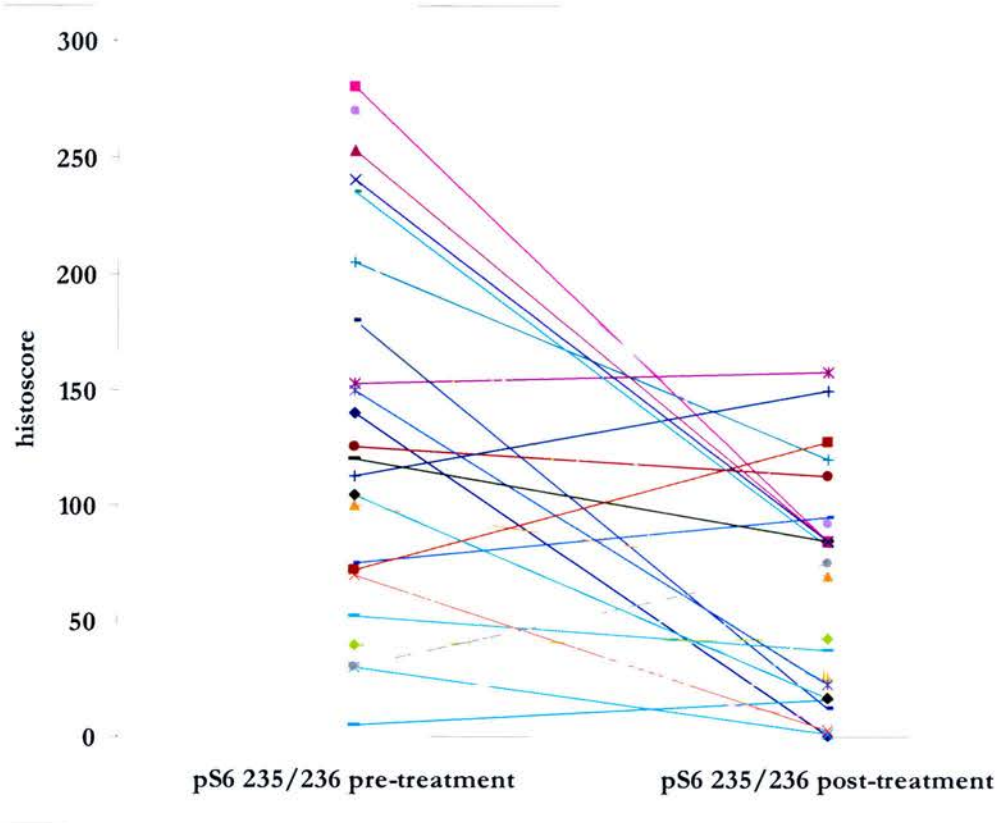


Figure 35
Individual results for p-S6 (ser 235/236) before and after treatment with RAD001 demonstrating a significant reduction in p-S6 with RAD001 treatment ($p<0.0001$).

Patient number	Days of treatment completed	Pre-treatment S6 ser 235/236	Post-treatment S6 ser 235/236	Change in S6 ser 235/236
1	14	140	0.5	-139.5
2	10	280	85	-195
3	14	180	19.5	-160.5
4	14	30	1.5	-28.5
5	14	152.5	157.5	5
6	14	125	112.5	-12.5
7	14	205	120	-85
8	14	180	12.5	-167.5
9	14	5.5	16	10.5
10	14	55	47.5	-7.5
11	14	195	65	-130
12	14	22	22.5	0.5
13	9	80	25	-55
14	14	40	7.5	-32.5
15	14	270	92.5	-177.5
16	14	210	17.5	-192.5
17	14	75	95	20
18	13	52.5	37.5	-15
19	14	40	42.5	2.5
20	14	187.5	26.5	-161
21	14	100	70	-30
22	14	70	3	-67
23	14	150	22.5	-127.5
24	14	30	75	45
25	11	112.5	150	37.5
26	4	235	82.5	-152.5
28	14	120	85	-35
29	14	105	17	-88
30	14	72.5	127.5	55
31	14	252.5	85	-167.5
32	7	240	85	-155

Table 11
Individual values for phospho-S6 ser (235/236) pre and post treatment (responders (highlighted) and non-responders by Ki67)

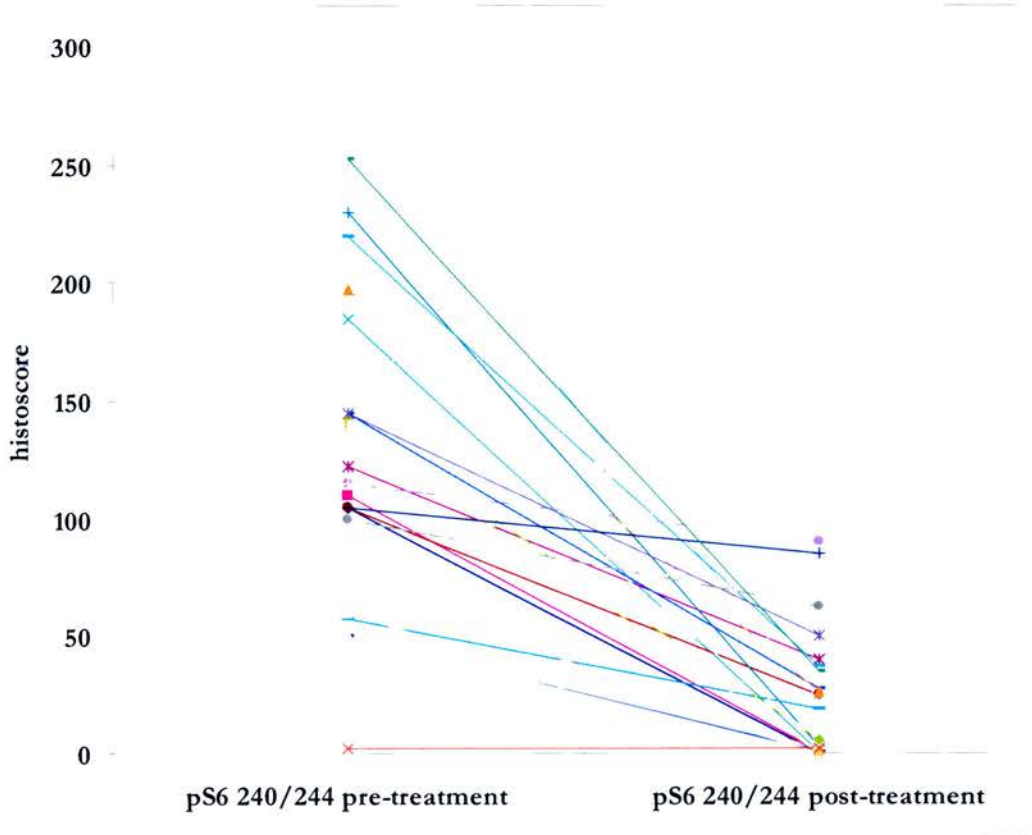


Figure 36
Individual results for p-S6 (ser 240/244) before and after treatment with RAD001 demonstrating significant reduction in S6 with RAD001 treatment ($p<0.0001$).

Patient number	Days of treatment completed	Pre-treatment S6 ser 240/244	Post-treatment S6 ser 240/244	Change in S6 ser 240/244
1	14	105	0	-105
2	10	270	195	-75
3	14	185	0	-185
4	14	110	0.5	-109.5
5	14	185	0.5	-184.5
6	14	122.5	40	-82.5
7	14	105	25	-80
8	14	230	3	-227
9	14	50	1	-49
10	14	57.5	19	-38.5
11	14	175	5	-170
12	14	60	0	-60
13	9	270	5	-265
14	14	50	0	-50
15	14	265	22.5	-242.5
16	14	72.5	1.5	-71
17	14	115	90	-25
18	13	115	57.5	-57.5
19	14	145	27.5	-117.5
20	14	220	37.5	-182.5
21	14	142.5	5.5	-137
22	14	140	0	-140
23	14	197.5	26.5	-171
24	14	2	2.5	0.5
25	11	52.5	35	-17.5
26	4	200	7.5	-192.5
28	14	145	50	-95
29	14	100	62.5	-37.5
30	14	105	85	-20
31	14	252.5	35	-217.5
32	7	157.5	135	-22.5

Table 12
Individual values for phospho-S6 at ser 240/244 pre and post treatment (responders (highlighted) and non-responders by Ki67).

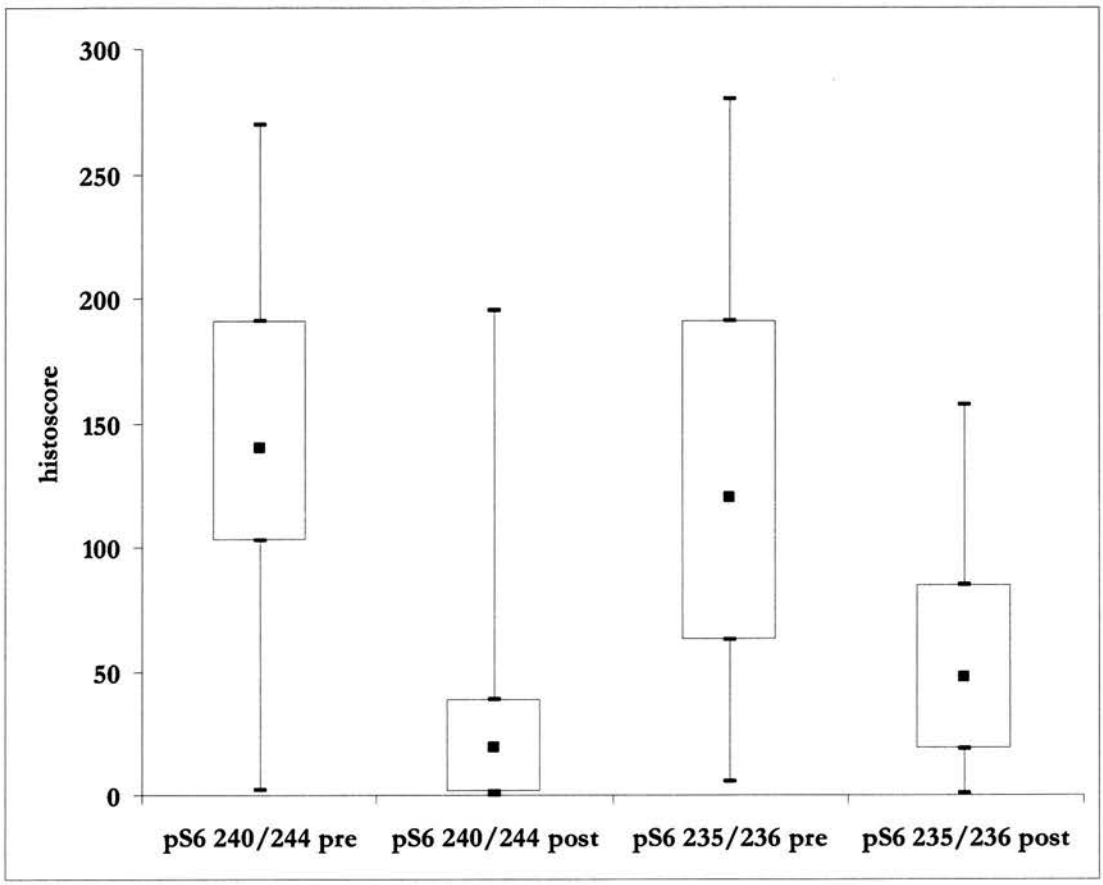
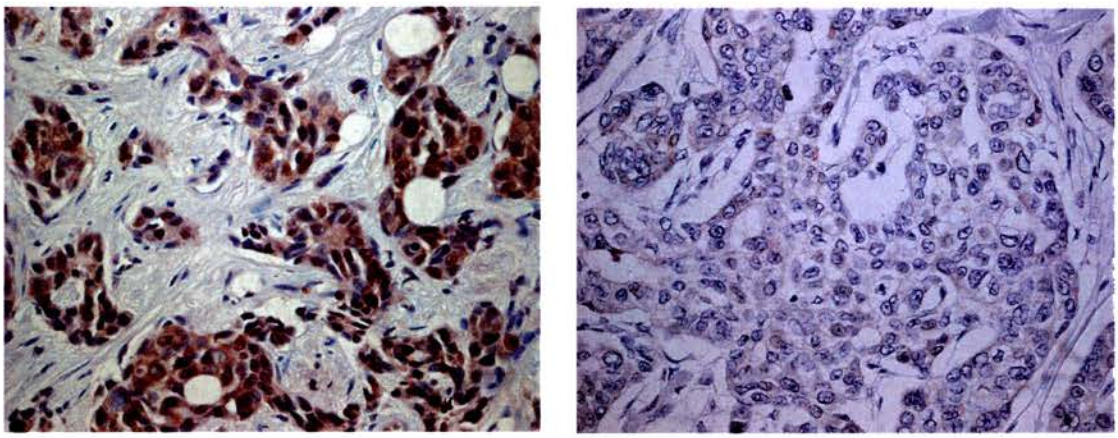


Figure 37
Change in phospho-S6 after 2 weeks treatment with RAD001 for all patients presented as box plots, demonstrating significant reduction for both sites of phosphorylation ($p<0.001$).

phospho-mTOR

There was no significant change in phospho-mTOR (p-mTOR) in the ITT group. **Figure 38; Table 13** There was no significant difference in change in p-mTOR with RAD001 treatment between tumours that had high scores for p-mTOR pre-treatment and those with low p-mTOR. Pre-treatment levels of p-mTOR did not correlate with either Ki67 at baseline or change in Ki67 with treatment. There was however evidence for a relationship between the Ki67 pre-treatment and the change in p-mTOR with treatment (r 0.43, p=0.0168). These data suggest that tumours that were highly proliferative had a decrease in phosphorylation of mTOR after RAD001 treatment. **Figure 39** There was no difference in scores at baseline, nor change in p-mTOR between responders and non-responders.

Multivariable regression analysis was performed to determine prevariables that may influence the change in Ki67. From this pre-treatment mTOR as a predictor for change in Ki67 (R-squared adjusted 16.1%, p=0.088) was not significant.



(a) (b)

Figure 38
Example of staining of p-mTOR pre- (a) and post-(b) treatment with RAD001.

Patient number	Days of treatment completed	Pre-treatment phospho-mTOR	Post-treatment phospho-mTOR	Change in phospho-mTOR
1	14	127.5	55	-72.5
2	10	135	255	120
3	14	255	212.5	-42.5
4	14	57.5	215	157.5
5	14	170	115	-55
6	14	155	75	-80
7	14	180	130	-50
8	14	140	175	35
9	14	105	115	10
10	14	190	300	110
11	14	200	135	-65
12	14	85	122.5	37.5
13	9	62.5	20	-42.5
14	14	160	245	85
15	14	122.5	35	-87.5
16	14	110	52.5	-57.5
17	14	120	112.5	-7.5
18	13	185	55	-130
19	14	240	75	-165
20	14	145	130	-15
21	14	145	25	-120
22	14	135	120	-15
23	14	140	177.5	37.5
24	14	105	210	105
25	11	135	225	90
26	4	75	245	170
28	14	235	220	-15
29	14	75	52.5	-22.5
30	14	100	230	130
31	14	90	160	70
32	7	170	280	110

Table 13
Individual histoscore values for phospho-mTOR pre and post treatment (responders (highlighted) and non-responders by Ki67).

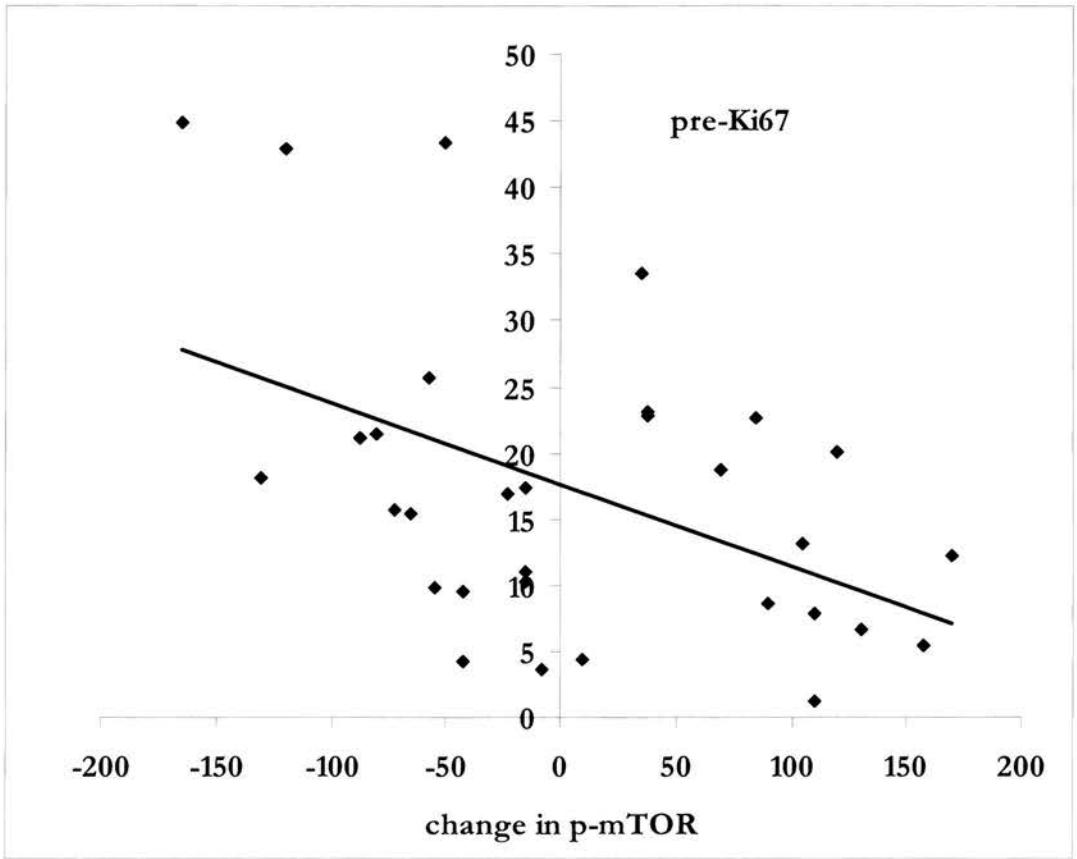


Figure 39
Correlation between p-mTOR change with treatment and pre-treatment Ki67% showing to the left a reduction in p-mTOR with RAD001 treatment in those with high pre-treatment Ki67, but to the right an increase in p-mTOR in those with low pre-treatment Ki67 (r=0.43, p=0.0168).

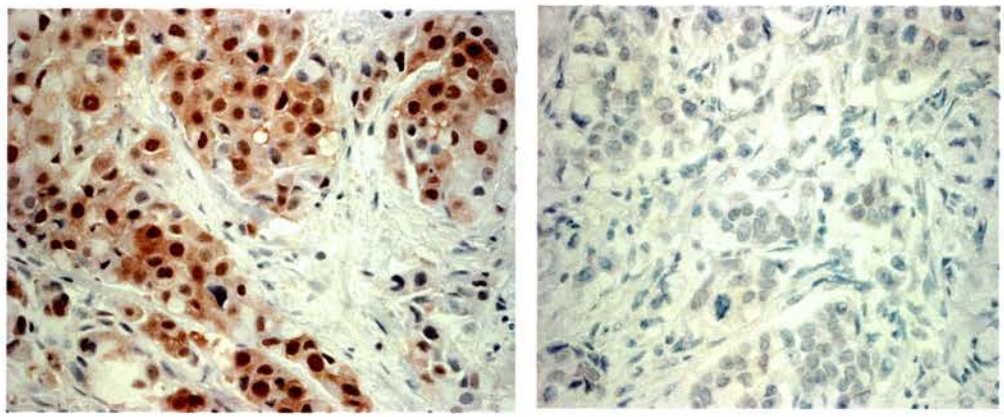
phospho-Akt

Staining for p-Akt was both nuclear and cytoplasmic, and separate scores were calculated for both types of staining. **Figure 40** There was a significant reduction in nuclear phosphorylation of Akt (mean fall 20.9, 95%CI 5.02, 36.79, p=0.012) with RAD001 treatment. **Figure 41** In those that had any nuclear staining before treatment (n=18), only 5 had residual nuclear staining for p-Akt after treatment with RAD001. **Table 14** Those with high pre-treatment p-Akt nuclear staining had a significantly greater fall in nuclear p-Akt with RAD001 (p=0.0021). There was no correlation between pre-treatment p-Akt nuclear staining and Ki67 at baseline or after treatment. Change in nuclear p-Akt did not correlate with Ki67 at baseline nor change in Ki67. There was no difference in either baseline or change in nuclear p-Akt between responders and non-responders to RAD001 treatment.

There was no significant change in levels of phosphorylation of cytoplasmic Akt pre and post treatment with RAD001. **Table 15** Patients with a pre-treatment score above the median (70) fell by an average of 58.6 (95% CI 25.0, 92.1) with treatment, while the p-Akt scores of patients with pre-treatment p-Akt below median 70 had no significant change with treatment (mean rise 12.87, 95% CI fall of 21.7 to rise of 47.5, NS). Thus those with high pre-treatment p-Akt had a significant fall in p-Akt with treatment, and those with low pre-treatment scores had no change. The change between the two groups was significant (p=0.0051). There was a correlation between high pre-treatment Ki67 and high pre-treatment cytoplasmic p-Akt (r=0.45, p=0.0105), suggesting that highly proliferative tumours have high cytoplasmic phosphorylation of Akt. **Figure 42** There was also a correlation between the change in Ki67 and change in cytoplasmic p-Akt with treatment (r=0.45, p=0.0106),

suggesting that these tumours are those that have the greatest reduction in Ki67, with evidence to suggest a correlation between pre-treatment Ki67 and the change in p-Akt ($r=0.35$, $p=0.0505$). However in the multivariable analysis, pre-treatment cytoplasmic p-Akt was not identified as a predictor for change in Ki67. There was no difference between those with high cytoplasmic p-Akt and low cytoplasmic p-Akt (data split about the median) in terms of the change in Ki67.

There was a significant reduction in cytoplasmic p-Akt in those tumours that were classified as responding to RAD001 (mean fall 52, 95%CI 19.7, 84.4) compared with those who did not respond, in which there was no significant change (mean rise 14.8, 95%CI fall 23.2, rise 52.9). The difference between the groups was significant ($p=0.0105$). **Figure 43**



(a) **(b)**

Figure 40
Example of staining for p-Akt showing nuclear and cytoplasmic staining pre- (a) and post- (b) treatment with RAD001.

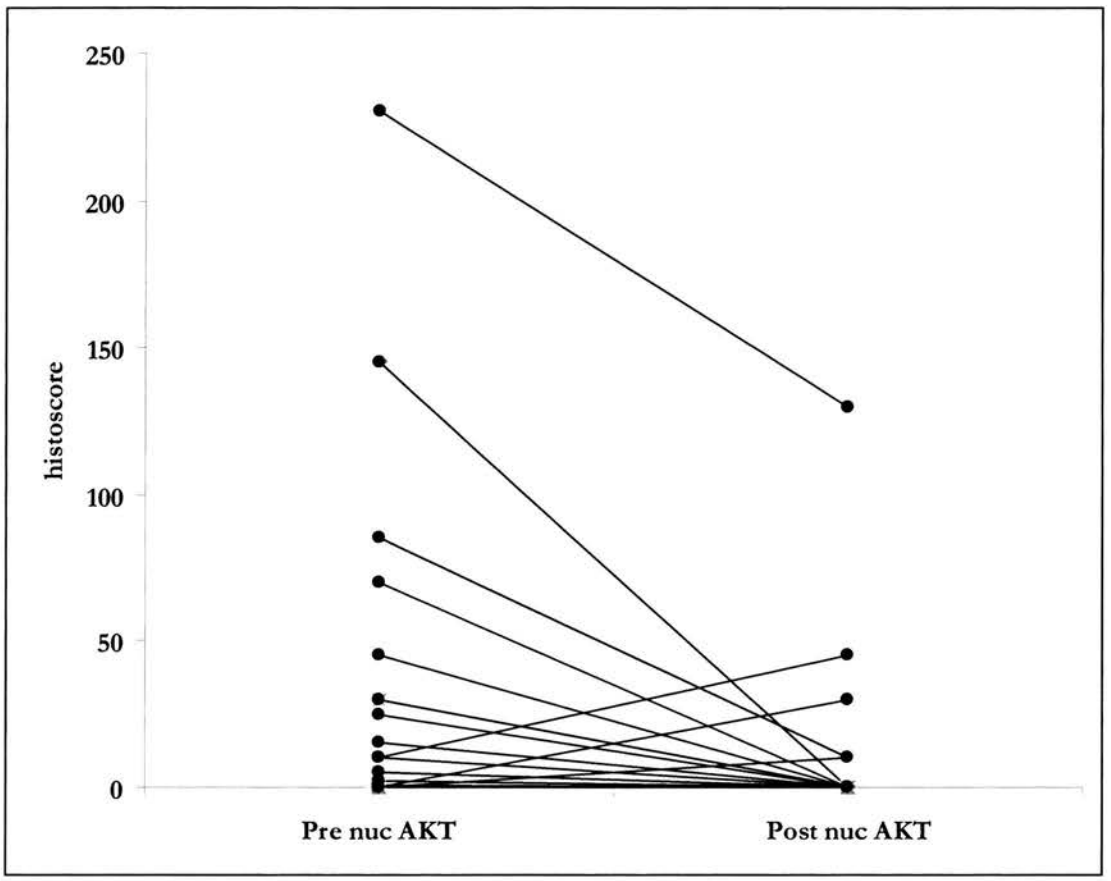


Figure 41
Histoscore results for nuclear p-Akt before and after RAD001, showing a significant reduction in scores after treatment (p=0.012).

Patient number	Days of treatment completed	Pre-treatment nuclear p-Akt	Post-treatment nuclear p-Akt	Change in nuclear p-Akt
1	14	0	0	0
2	10	230	130	-100
3	14	5	0	-5
4	14	5	0	-5
5	14	1	0	-1
6	14	0	0	0
7	14	0	0	0
8	14	145	0	-145
9	14	0	0	0
10	14	70	0	-70
11	14	0	0	0
12	14	0	10	10
13	9	145	0	-145
14	14	30	0	-30
15	14	10	0	-10
16	14	10	0	-10
17	14	0	30	30
18	13	0	0	0
19	14	0	0	0
20	14	0	0	0
21	14	45	0	-45
22	14	0	0	0
23	14	85	10	-75
24	14	10	0	-10
25	11	0	0	0
26	4	10	45	35
28	14	25	0	-25
29	14	2	0	-2
30	14	15	0	-15
31	14	30	0	-30
32	7	0	0	0

Table 14
Individual histoscores for nuclear phospho-Akt pre and post treatment (responders (highlighted) and non-responders by Ki67).

Patient number	Days of treatment completed	Pre-treatment cytoplasmic -Akt	Post-treatment cytoplasmic p-Akt	Change in cytoplasmic p-Akt
1	14	27.5	5	-22.5
2	10	175	100	-75
3	14	215	170	-45
4	14	10	1	-9
5	14	35	70	35
6	14	125	195	70
7	14	45	155	110
8	14	115	20.5	-94.5
9	14	2.5	10	7.5
10	14	57.5	50	-7.5
11	14	29	7.5	-21.5
12	14	60	50	-10
13	9	45	7.5	-37.5
14	14	115	7.5	-107.5
15	14	95	1.5	-93.5
16	14	24.5	25	0.5
17	14	40	122.5	82.5
18	13	172.5	102.5	-70
19	14	205	83	-122
20	14	24	110	86
21	14	235	92.5	-142.5
22	14	137.5	0	-137.5
23	14	145	90	-55
24	14	210	55	-155
25	11	2.5	45	42.5
26	4	112.5	57.5	-55
28	14	98	182.5	84.5
29	14	55	0	-55
30	14	62.5	54.5	-8
31	14	79	50	-29
32	7	70	160	90

Table 15
Individual histoscores for cytoplasmic phospho-Akt pre and post treatment (responders (highlighted) and non-responders by Ki67).

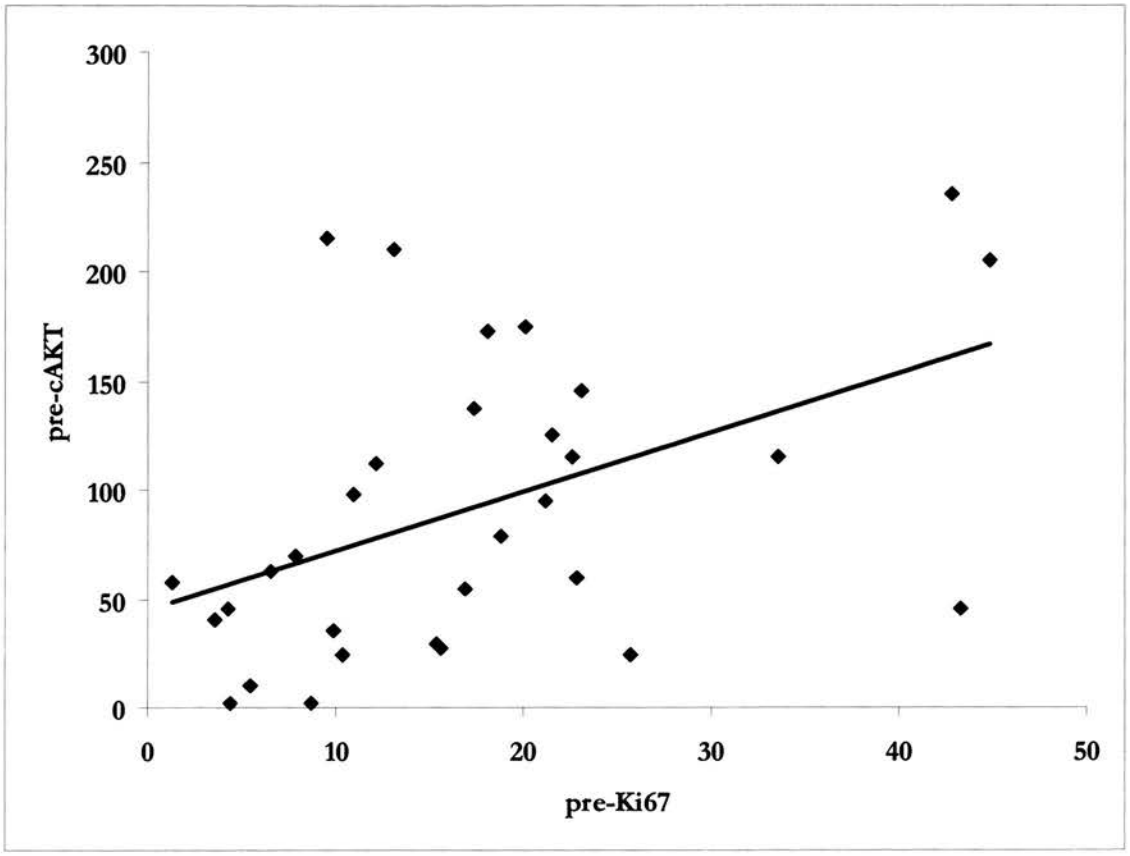


Figure 42
Correlation between pre-treatment scores for cytoplasmic p-Akt and proliferation counts, demonstrating that tumours high in p-Akt tended to also be highly proliferative (r=0.45, p=0.0105).

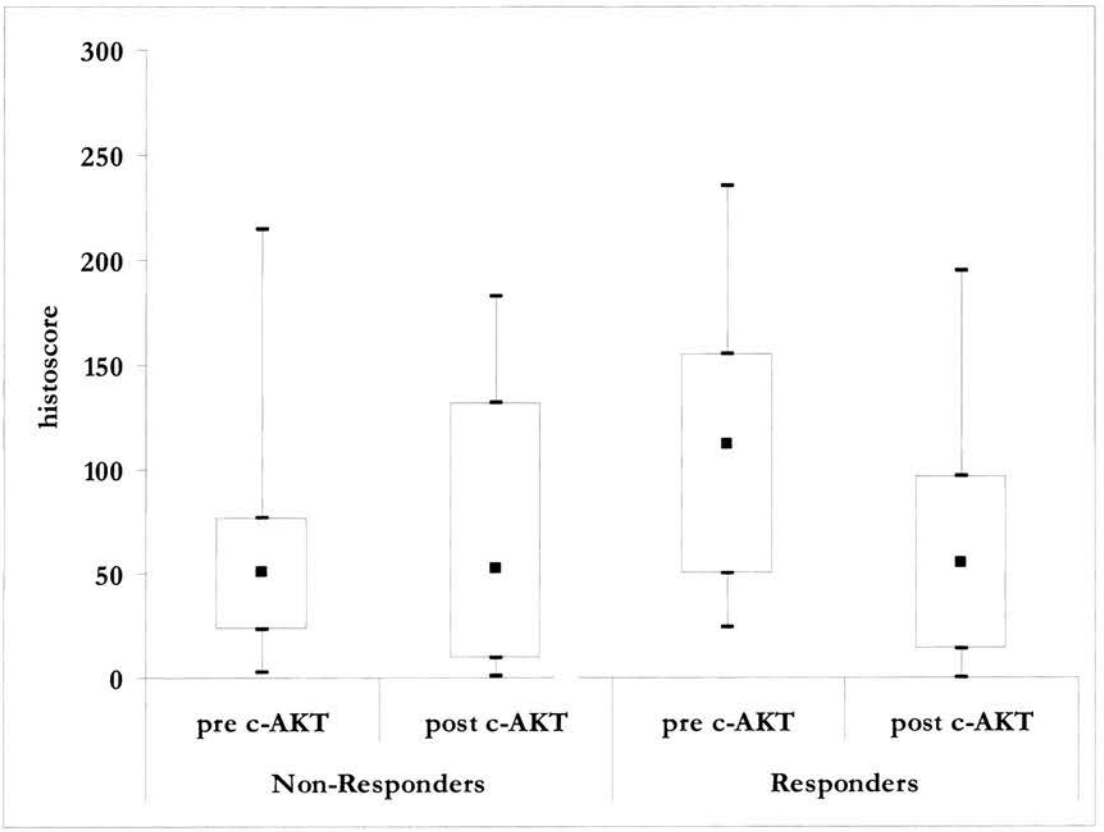


Figure 43
Change in cytoplasmic p-Akt after 2 weeks treatment with RAD001, divided into non-responders (left) and responders (right), demonstrating a significant difference between the two groups, with responding tumours to RAD001 having a significant reduction in score after treatment (p=0.0105), but no change in those that did not respond to RAD001.

Apoptosis

There was no significant change in levels of apoptosis measured by active caspase-3 with RAD001 treatment for the ITT population (median pre-treatment 0.6%; 95%CI 0.46, 0.81; median post-treatment 0.22%; 95%CI 0.15, 0.68; p=NS). **Figure 44; Table 16** In those tumours that had high pre-treatment Ki67 there was evidence to suggest an increase in apoptosis with RAD001 treatment (r=0.37, p=0.04). **Figure 45** There was no significant difference between responders and non-responders for pre-treatment levels of apoptosis or change in apoptosis with treatment.

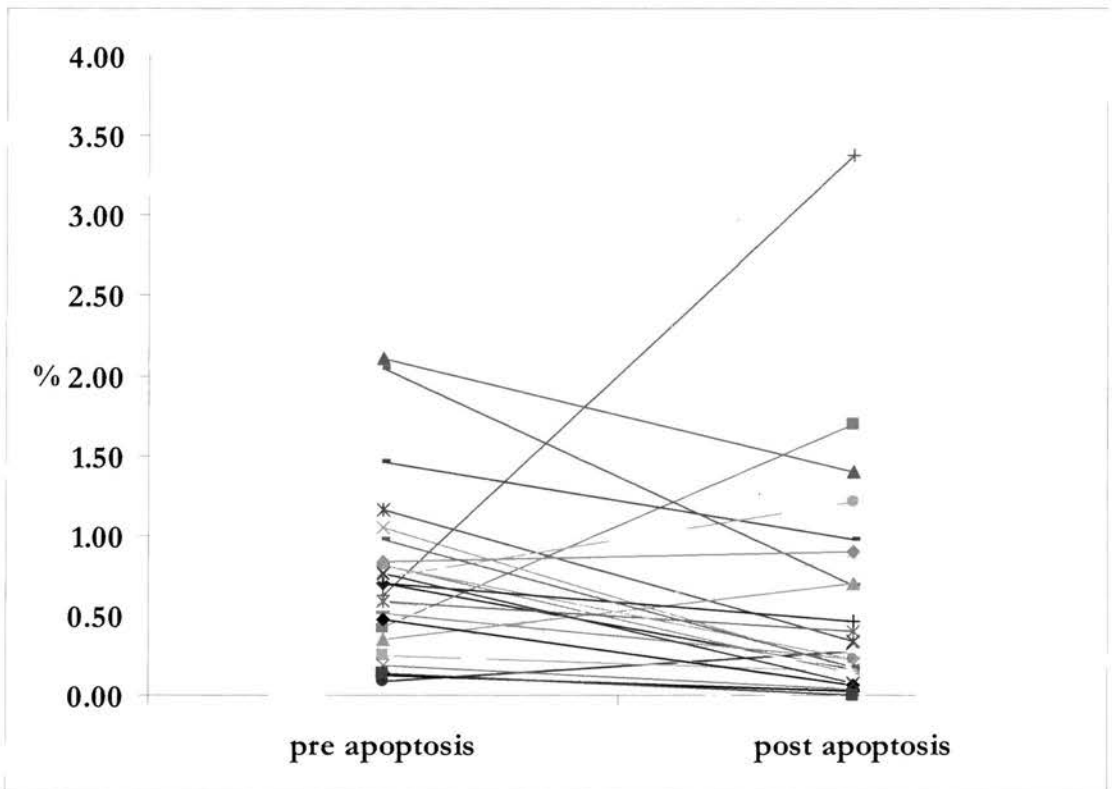


Figure 44
Individual results for apoptosis (caspase-3) before and after treatment with RAD001 (p=NS)

Patient number	Days of treatment completed	Pre-treatment apoptosis	Post-treatment apoptosis	Change in apoptosis
1	14	0.70	0.17	-0.53
2	10	0.42	1.69	1.27
3	14	0.60	0.03	-0.57
4	14	1.05	0.16	-0.89
5	14	1.16	0.34	-0.82
6	14	0.09	0.27	0.19
7	14	0.62	3.37	2.75
8	14	1.46	0.98	-0.48
9	14	0.81	0.14	-0.67
10	14	0.34	0.27	-0.07
11	14	0.48	0.06	-0.42
12	14	0.54	0.02	-0.52
13	9	0.26	0.87	0.61
14	14	2.60	2.12	-0.48
15	14	0.73	1.20	0.47
16	14	0.58	0.17	-0.41
17	14	2.04	0.68	-1.36
18	13	0.51	0.22	-0.29
19	14	0.83	0.90	0.07
20	14	0.25	0.16	-0.09
21	14	0.35	0.70	0.35
22	14	0.19	0.03	-0.16
23	14	0.58	0.40	-0.18
24	14	0.81	0.22	-0.59
25	11	0.70	0.46	-0.24
26	4	0.97	0.18	-0.79
28	14	0.13	0.03	-0.10
29	14	0.47	0.06	-0.41
30	14	0.14	0.00	-0.14
31	14	2.10	1.39	-0.71
32	7	0.76	0.08	-0.68

Table 16
Individual values for apoptosis (%) pre and post treatment (responders (highlighted) and non-responders by Ki67).

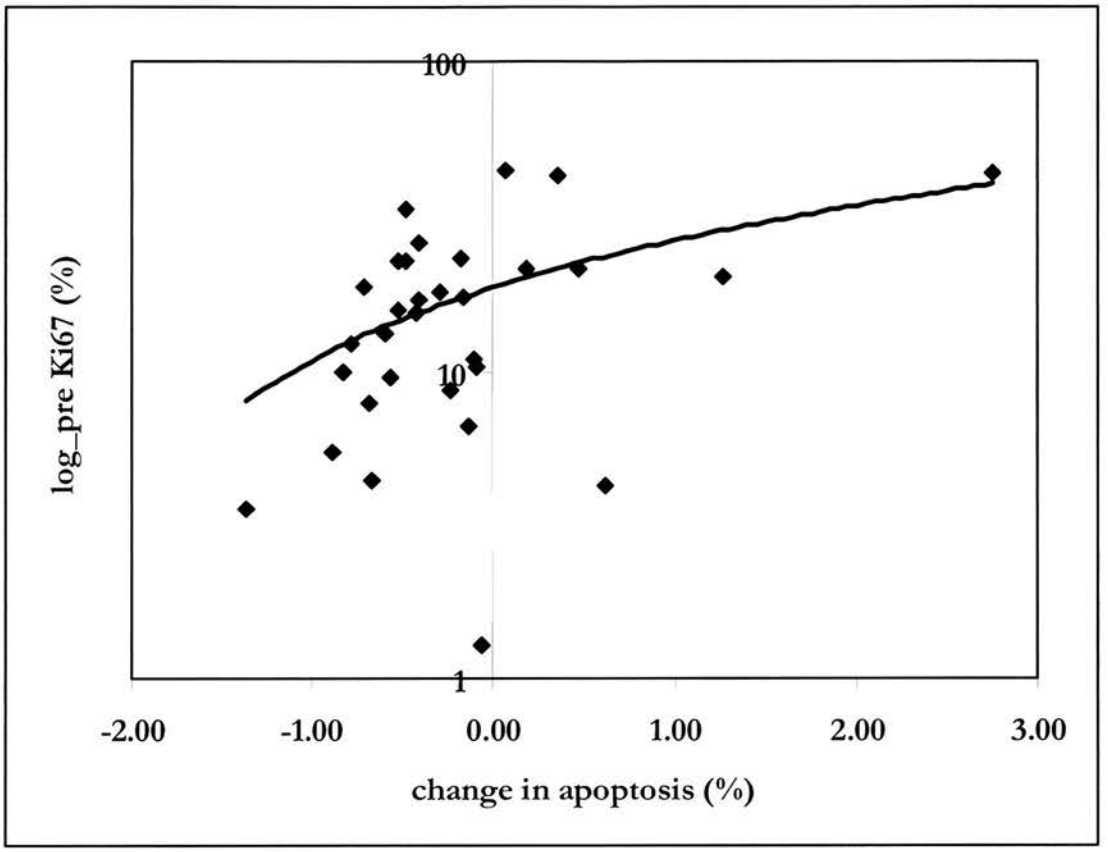


Figure 45
Correlation between pre-treatment proliferation (Ki67%) and change in apoptosis by caspase-(%) with RAD001 treatment suggesting that those tumours with higher pre-treatment proliferation had a greater increase in apoptosis with RAD001 treatment (r= 0.37; p=0.04).

phospho-PTEN

At the time of completion of this thesis, several attempts had been made to optimise the immunohistochemical protocol for phospho-PTEN without success. No results are available for the PTEN status of the tumours in this study. The results of a study of PI3KCA mutation status of the tumours from this RAD001 study but not carried out directly by the candidate will be discussed below.

Discussion

The primary outcome measured in this pre-operative study was the change in proliferation with treatment with RAD001 as measured by Ki67. There was a significant reduction in proliferation with up to 14 days treatment with RAD001. Those that had the highest Ki67 pre-treatment had the greatest reduction in proliferation, which may be expected as those tumours have the greatest potential fall, but the log transformed data suggest a significant absolute fall in response to RAD001 treatment in the highly proliferative tumours in this study group. These highly proliferative tumours were also those that had the greatest increase in apoptosis with RAD001 treatment, and also tended to be ER negative and of higher tumour grade. No correlation was found between proliferation and HER-2 positivity. The measurement of Ki67 has attracted interest recently as a possible predictor of response to endocrine therapy. [117] Previous pre-operative studies performed in Edinburgh have used Ki67 as an outcome measure to allow comparison between agents and the individual efficacy of agents as anti-proliferative agents. [56, 203] From a study of letrozole and anastrozole given for 2 weeks pre-operatively there was a median reduction in Ki67 of 5.8%. [203] The median Ki67 pre-treatment was 6.68%, which was lower than the pre-treatment median for the patients in this RAD001 study (18.5%), which might in part account for the absolute fall in Ki67 by 8.5% with RAD001 treatment. In the letrozole and anastrozole study the tumours were all ER positive and were smaller, mainly screen detected cancers.

Of note from the above results for proliferation, there was one tumour (patient number 17) that had an unusual change in proliferation, with a rise in Ki67 from 3.60 % to 47.20%. The immunohistochemical staining was repeated on both samples and

rescored with similar results. It was noted that there was a large proportion of inflammatory cells in the excision specimen. The pathology of this tumour was ER+/PR-/HER-2 negative invasive ductal carcinoma (no specific type), grade 3 on both core and excision specimens, with a whole tumour size 35mm, lymphovascular invasion and 15 of 21 lymph nodes positive after mastectomy and axillary clearance (NPI 6.7). Results for the other markers for this patient's tumour in response to RAD001 reveal a significant reduction in ER score (histoscore pre 230, post 15), no change in PR, increase in cytoplasmic p-Akt (pre 40, post 122.5), increase in nuclear p-Akt (pre 0, post 30), no reduction in p-S6 (s240/244 pre 115, post 90; s235/236 pre 75, post 95) and no change in p-mTOR (pre 120, post 112.5). The above combination of results would represent a tumour that has not had inhibition of mTOR and that rather than just maintain its rate of proliferation has dramatically increased its rate of proliferation. This patient was not noted to have clinical evidence of an inflammatory cancer but the findings of a significant increase in proliferation in this particular tumour is concerning as it raises the possibility that there are tumour types that are resistant to mTOR inhibition, inflammatory tumours in particular. This was the only tumour noted to have such a lack of response to RAD001. This tumour was HER-2 negative, and it has previously been found with a different mTOR inhibitor, CCI-779, in metastatic breast cancer that there were no responses seen in HER-2 negative tumours. [184] In this RAD001 study, there appeared to be a greater response in HER-2 positive tumours (87.5% of HER-2 positive tumours were responders by Ki67%) than in HER-2 negative tumours (47.8% responders), although responses were seen in both HER-2 positive and negative tumours, in comparison to the findings with CCI-779.

In this RAD001 study those tumours with a high proliferation rate before treatment were also found to have high levels of cytoplasmic phospho-Akt before treatment. This would support previous findings that poor prognosis or resistant cancers tend to have higher proliferation rates, and both greater expression of p-Akt and activity of mTOR. [147] The above results reinforce previous findings from studies in ovarian cancer in which RAD001 reduced proliferation in mice with advanced malignancy and in combination with cisplatin there was enhanced inhibition of proliferation particularly in those with high p-Akt and mTOR signalling pre-treatment. [178, 204] High pre-treatment levels of Ki67 significantly influenced the change in Ki67 with treatment, but no other variables were found to predict strongly for response to treatment with RAD001 as assessed by reduction in proliferation. All tumours had a reduction in S6 phosphorylation independent of levels of proliferation. The correlation between high proliferation pre-treatment and change in cytoplasmic p-Akt did not reach significance but there was a significant difference in tumours that responded to RAD001 (as assessed by Ki67) with a reduction in cytoplasmic p-Akt compared with those that did not have response to RAD001.

Previous studies assessing levels of the downstream activity of RAD001 on the mTOR pathway have most commonly used phospho-p70S6 kinase (either one of two isoforms) antibodies as an outcome measure. It has since been shown that the most commonly used commercially available antibody for p-p70S6kinase is not phospho-specific to allow accurate immunohistochemistry assessments, thus making previous results difficult to interpret. For this study it was decided to assess the cytoplasmic levels of the ribosomal protein S6 (S6) that is phosphorylated by the p70S6kinase, which was considered to directly reflect the level of activity. At both sites of

phosphorylation there was a clear reduction in phosphorylation of S6, this being more marked at sites 240/244, indicating reduction of activity of the downstream effects of mTOR with treatment with RAD001. The extent of reduction at either site did not correlate with either the reduction in Ki67 or with the changes in p-Akt nor p-mTOR.

While there was no significant change in phosphorylation of mTOR (s2448) overall, there was some evidence to suggest that those tumours that had a high Ki67 pre-treatment were more likely to have an decrease in p-mTOR after RAD001 treatment. There is debate from published data as to the mechanisms behind phosphorylation of mTOR at ser2448, but the most recent data would suggest that ser2448 is predominantly a site for mTORC1 and ser 2481 for TORC2, with p-S6 functioning as the kinase for ser2448. [167] In this case one would expect some correlation between S6 activity and mTOR activity but no such relationship could be identified from this data. It appears to be tumours that are highly proliferative that have the greatest reductions in proliferation, rise in apoptosis and reduction in p-mTOR, implying that these are the tumours that will best respond to RAD001.

There was a significant reduction in ER scores with RAD001 treatment, with a significantly greater fall in ER in those tumours that were lower in ER pre-treatment. This downregulation in ER-poor tumours may be of biological significance in the study of endocrine resistance. As discussed earlier, current understanding of resistance to endocrine therapy is of a cross-talk between signal transduction pathways such as mTOR and an ER signalling shift between nuclear and peri-nuclear activity. [119, 144, 205-207] Further discussion of the role of mTOR inhibitors in combination with endocrine agents and results from clinical trials will follow. The

concern regarding the reduction in ER is highlighted by results from the IMPACT study that had low ER scores after 2 weeks was predictive of poorer recurrence-free survival at median 37 months follow-up (HR 0.78; 95%CI 0.62, 0.99; p=0.04), but these results were for absolute ER value at 2 weeks rather than a reduction from baseline, and may therefore be indicative of the known intrinsic value of ER for response to aromatase inhibitors rather than the effect of reducing ER. [117] No such predictive value was seen in the Edinburgh study of neoadjuvant letrozole for ER and pathological or clinical response, with no significant change in ER seen with treatment. [118] What is not clear is the mechanism by which RAD001 reduces ER expression, and there is thus a possibility that the reduction in ER expression may render tumours less sensitive to the effects of aromatase inhibition.

Current understanding of the mTOR pathway and the two distinct complexes formed by mTOR with raptor (mTORC1) and rictor (mTORC2) has been based upon work with rapamycin, hence the naming of raptor (rapamycin associated companion to mTOR) and rictor (rapamycin insensitive companion to mTOR). One of the aims of this study was to determine the action of RAD001 upon mTOR and whether rictor is indeed insensitive to rapamycin-like mTOR inhibitors in vivo. The result that would be anticipated from understanding of the current literature is that if one is inhibiting mTOR raptor only one would expect downregulation of mTOR, p-S6 and upregulation of Akt by negative feedback. The negative associations of upregulation of Akt include resistance to apoptosis, increased cell growth and proliferation, increased resistance to endocrine treatment and increased risk of metastasis and reduced survival. [146, 147, 175, 176] If however, an mTOR inhibitor inhibits both mTOR

raptor and mTOR rictor then one would expect downregulation of mTOR and p-S6k, and also downregulation of p-Akt.

In this study it was found that in all tumours there was a significant reduction in nuclear p-Akt after treatment with RAD001, indicating that both raptor and rictor complexes of mTOR are inhibited. Similarly, tumours with high cytoplasmic p-Akt pre-treatment had a reduction in p-Akt in the cytoplasm after treatment with RAD001. However, tumours with a low p-Akt cytoplasmic score treated with RAD001 had no change or an increase in p-Akt cytoplasmic score with RAD001 treatment, indicating that tumours without high p-Akt may respond less well to RAD001. In those tumours that responded to RAD001 with a reduction in Ki67 there was a significant decrease in cytoplasmic p-Akt with treatment, indicating that in tumours where RAD001 is effective at inhibiting the mTOR pathway, both complexes of mTOR are inhibited.

These results are in keeping with a recent study of RAD001 and CCI-779 in acute myeloid leukaemia (AML), in which blood samples from patients treated with CCI-779 demonstrated reduction in mTORC1 and mTORC2 activity, and levels of p-Akt (ser 473) were reduced with both CCI-779 and RAD001. [208]

It was disappointing to be unable to ascertain the PTEN status of these tumours due to problems with the immunohistochemical staining methodology. This information would have allowed further interpretation of the influences acting upon the mTOR pathway. PTEN acts as a brake upon the activity of Akt and thus mTOR and the hypothesis is that patients who have PTEN deficient tumours are more likely to belong to the group who had higher Akt, mTOR and Ki67, and thus be more responsive to the activity of an mTOR inhibitor. One study assessed the

antiproliferative activity of RAD001 in tumour cell lines with varying expression of PTEN and found that while there was a trend towards increased sensitivity to RAD001 in PTEN null glioblastoma cell lines compared with PTEN expressing cell lines, no such trend was observed in breast or prostate cell lines. [209] In the same study however, there was evidence to suggest an association between higher p-Akt levels and the antiproliferative response to RAD001, as found herein.

Further work has been carried out on the paraffin sections available from this study to characterise the upstream influences of the PTEN pathway upon tumour response to RAD001. [210] The aim was to assess the *PI3KCA* mutational status, as described in a significant proportion of breast cancers. DNA was extracted from the post-treatment paraffin-embedded tissues and analysed for the 3 most commonly occurring *PIK3CA* mutations (H1047R, E542K, E545K) using a multiplex QPCR assay using methods previously described. [211] Eight of 31 (25.8%) samples were found to contain *PI3KCA* mutations: 5 - E545K; 2 - H1047R; 1 – E542K. Although cytoplasmic p-Akt scores were higher in pre-treatment tumours containing *PI3KCA* mutations than in those without mutations (median histoscores - 113.8 and 70, respectively), the difference was not statistically significant. Five mutations were found in tumours from 25 patients who completed treatment of which 15 patients were classified as responders to RAD001 and 10 patients as non-responders (responders were defined by a fall in % Ki-67 positive cells). 2/15 (13%) responders and 3/10 (30%) non-responders exhibited *PI3KCA* mutations. However, no significant association between *PI3KCA* mutations and response was observed in this study.

Transcriptional profiling study

Aims

The aim of this transcriptional profiling study was to characterise the effects of pre-operative RAD001 treatment in primary breast cancer, and to determine whether these effects correlated with those found in the immunohistochemical analysis of fixed tumour samples from the same patient tumours, and if there is a potential molecular profile of tumours that respond to RAD001.

Methods

During the clinical phase of this study, fresh frozen tumour biopsies before and after RAD001 treatment were collected and stored in liquid nitrogen. The results discussed below have been presented at the San Antonio Breast Cancer Symposium. [212] Frozen sections were removed, sectioned and stained with haematoxylin and eosin to assess tumour content. Approximately 100mg tissue from pre- and post-treatment biopsies from 27 patients who completed RAD001 treatment were placed in RNAlater® ICE (Ambion, Warrington, United Kingdom) overnight at -20°C prior to RNA extraction.

RNA was extracted using the RNeasy Mini Kit, including RNase-Free DNase treatment (Qiagen), amplified using Illumina® TotalPrep RNA Amplification Kit (Ambion), and analysed in duplicate using Illumina Human Ref.8 chip v2. Stratagene Universal Reference RNA (UHRR) was added to each chip to enable inter-run variation to be assessed. Gene expression changes were compared before and after RAD001 treatment between the responders and non-responders using Bioconductor programmes [213] implemented in the R statistical programming language. [214] Illumina probe profile expression data were normalised by quartile normalisation within the

beadarray package [215] and corrected for batch processing effects using the combat tool. [216] Differentially expressed genes and false discovery rate (FDR) were determined using 'Significance Analysis of Microarrays' (SAM) implemented within the siggenes package. [217] An alternative approach used to look at the effect of RAD001 was to compare the relative change in gene expression of each probe calculated between the duplicate pre- and post-treatment samples and find the mean of the four values. Centred average linkage clustering was performed using the Cluster [218] and TreeView programs. [219]

RNA was available from 21 matched biopsies and 23 pre-treatment samples. Patients were classified as responders to RAD001 by a significant (95% CI) fall in %Ki67 as discussed previously (n=13 matched; n=14 pre-treatment), or by a significant (95% CI) fall in cytoplasmic p-Akt positive cells (n=7 matched; n=7 pre-treatment). The remaining patients were considered as non-responders.

Results

Effect of RAD001 treatment on gene expression

Duplicate samples were highly consistent although subtle variations were noted. Viewing the differences in the overall transcriptome before and after RAD001 treatment using multidimensional scaling, the responders tend to group together suggesting a difference between responders and non-responders. **Figure 46** The majority of the post-treatment paired samples (n=21) are to the right of the pre-treatment samples suggesting some consistency in the response to RAD001. Of the top 500 most significantly changed probes following treatment in each set of replicates, there were 220 probes in common (intersect of top 500, false discovery rate (FDR) 15%, **Figure 47**). Of the 13 responders with significant reductions in proliferation, there were 203 genes which demonstrated a consistent change (FDR 14%), with those genes representing cell cycle (e.g. CDK2, CDC2) significantly changed with RAD001 treatment. In the non-responders, there was a lack of consistency in change in gene expression (FDR 71%). Similarly, when grouped for response according to p-Akt change (n=7), there was a consistent change in gene expression of 121 genes in responders (FDR 17%), but a lack of consistent change in non-responders (FDR 57%). The functional (KEGG) pathways determined to be most associated with RAD001 treatment when all tumours were analysed together were the complement and coagulation cascades ($p = 0.002$; predicted FDR = 1.99), demonstrating the impact of mTOR inhibition on the cell cycle and also upon the immune system. RAD001 is currently licensed for use in transplant immunosuppression. This is also in agreement with the clinical study findings of significant reductions in white cells, neutrophils and platelets.

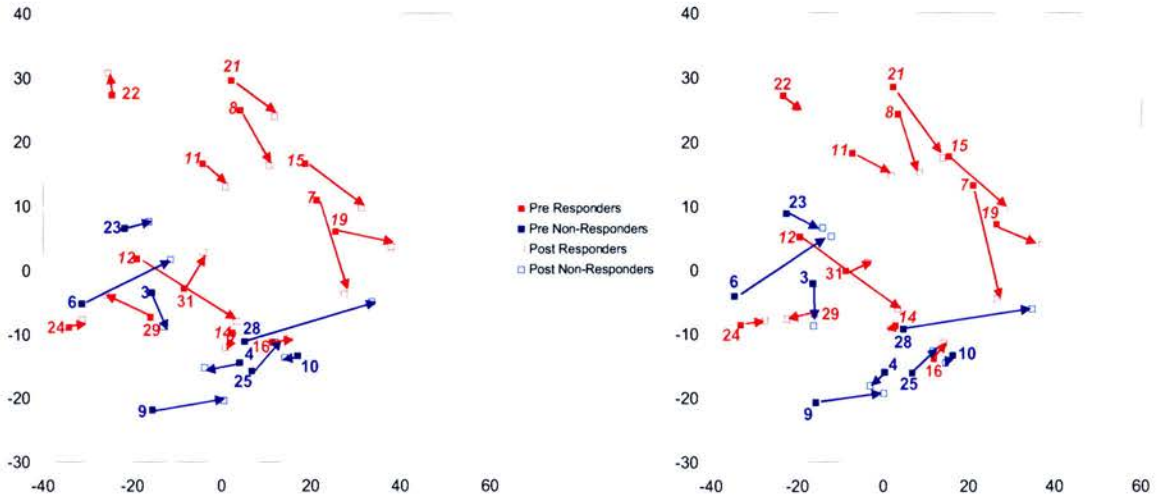


Figure 46
Differences in overall transciptome before and after RAD001 showing consistency of duplicates, and post-treatment samples generally to the right of the pre-treatment (response defined by Ki67%). Numbers represent sample number.

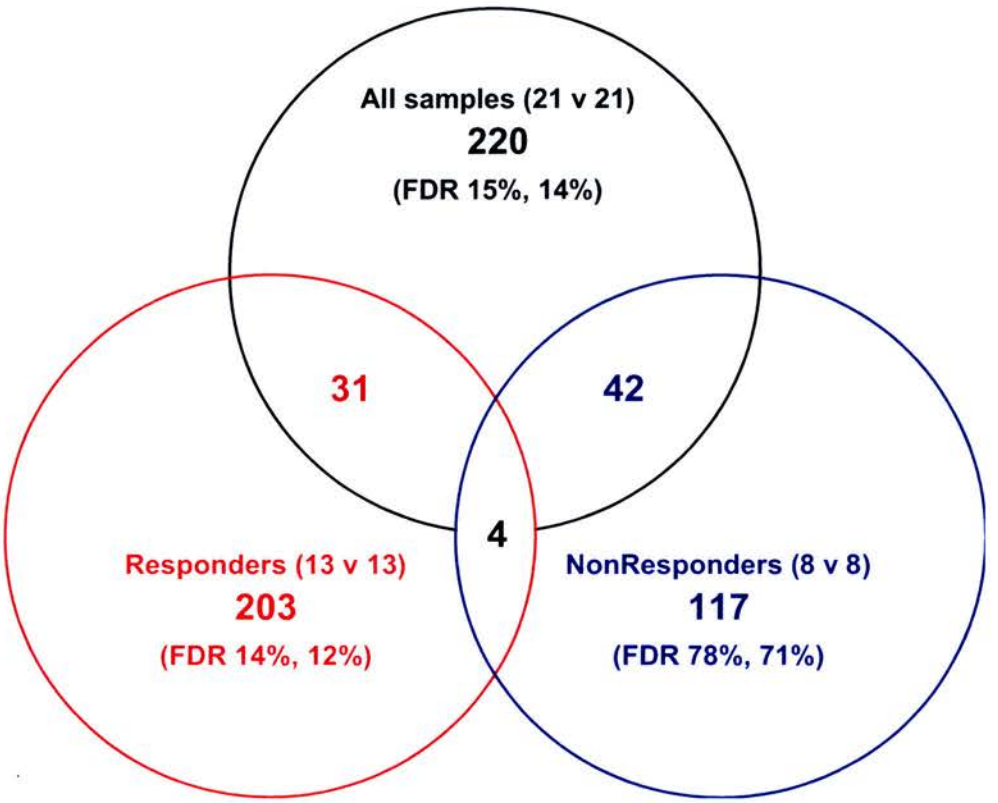


Figure 47
Common differentially expressed genes following RAD001 treatment (intersect of top 500) in all responding and non-responding tumours (by Ki67%). (FDR = false discovery rate).

Effect of RAD001 treatment on mTOR pathway, proliferative genes and oestrogen dependent gene expression

Many cell cycle genes eg. CDK2 (cyclin-dependent kinase 2) and CDC2 (cell division cycle 2, G1 to S and G2 to M) were significantly differentially expressed following RAD001 treatment. **Figure 48** CDC2, expression of which appears to be down-regulated in the responding tumours, has also been reported to be decreased in B-cell lymphoma cell lines and pancreatic cells after rapamycin treatment. [220, 221] There was a significant reduction in expression of proliferative genes after RAD001 treatment in those that had responded to RAD001, which may in part be explained by the definition of response based upon reduction in proliferation, but as with the immunohistochemical (IHC) finding, there was greater reduction in proliferation genes in those that were highly proliferative pre-treatment. There was a significant correlation between the change in expression of cyclin A2 (CCNA2) and change in Ki67 ($R=0.79$; $p<0.0001$).

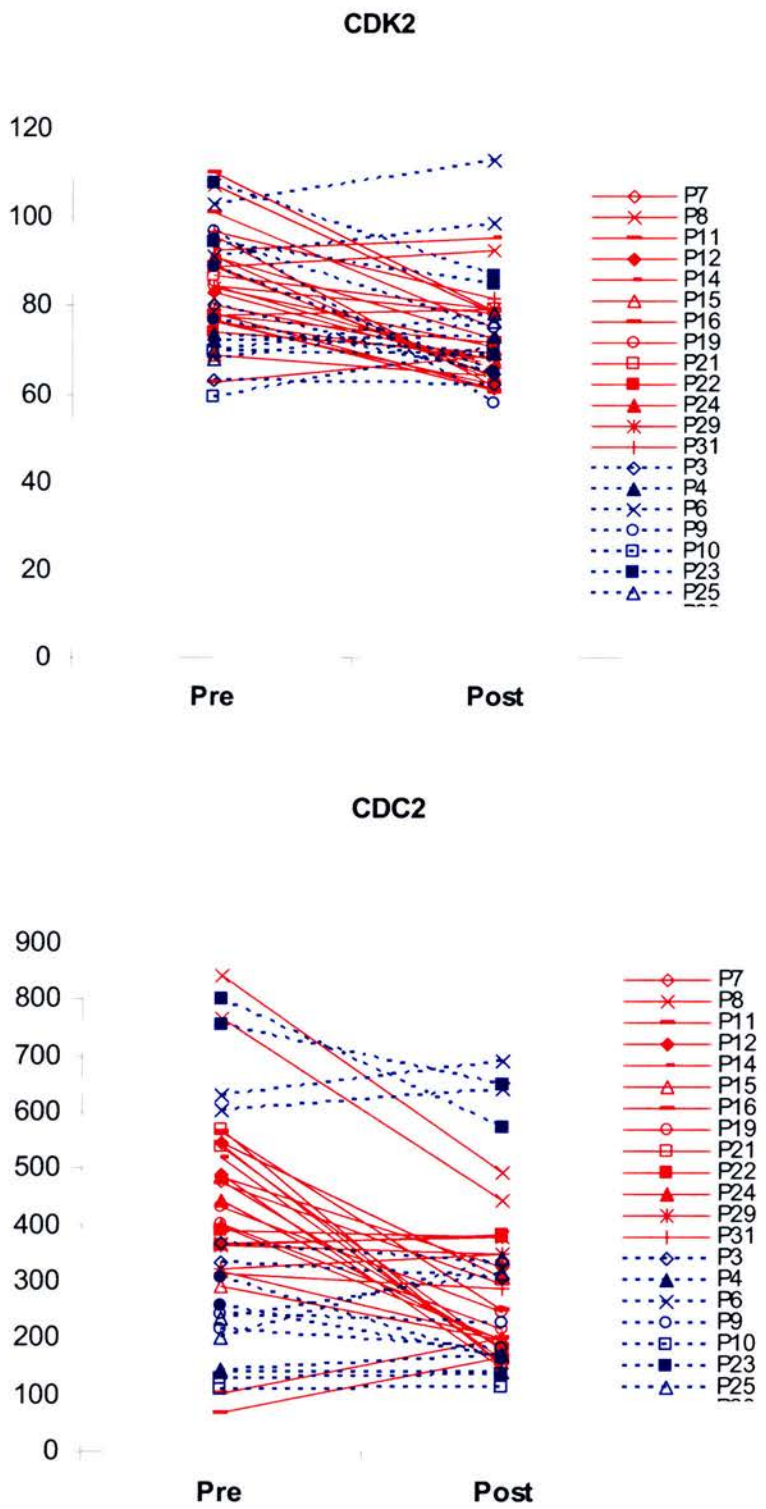


Figure 48
Change in expression of CDK2 (above) and CDC2 (below) with RAD001 treatment, for responders (red) and non-responders (blue). For both CDK2 and CDC2 there is a reduction in expression in the responders.

Of those genes related to oestrogen dependence, two genes were also found to be down-regulated after RAD001 treatment: KIAA0101 in matched samples for those that responded to RAD001 by Ki67 and SERPINA3 in those that responded defined by p-Akt.

Genes involving the mTOR pathway such as mTOR and BAD were not changed following RAD001 treatment. There was a significant fall in expression of GSK3B in all tumours with RAD001 treatment (p=0.003), but no difference between responders and non-responders. With transcriptional profiling there was a trend toward higher expression of mTOR in responders than in non-responders after treatment with RAD001 (p=0.01). In the immunohistochemical RAD001 study there was a trend toward those tumours that were highly proliferative pre-treatment having a greater decrease in p-mTOR. This discordance may reflect different forms of mTOR, in that the immunohistochemical findings reflect the actions of mTORC1 at ser2448, with p-S6 functioning as the kinase for ser2448, [167] and the expression of mTOR may represent mTOR dissociated from either mTORC1 or mTORC2.

Pre-treatment gene expression predicting for response

To determine whether there was a gene expression profile that predicts for response to RAD001, pre-treatment gene expression of responders (by %Ki67; n=14) and non-responders (n=9) was compared. No significant difference between the common differentially expressed genes (intersect of top 500 genes) was found (FDR 50%). Clustering of 248 significantly differentially expressed genes showed responders clustered into 2 groups, one group with high expression of proliferative genes by both %Ki67 and genomic grade index (GGI), [222] and the other group of responders less distinct. **Figure 49** These results may be attributable to the definition of response versus non-response calculated upon reduction in %Ki67 as an absolute rather than relative value, but allow correlation of the immunohistochemical and transcriptional profiling finding that those with higher pre-treatment proliferation were more likely to respond to RAD001. Using a 52-gene consensus prognostic classifier for ER-positive tumours, these highly proliferative tumours would be predicted to have a poor prognosis. [223]

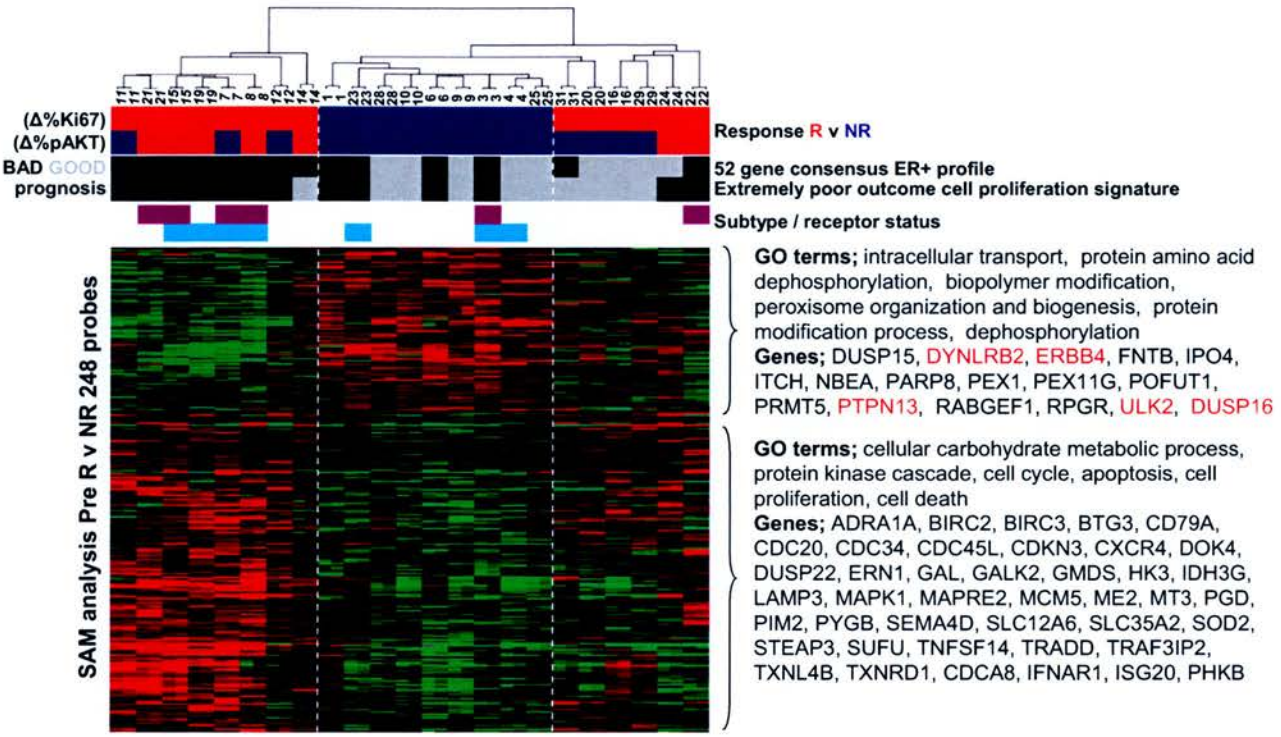


Figure 49

Hierarchical clustering of 248 significantly differentially expressed probes by responders and non-responders (by %Ki67). Grey and black bars signify which tumours have a good and bad prognosis based upon the 52-gene consensus classifier for ER-positive tumours [223] and the extremely poor outcome proliferation signature. [224] Light blue and purple bars signify which tumours were PR- and HER-2+ respectively. Probes highlighted in red were amongst the 238 differentially expressed probes identified between responding and non responding patients by a significant reduction in phospho-Akt. The heatmap represents differential expression of genes (fold changes) in rows and patients in columns. Red is high expression and green is low expression on a relative (mean-centred) scale.

There were differences in expression of specific mTOR pathway genes between responders and non-responders. Expression of BAD was higher in non-responders than in responders both pre (p=0.05) and post (p=0.003) treatment with RAD001, and pre-treatment expression of TSC2 was higher in non-responders than responders (p=0.02). **Figures 50 and 51** These findings are suggestive of a gene expression profile of high expression of BAD and TSC2 predictive of a lack of response to RAD001, rather than an expression profile that predicts for response.

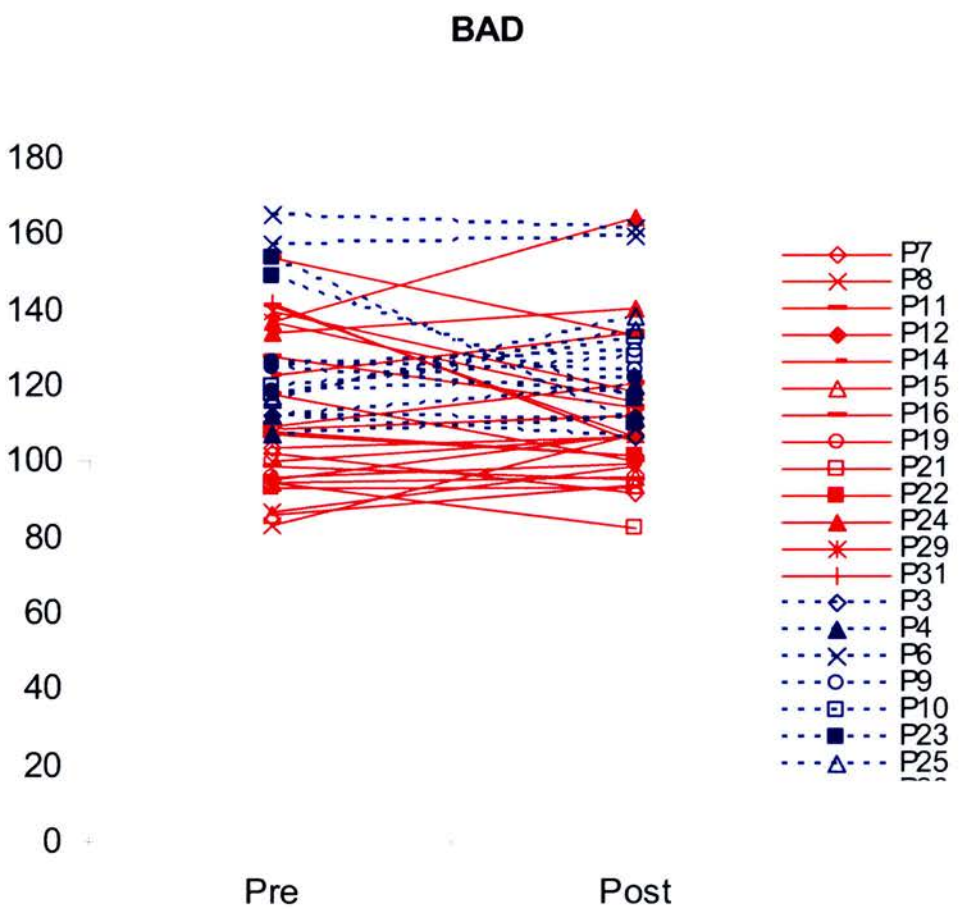


Figure 50
Differential expression of BAD with RAD001 treatment in responders (red) and non-responders (blue) showing higher pre and post treatment expression in non-responders.

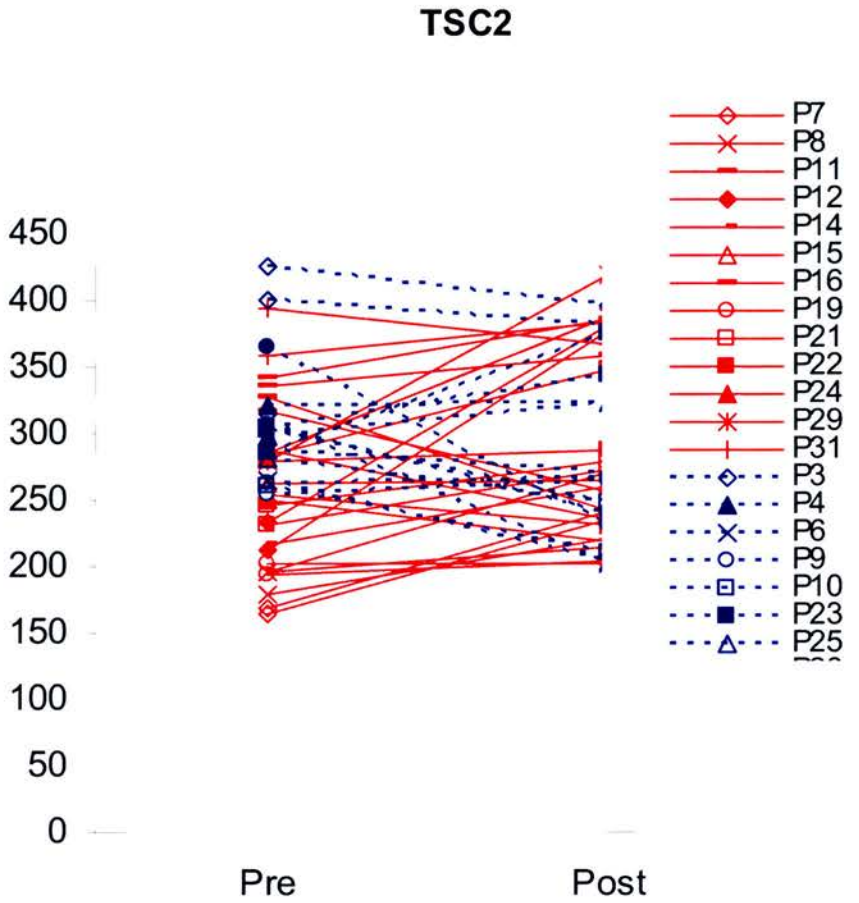


Figure 51
Differential expression of TSC2 in responders (red) and non-responders (blue) pre and post treatment, showing higher pre-treatment expression of TSC2 in non-responders.

Discussion

The findings of homogeneity within the group of responders compared with a heterogenous group of non-responders are comparable with results from gene expression profiling of letrozole treated tumours from an Edinburgh series. [225] In this study it was found that those tumours that had a clinical response to treatment with letrozole had at 14 days a similar genetic profile as a group that was dissimilar to non-responding cancers, and that the non-responding cancers were distinctive from each other.

It has previously been shown that a particular subpopulation of breast cancer patient groups can be identified by a "proliferation signature" that has an extremely poor outcome. [224] However, our study suggests that highly proliferating tumours with poor prognosis are amongst those that respond to RAD001, at least in the short term, with reductions in expression of oestrogen regulated genes and cell cycle genes.

In the above mentioned letrozole study, genes identified to have been down-regulated with letrozole treatment could be categorised as those related to oestrogen dependence or proliferation, [226] with tumours grouped into those in which both oestrogen-dependent and proliferation genes were decreased, those in which markers were changed only marginally and those in which oestrogen-dependent genes were decreased but proliferation genes were increased, in keeping with the action of letrozole as a specific aromatase inhibitor to induce oestrogen deprivation. Similarly, in a study of gene expression after 14 days treatment with anastrozole or letrozole, complex changes were found in oestrogen responsive pathways, proliferation and matrix remodelling. [227]

The findings of down-regulation of oestrogen dependence with RAD001 are particularly interesting given the IHC finding of a significant reduction in ER after 2 weeks treatment with RAD001. Similar findings have been reported from a study of gene expression correlated to response to combination doxorubicin/ cyclophosphamide (discussed below) in which oestrogen-regulated genes (ESR1, IRS1) were over-expressed in sensitive tumours compared with resistant tumours before treatment. [228] In the chemotherapy study, about half the patients were premenopausal, and some patients had recently stopped hormone replacement therapy, which may compound these findings.

In the previously mentioned study of gene expression after letrozole treatment no single gene changed consistently with treatment in all 58 cases, and using the criterion of change in at least 50 cases, only 7 genes were identified. [225] This was felt to be in keeping with the known heterogeneity of breast cancer. By employing the SAM analysis as employed in the RAD001 study, and considering the incidence and degree of change, 58 genes were down-regulated and 6 up-regulated after letrozole treatment. Analysis of the letrozole treated tumours failed to discriminate totally between responding and non-responding patients, other than to note that responding tumours displayed a high degree of similarity as a group, in comparison to non-responding tumours.

Previous studies of microarray expression analysis of pre-treatment biopsies have had positive findings for predicting response to treatment with combination doxorubicin/ cyclophosphamide treatment, [228] and for docetaxel therapy. [229] In the docetaxel study, pre-treatment samples from 24 patients were correlated with clinical response after 4 cycles, and it was found that, in general, resistant tumours

overexpressed genes associated with protein translation, cell cycle, and RNA transcription functions, whereas sensitive tumours overexpressed genes involved in stressor apoptosis, cytoskeleton, adhesion, protein transport, signal transduction, and RNA splicing or transport, and apoptosis, similar to our findings with RAD001.

In the 40 patients undergoing combination doxorubicin/ cyclophosphamide gene expression from pre-treatment biopsies were analysed and correlated with response after 6 cycles of treatment. [228] Two hundred fifty three differentially expressed genes were identified, with a 1.5–8.0-fold decreased or 1.5–4.2-fold increased expression in resistant versus sensitive tumours. Of the 75 genes overexpressed in resistant tumours, major categories were transcription (GTF3C1, ILF3), differentiation (ST14, CTNNBIP1), signal transduction (EIF1AX, EIF4EBP1), amino acid metabolism (SRM, PLOD1, PLOD3), and genes of unknown function. Of the 178 gene overexpressed in sensitive tumours major categories were cell cycle (BUB3, CDKN1B), survival (BCL2, BAG1, BIRC1, STK39, WEE1), stress response (CYP2B6, MAPK14), and oestrogen-regulated genes (ESR1, IRS1). In general, the AC-sensitive tumours had increase in expression of genes involved in metabolism, stress response, survival, and cell cycle relative to tumours resistant to therapy.

For both studies it was recommended that larger validation studies are necessary to define unique expression patterns for different therapeutic agents. From a pilot retrospective study in tamoxifen treated patients, a 44-gene expression signature was found to better predict anti-oestrogen therapy resistance than ER, the currently used clinical factor used to predict response (77% predictive value versus 50-60% for ER). [230] These findings promise regimen-specific gene expression-based predictors for

therapy, allowing optimisation of systemic therapy for individual breast cancer patients.

Cell line study

Aims

The aim of the cell line study was to investigate the effect of RAD001 on breast cancer cell lines with varying expression of phospho-Akt. From the immunohistochemical analyses described above it was apparent that nuclear p-Akt was significantly reduced by RAD001 treatment, and there was a significant difference between cytoplasmic phosphorylation of Akt after RAD001 in responders and non-responders. Current understanding of the action of RAD001 and thus understanding of the mTOR pathway is that the two mTOR complexes act in different ways upon Akt, but there is newer evidence to suggest that both mTORC1 and mTORC2 are sensitive to mTOR inhibition rather than mTORC2 being insensitive, and that this is influenced by treatment duration. [231] By determining the effect of RAD001 in cells expressing different levels of activity of p-Akt due to stimulation with heregulin, it was anticipated that it could be determined if both complexes of mTOR are inhibited by RAD001 with both short and long term treatment.

Materials and methods

Three breast cancer cell lines were selected for ER and PTEN methylation pairing from the cell line bank of JMSB group: MDA-MB-453, MDA-MB-361, MCF-7. Cells were cultured in DMEM (Invitrogen) with 10% foetal calf serum, 50μ/ml penicillin, 50μg/ml streptomycin and 0.29mg/ml L-Glutamine. Prior to introduction of drug to cells, flasks were incubated for 24 hours in serum-free media with 50μ/ml

penicillin, 50µg/ml streptomycin and 0.29mg/ml L-Glutamine. All experiments were conducted in duplicate under sterile conditions.

In order to determine the optimum concentration of RAD001 for use in the time point experiment IC50s were obtained for each cell line using the following method. Once cells had reached 50-60% confluence they were incubated overnight in serum-free media as described above in T-25 flasks. After washing in PBS (Invitrogen), cells were incubated with RAD001 at four different concentrations with heregulin (HRG) 10nmol/L, DMSO and serum-free media for 15 minutes. Controls for DMSO and DMSO-free and for heregulin were also used as shown below.

2x DMSO-free control
2x DMSO control
2x10nmol/l HRG DMSO control
2x10nmol/l HRG DMSO + 1 nmol RAD
2x10nmol/l HRG DMSO + 3 nmol RAD
2x10nmol/l HRG DMSO + 10 nmol RAD
2x10nmol/l HRG DMSO + 30 nmol RAD

} for each cell line

Cells were then washed with cold PBS, 500µl lysis buffer added and cells scraped and transferred to eppendorfs and stored on ice. After centrifugation for 10 minutes at 10,000 rpm at 4°C, the supernatant was transferred to a fresh eppendorf and stored at -80°C. To quantify the protein extraction, a Bradford Protein Assay was performed using a Biorad SmartSpec Plus Spectrophotometer, and concentrations for each sample obtained.

In order to determine the level of downregulation of the mTOR pathway by RAD001, Luminex assays were performed for each cell line for p-S6 (ser 235/236) by the following method. Lysates were diluted to a concentration of 600µg/ml using the quantification given in the Bradford Protein Asssay with lysis buffer, and further diluted to 300µg/ml with assay buffer. The lysates were incubated with p-S6 (s235/236) beads in a 96-well plate overnight on a microplate shaker. The lysates were then incubated with detection antibody for 30 mins, followed by streptavidin for 10 mins. After washing with resuspension buffer, quantification was performed by the Biorad Bio-Plex to provide p-S6 concentrations.

In order to determine the effects of RAD001 at its optimum concentration in each cell line with varying levels of Akt activation, the IC50 for RAD001 calculated as above was used for the following experiments. All experiments were conducted in duplicate under sterile conditions. Cells were cultured in DMEM (Invitrogen) with 10% foetal calf serum, 50µ/ml penicillin, 50µg/ml streptomycin and 0.29mg/ml L-Glutamine. Once cells had reached 50-60% confluence they were incubated for 24 hours in serum-free media with 50µ/ml penicillin, 50µg/ml streptomycin and 0.29mg/ml L-Glutamine.

After washing in PBS (Invitrogen), cells were incubated with either RAD001 at IC50 or heregulin at 1 or 10nmol/l or both as shown below for 15, 30, 60 minutes and 24 hours. Controls for DMSO and DMSO-free media were used. Cells incubated with RAD001 for the required time point, then washed with warm PBS before treatment with heregulin at the required concentration for 15 minutes. Those that were treated with heregulin were incubated initially in DMSO media for the same time period as those in RAD001 before heregulin incubation.

2x DMSO-free control

2x DMSO control

2x DMSO + IC50 for RAD + 1nmol/l HRG

2x DMSO + IC50 for RAD + 10nmol/l HRG

2x DMSO + IC50 for RAD

2x DMSO + 1nmol/l HRG

2x DMSO + 10nmol/l HRG

} for each cell line for each time point

Cells were then washed with cold PBS, 500µl lysis buffer added and cells scraped and transferred to eppendorfs and stored on ice. After centrifugation for 10 minutes at 10,000 rpm at 4ºC, the supernatant was transferred to a fresh eppendorf and stored at -80ºC.

To quantify the protein extraction, a Bradford Protein Assay was performed using a Biorad SmartSpec Plus Spectrophotometer, and concentrations for each sample obtained.

In order to determine the downstream effects of RAD001 on the mTOR pathway in the presence and absence of heregulin stimulation, levels of p70 S6kinase were assessed using western blot analysis using the protocol described below. To determine the effects RAD001 upon p-Akt in the presence and absence of heregulin stimulation, p-Akt (ser 473) was assessed. To allow normalisation to the test primary antibody and to control for protein loading reprobing with actin was performed.

Western blotting method for p-Akt and actin

The protein lysates were thawed and diluted with lysis buffer and gel loading buffer added. The samples were then heated to 100°C (hot block) for 5 minutes, returned to ice to cool and spun. The gels were then loaded and run for one hour. The PDVF Immobilon P Millipore membrane was prepared for transfer in methanol, dH2O then transfer buffer. Gels were transferred overnight at 30 volts in the cold room. Membranes were removed and blocked using 5% milk in TBS for one hour at room temperature. Incubation with primary p-Akt antibody (1:10,000 in 5%milk) was performed overnight at 4°C on a rocking platform. Membranes were then blocked using 0.5% blocking agent in TBS for 2x5 mins after washing, followed by incubation with secondary antibody (biotin 1:2000 in 5% milk) for 1 hour at room temperature on a rocking platform. Membranes were then incubated with activated luminal substrate for 1 minute then transferred to developer cassette. The membranes were exposed several times for different periods until optimal exposure achieved. Membranes were then reprobed with actin by re-blocking in 5% milk in TBS then adding the actin primary antibody (1:200,000 in 5% milk in TBS) and incubating overnight at 4°C. Membranes were then washed and incubated in 5% milk in TBS 2x5mins, then incubated in actin secondary antibody (1:20,000 in 5%milk in TBS) for 1 hour at room temperature. After washing, membranes were incubated with luminol and developed as above.

Western blotting method for p70S6k

Gels were loaded and run, then transferred to membrane as described above. Membranes were removed and blocked using 5% milk in TBS for one hour at room

temperature. Incubation with primary p70S6k antibody (1:500 in 5%milk) was performed overnight at 4°C on a rocking platform. Membranes were then blocked using 0.5% blocking agent in TBS for 5 mins x2 after washing. Incubation with secondary antibody (biotin 1:2000 in 5% milk) was performed for 1 hour at room temperature on a rocking platform. Membranes were incubated with activated luminal substrate for 1 minute then transferred to developer cassette. The membranes were exposed several times for different periods until optimal exposure achieved. To quantify the western blot results, a public domain Java image processing program (Image J) was used. [232, 233]

Results

After completing initial studies with MDA-MB-361 cells to obtain an IC50 for RAD001 in this cell line, it was discovered that there was contamination of this cell line and it was not possible to use the data for further analysis. Due to time constraints and lack of a suitable stocked cell line that would pair for ER and PTEN status, it was decided to proceed with the two cell lines MCF-7 and MDA-MB-453. For each concentration analysed for both cell lines there were four wells consisting of two sets of duplicates of cells incubated with RAD001 and further duplication for the Luminex assay. A mean ratio compared to the standards used (Hela non-stimulated and PC12 stimulated cells) was calculated for each concentration analysed. The results were calculated using the heregulin control as standard, and the end point of phospho-S6 (ser 235/236) assessed using the Luminex method described above. The mean ratios for reduction in phospho-S6 are shown below. **Figure 52** The optimum concentration was calculated as being the concentration of RAD001 at which the p-S6 ratio was 50% of that of the heregulin control for each cell line. For ease of calculations of volumes for the next part of the study, these figures were rounded to 1.5nM for MCF-7 and 10nM for MDA-MB-453. This calculated concentration was then used for the following experiment looking at the effect of RAD001 treatment over time.

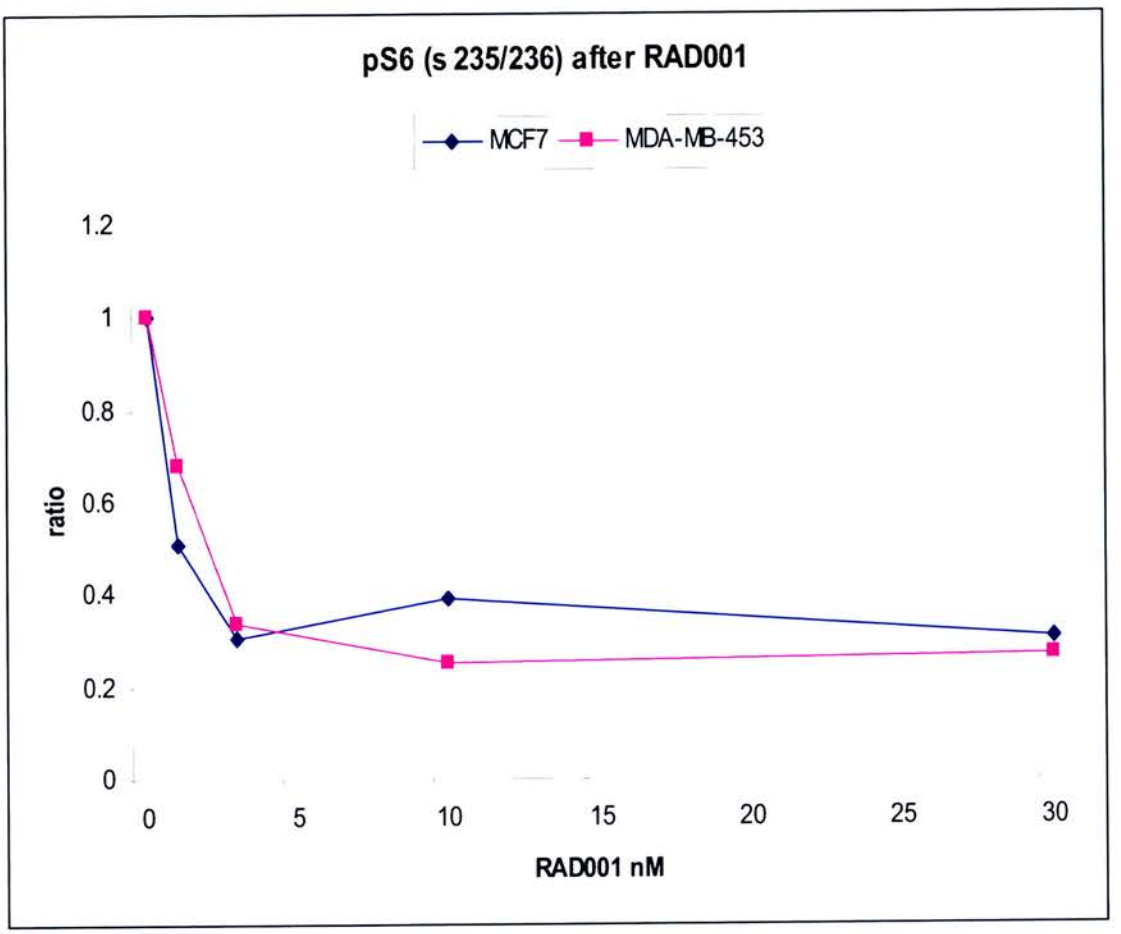


Figure 52
Results of experiment to determine IC50 for MCF7 and MDA-MB-453 cell lines treated with RAD001 at varying concentrations for reduction in p-S6 s235/236

Results are presented first for p70S6kinase for each cell line (MCF7 and MDA-MB-453) at the four time points evaluated (15, 30, 60 mins and 24 hours), then as above for p-Akt. All experiments were run in duplicate, thus results are for MCF7 2a and 2b, MDA-MB-453 2a and 2b etc for each time point. Two isoforms were detected for p70S6kinase (at 70kDa and 80kDa). Despite repeat experiments, the Western blots run for the MDA-MB-453 2a for p70S6kinase yielded no bands. The problem was identified as being during the incubation with p70S6k during the Western blot process rather than at the time of treatment of cells with RAD001, as reasonable results were obtained from the Western blots for MDA-MB-453 2a for p-Akt. There are therefore no results available as a duplicate for the p70S6k in the cell line MDA-MB-453 treated with RAD001. All blots were quantified as described above using Image J system to produce the results presented in graphs below.

Effect of RAD001 on p70S6kinase

MCF7 cell line

Figure 53 (A)

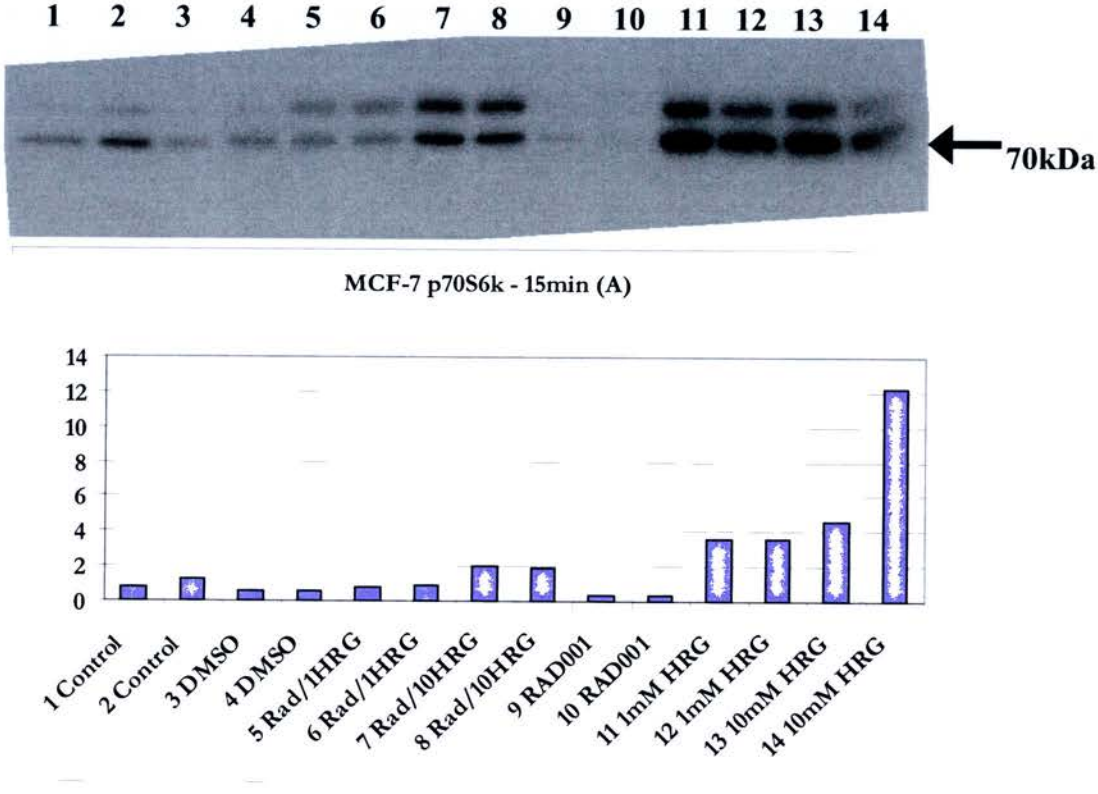


Figure 53 (B)

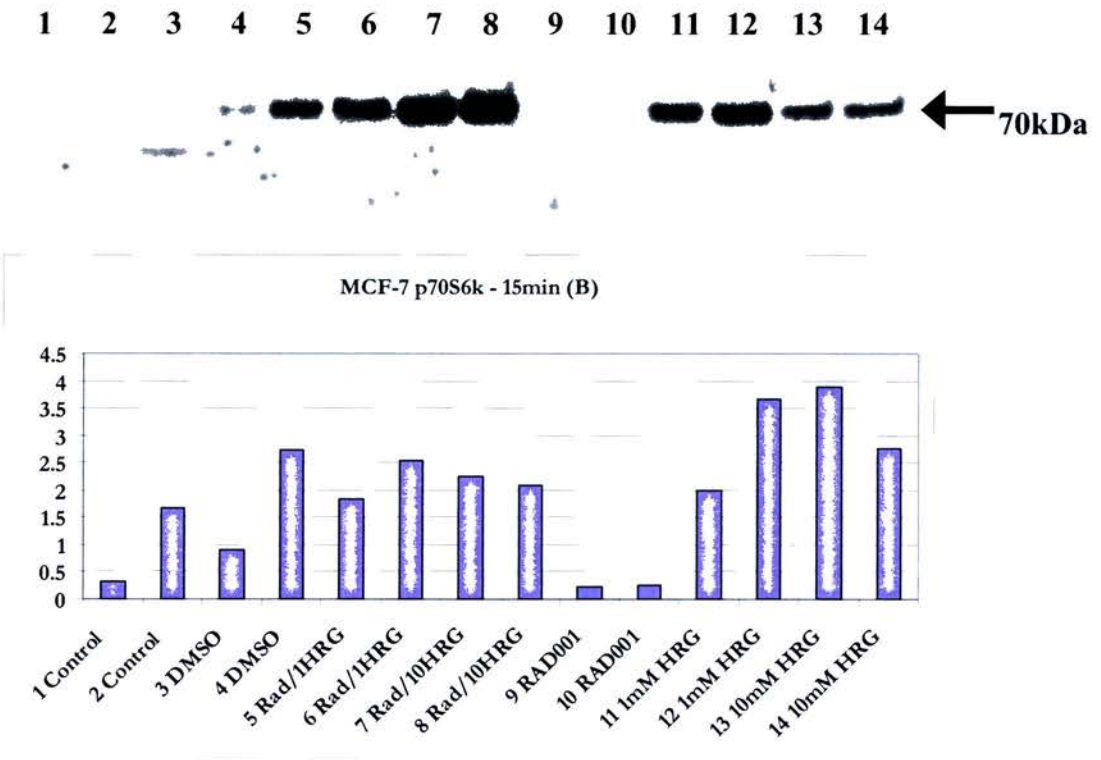
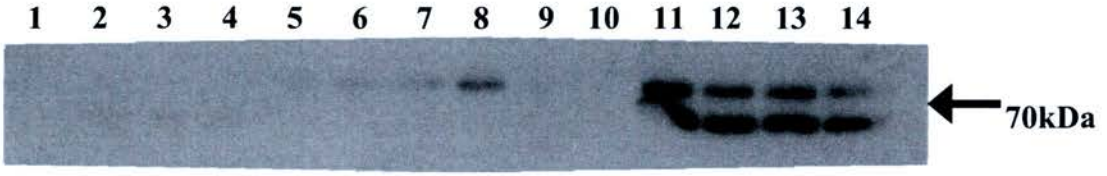


Figure 53
Western blots and Image J quantification for MCF-7 p70S6k treated for 15min in duplicate (Expt A & B) with quantification normalised to actin, demonstrating marked inhibition of p70S6kinase with RAD001 alone, but with RAD001 inhibition less marked in the presence of heregulin for duplicate experiments.

Figure 54 (A)


1 2 3 4 5 6 7 8 9 10 11 12 13 14
70kDa

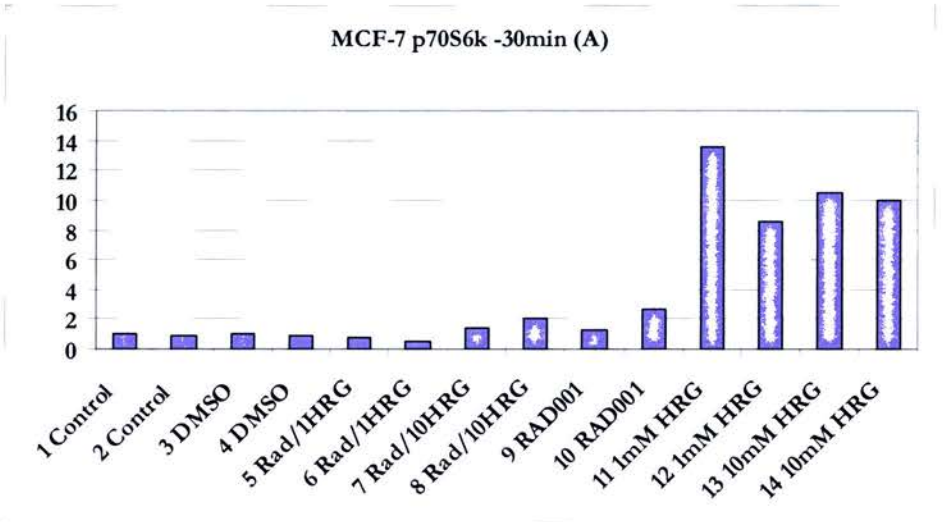

MCF-7 p70S6k -30min (A)
16
14
12
10
8
6
4
2
0
1 Control
2 Control
3 DMSO
4 DMSO
5 Rad/1HRG
6 Rad/1HRG
7 Rad/10HRG
8 Rad/10HRG
9 RAD001
10 RAD001
11 1mM HRG
12 1mM HRG
13 10mM HRG
14 10mM HRG

Figure 54 (B)

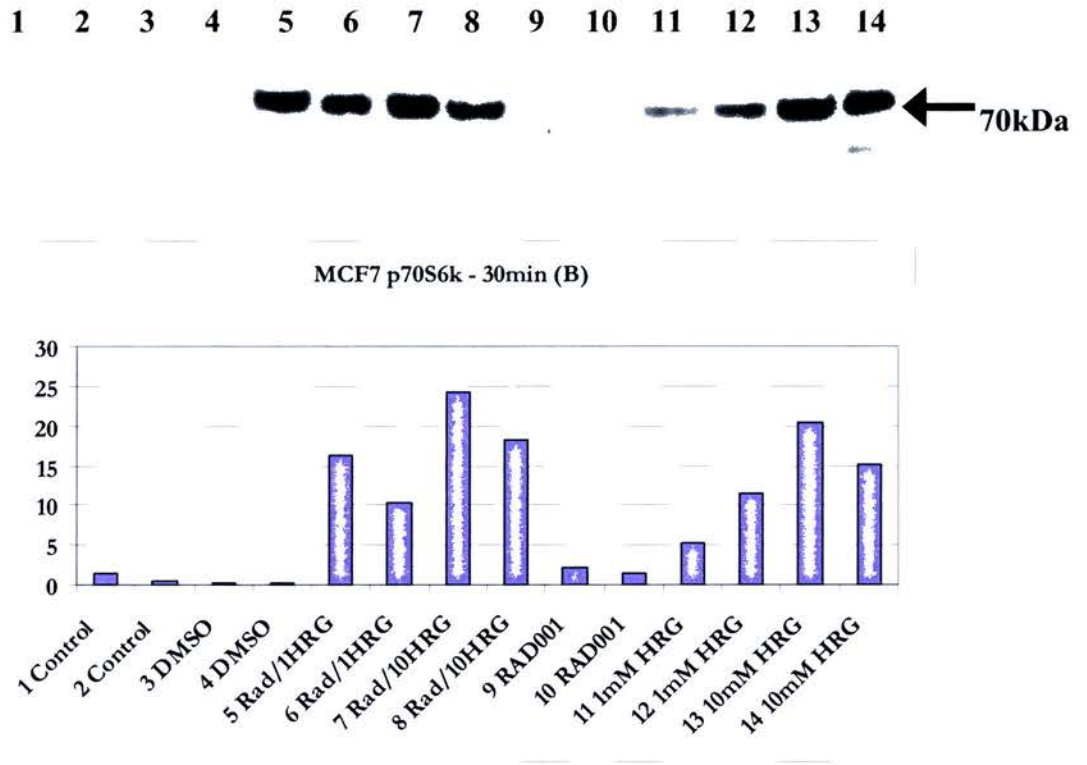
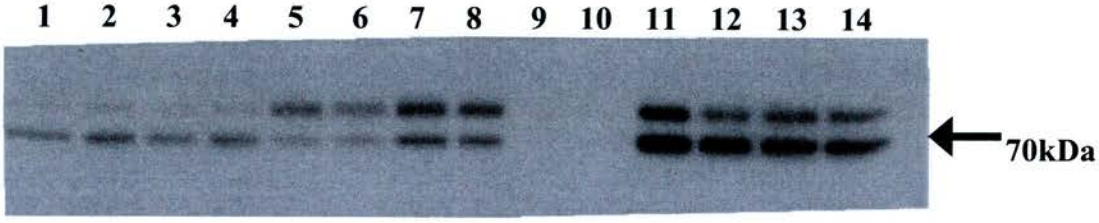


Figure 54
Western blots and Image J quantification for MCF-7 p70S6k treated for 30min in duplicate (Expt A & B) with quantification normalised to actin, demonstrating marked inhibition of p70S6kinase with RAD001 alone, and in one (Expt B) RAD001 inhibition less marked in the presence of heregulin, and in Expt A RAD001 inhibition even in the presence of heregulin.

Figure 55 (A)


1 2 3 4 5 6 7 8 9 10 11 12 13 14
70kDa

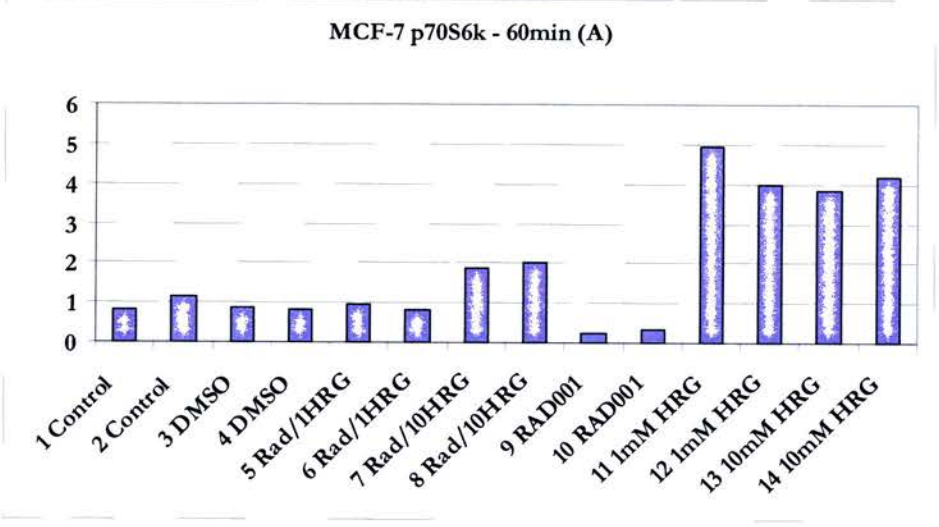
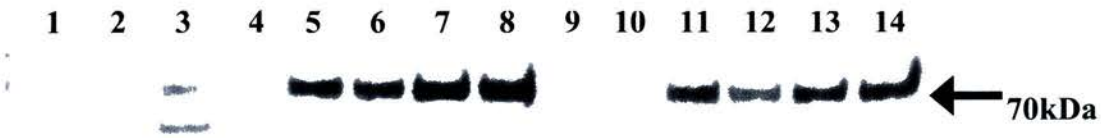

MCF-7 p70S6k - 60min (A)
6
5
4
3
2
1
0
1 Control
2 Control
3 DMSO
4 DMSO
5 Rad/1HRG
6 Rad/1HRG
7 Rad/10HRG
8 Rad/10HRG
9 RAD001
10 RAD001
11 1mM HRG
12 1mM HRG
13 10mM HRG
14 10mM HRG

Figure 55 (B)



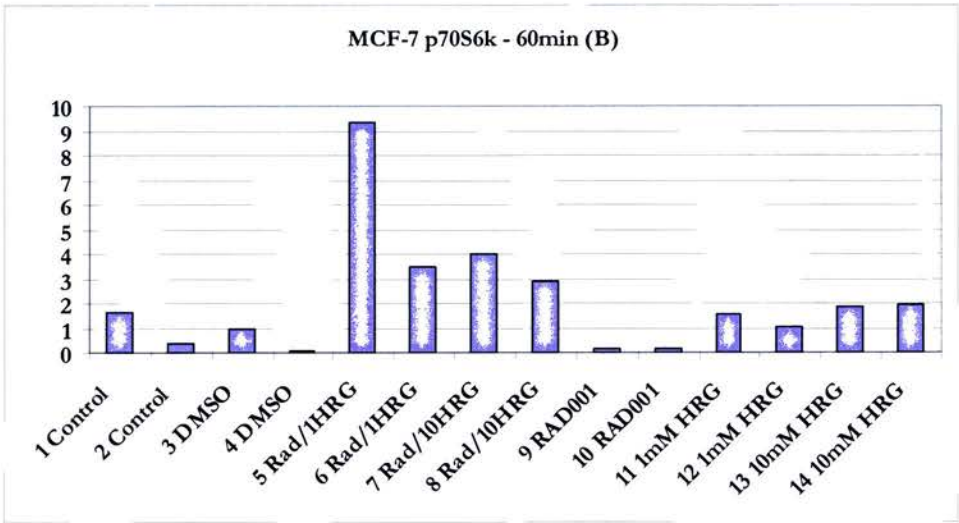
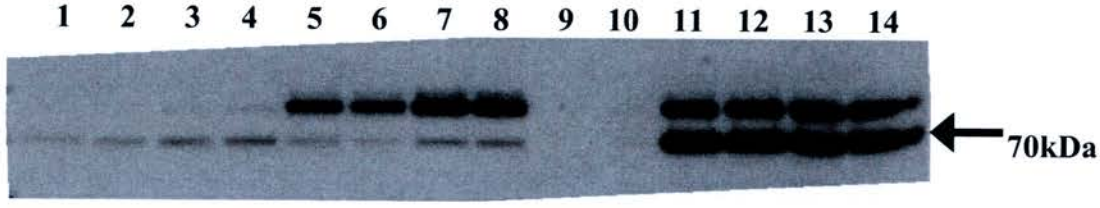


Figure 55
Western blots and Image J quantification for MCF-7 p70S6k treated for 60mins in duplicate (Expt A & B) with quantification normalised to actin, demonstrating marked inhibition of p70S6kinase with RAD001 alone, but not in the presence of heregulin.

Figure 56 (A)


1 2 3 4 5 6 7 8 9 10 11 12 13 14
70kDa

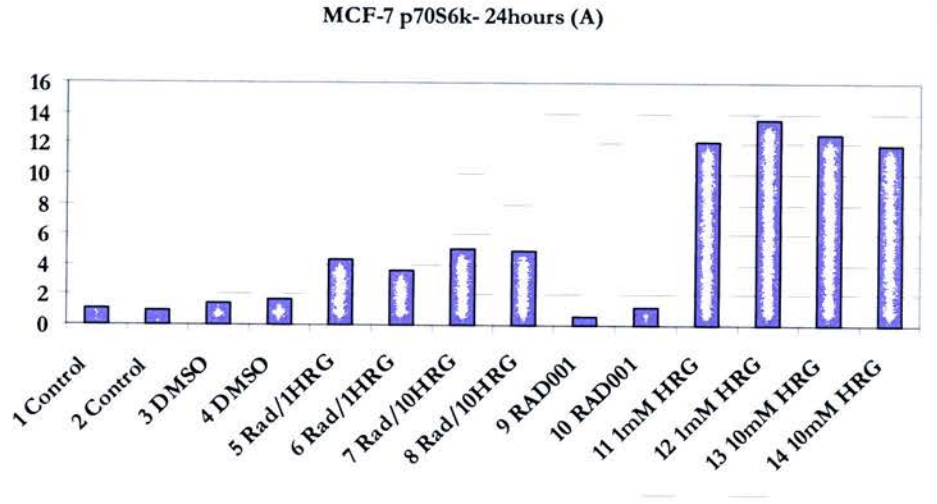

MCF-7 p70S6k- 24hours (A)
16
14
12
10
8
6
4
2
0
1 Control
2 Control
3 DMSO
4 DMSO
5 Rad/1HRG
6 Rad/1HRG
7 Rad/10HRG
8 Rad/10HRG
9 RAD001
10 RAD001
11 1mM HRG
12 1mM HRG
13 10mM HRG
14 10mM HRG

Figure 56 (B)

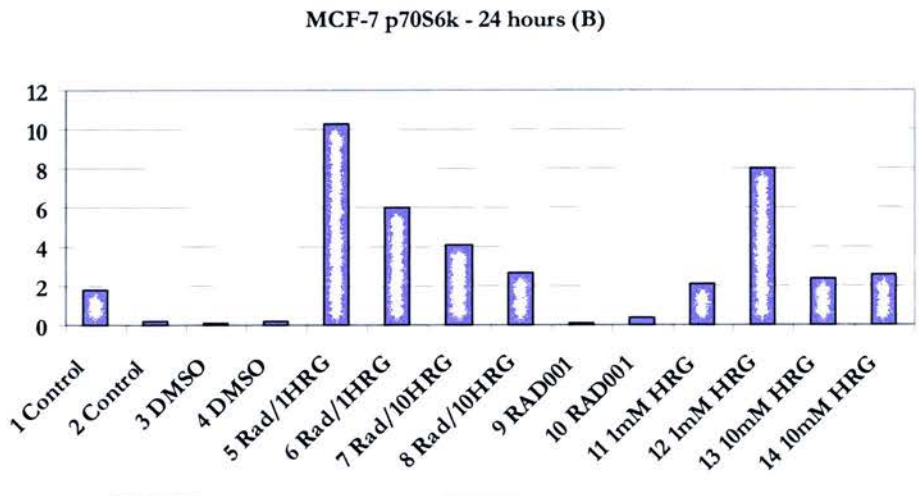


Figure 56
Western blots and Image J quantification for MCF-7 p70S6k treated for 24 hours in duplicate (Expt A & B) with quantification normalised to actin, demonstrating marked inhibition of p70S6kinase with RAD001 alone, but not in the presence of heregulin.

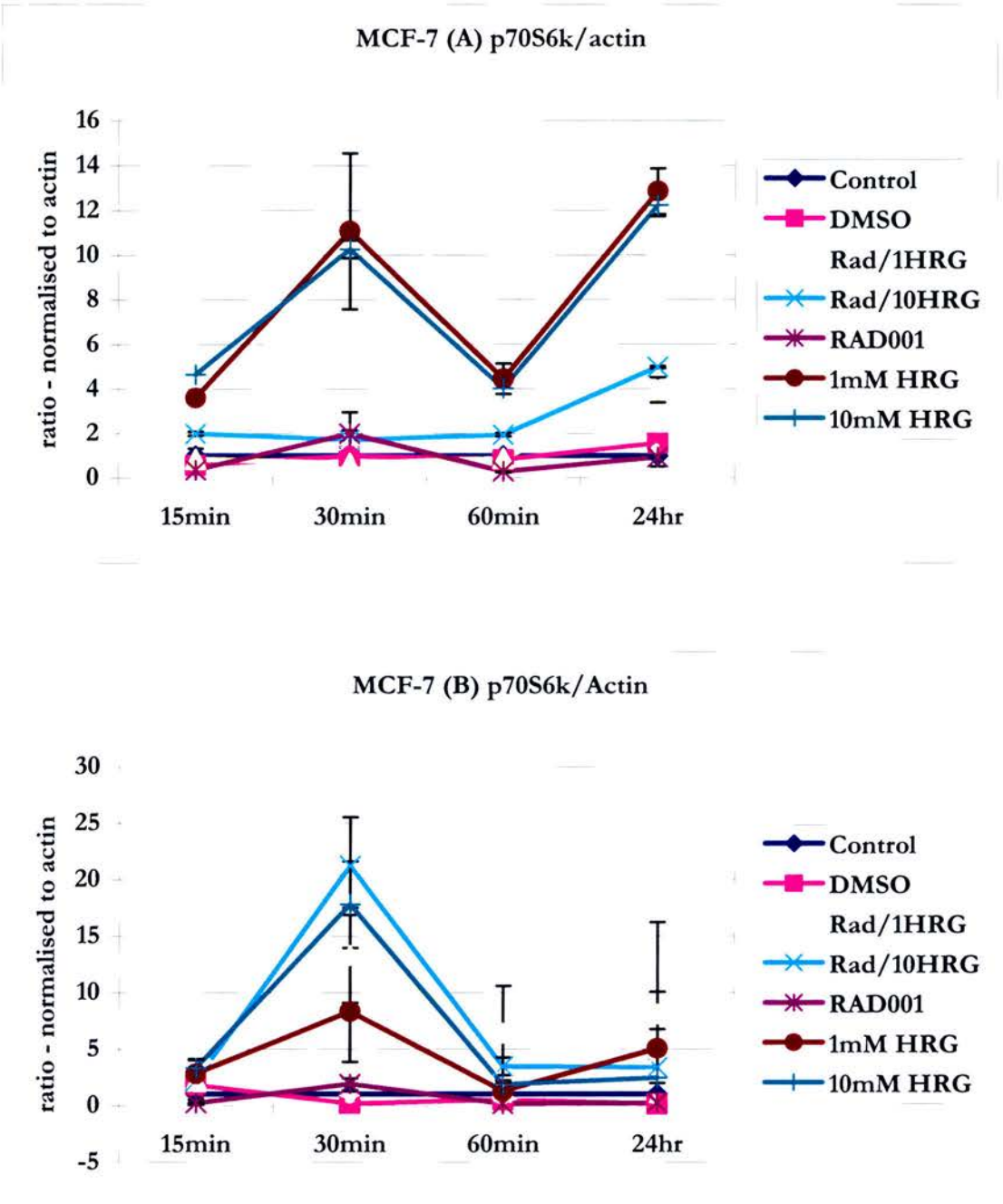


Figure 57
Effect over time of treatment with RAD001 on p70S6k in cell line MCF-7 for duplicate experiments (A & B). In (A) there is inhibition of p70S6k over the 24 hour period with RAD001 alone. In the presence of heregulin, RAD001 initially inhibits p70S6k, but there is evidence of some loss of inhibition of p70S6k at 24 hours. In (B), RAD001 alone inhibits p70S6k up to 24 hours, but not in the presence of heregulin up to 24 hours.

MDA-MB-453 cell line

It was not possible to obtain any quantifiable data from Western blots for MDA-MB-453 for p70S6k at any of the time points examined for the first of the experiments performed in duplicate. Results shown are for the second run performed as part of the duplication process (Expt B).

Figure 58

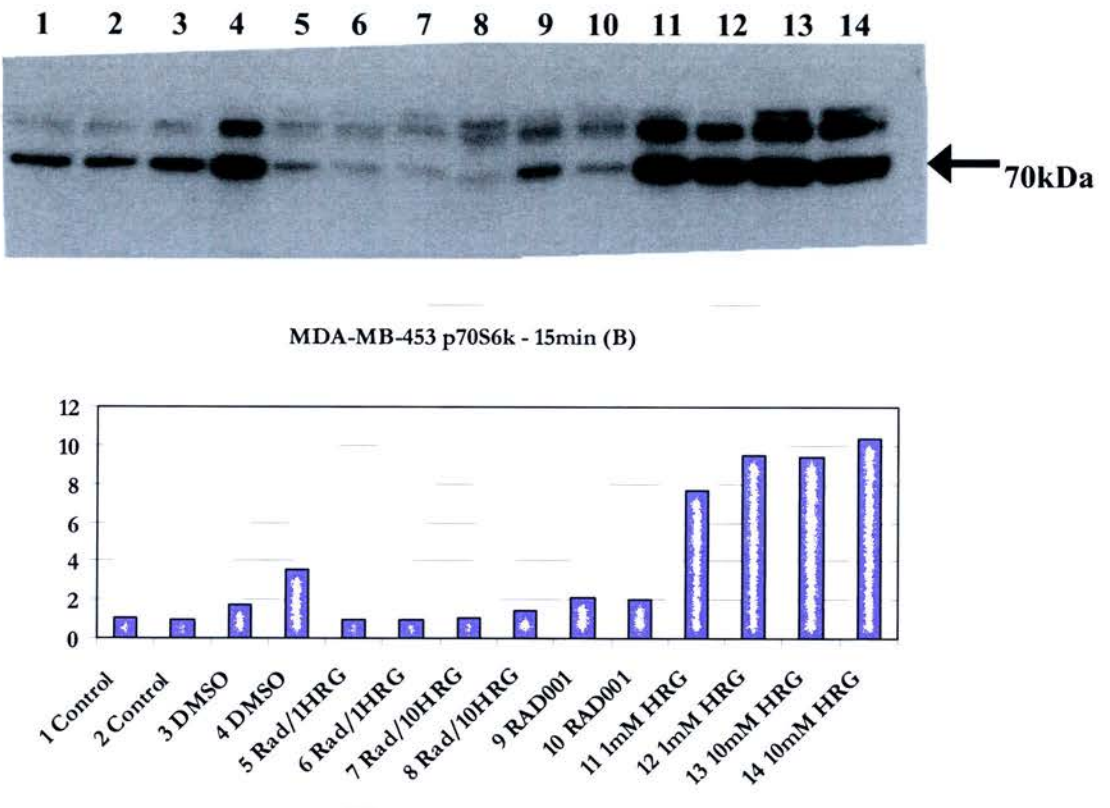


Figure 58
Western blots and Image J quantification for MDA-MB-453 p70S6k treated for 15min (Expt B) with quantification normalised to actin, demonstrating marked inhibition of p70S6kinase with RAD001 alone, and for RAD001 in the presence of heregulin.

Figure 59

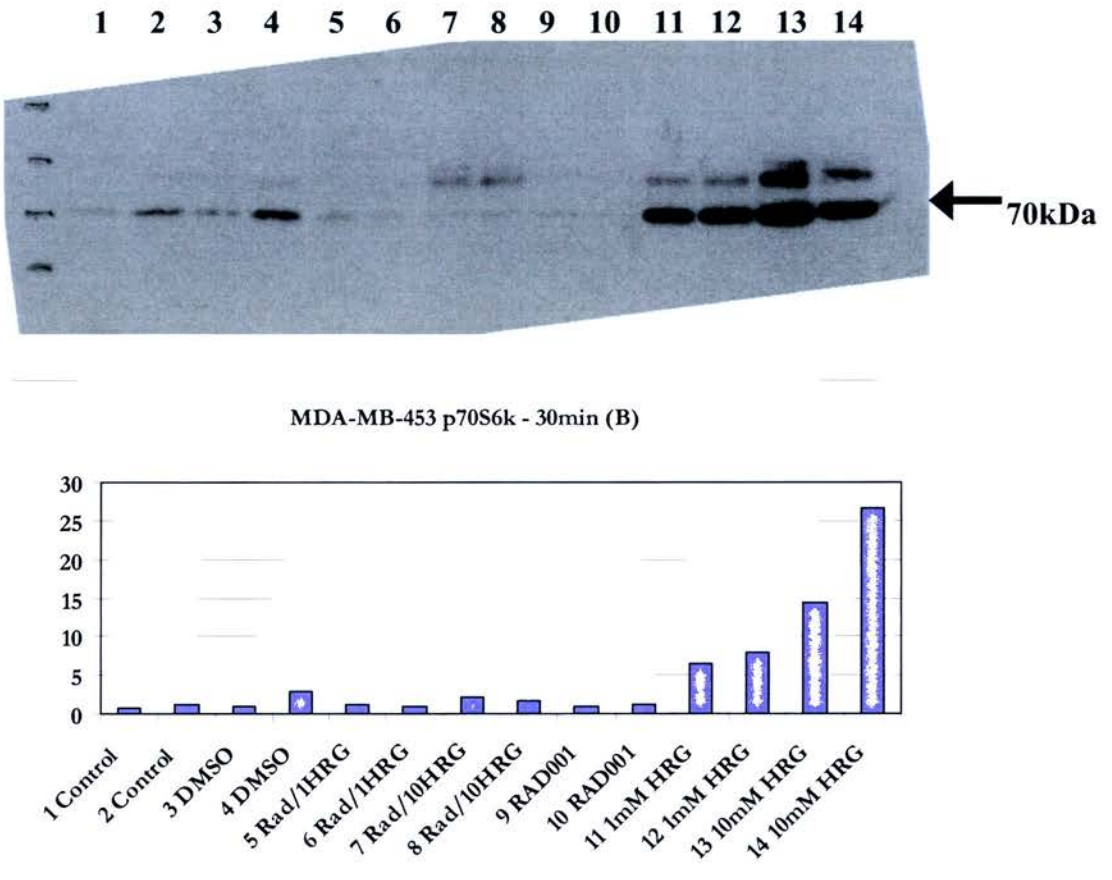


Figure 59
Western blots and Image J quantification for MDA-MB-453 p70S6k treated for 30mins (Expt B) with quantification normalised to actin, demonstrating marked inhibition of p70S6kinase with RAD001 alone, and for RAD001 in the presence of heregulin.

Figure 60

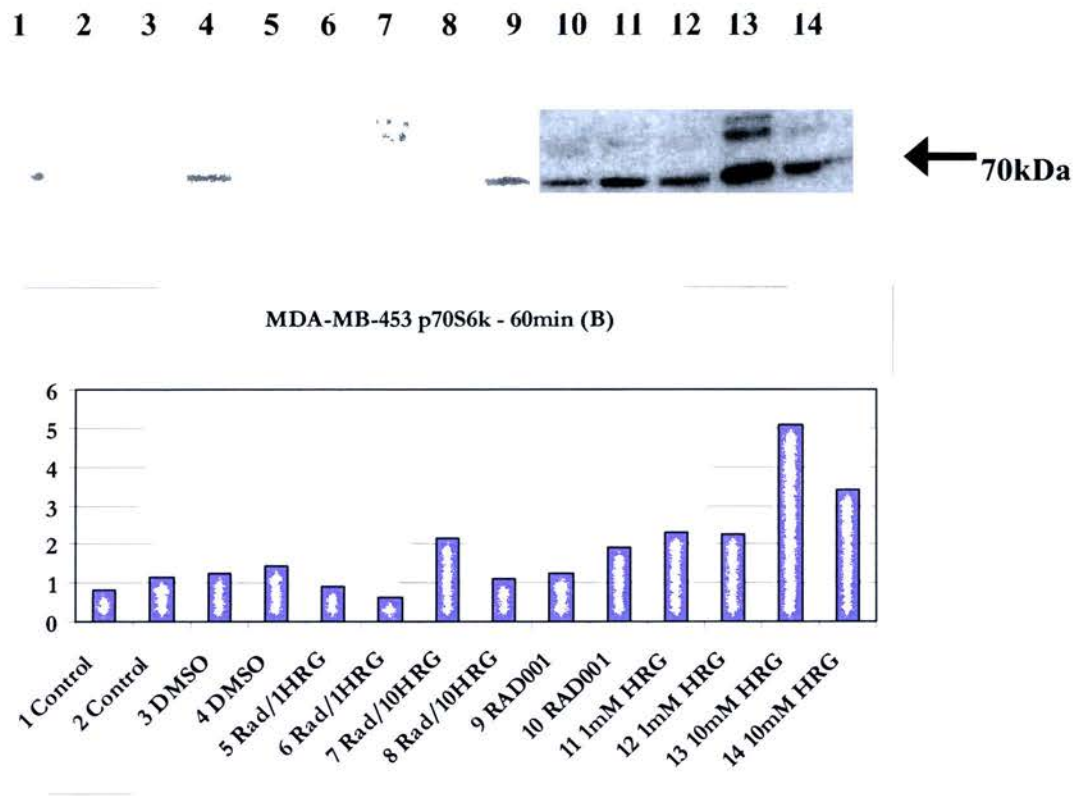


Figure 60
Western blots and Image J quantification for MDA-MB-453 p70S6k treated for 60mins (Expt B) with quantification normalised to actin, demonstrating some inhibition of p70S6kinase with RAD001 alone compared with heregulin alone, but less marked inhibition for RAD001 in the presence of heregulin.

Figure 61

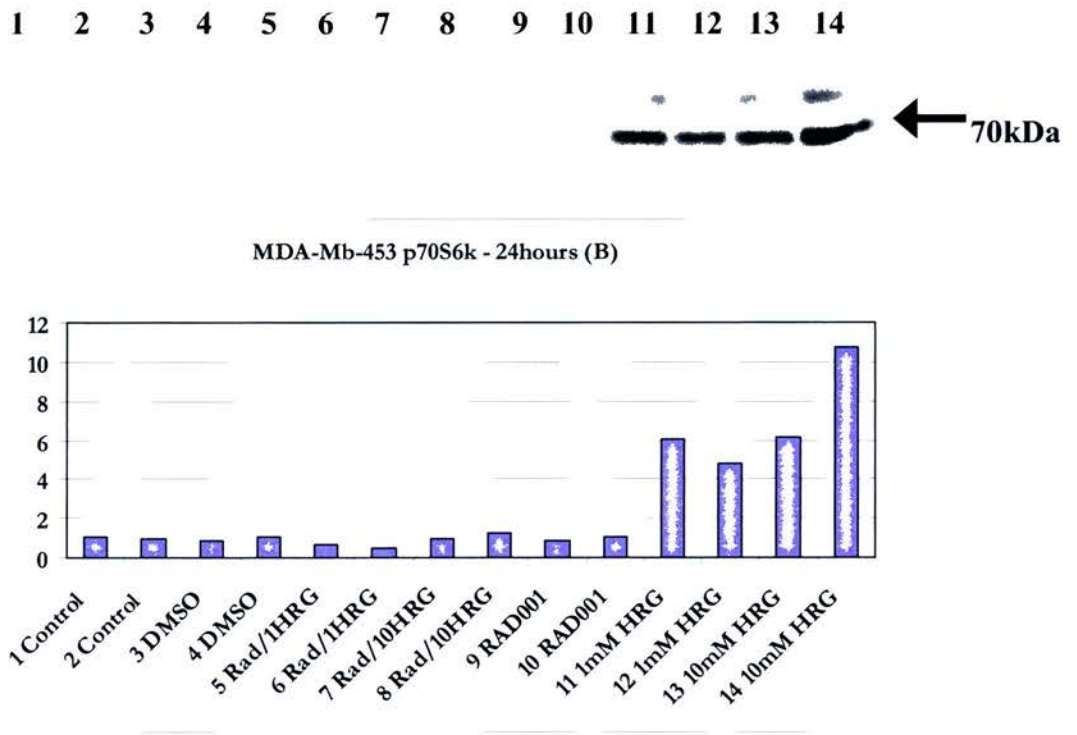


Figure 61
Western blots and Image J quantification for MDA-MB-453 p70S6k treated for 24 hours (Expt B) with quantification normalised to actin, demonstrating some inhibition of p70S6kinase with RAD001 alone compared with heregulin alone, and for RAD001 in the presence of heregulin.

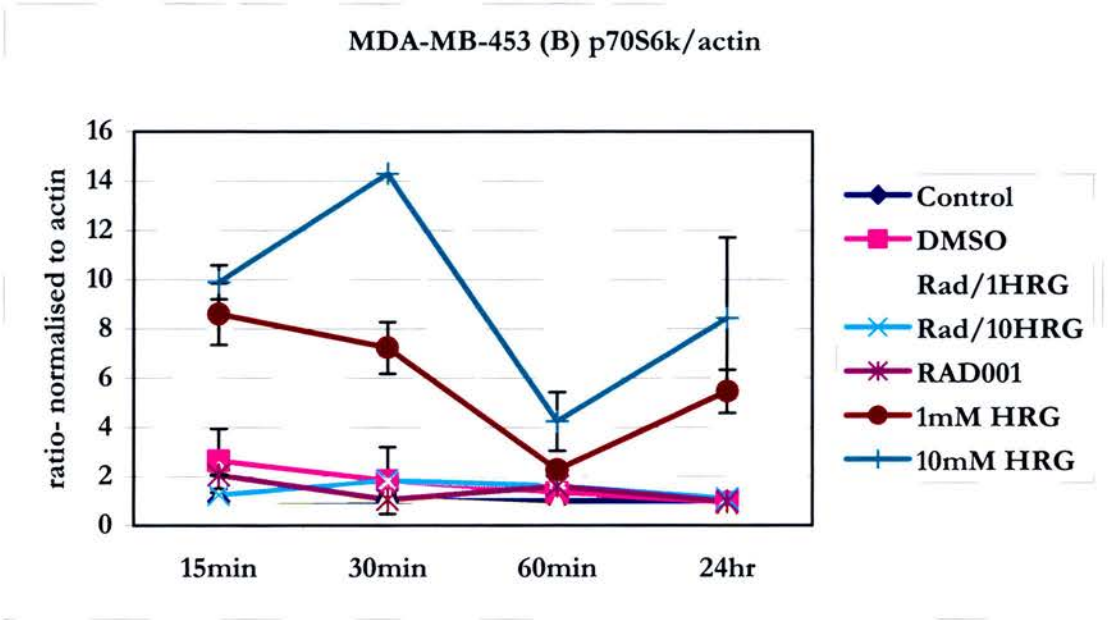
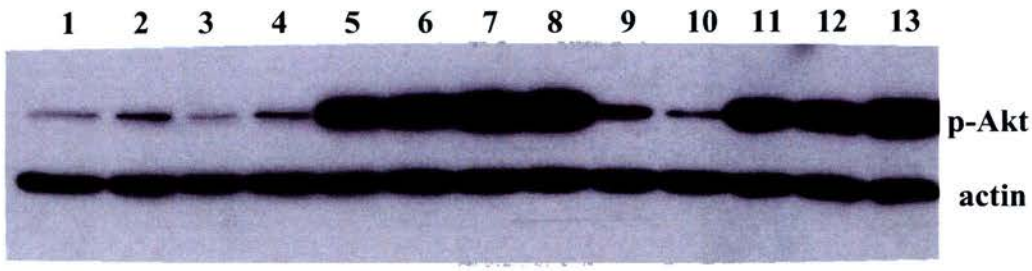


Figure 62
Effect over time of treatment with RAD001 on p70S6k in cell line MDa-MD-453 for experiments B. There is inhibition of p70S6k over the 24 hour period with RAD001 alone, and for RAD001 in the presence of heregulin compared with those treated with heregulin alone.

Effect of RAD001 on p-Akt

MCF7 cell line

Figure 63 (A)



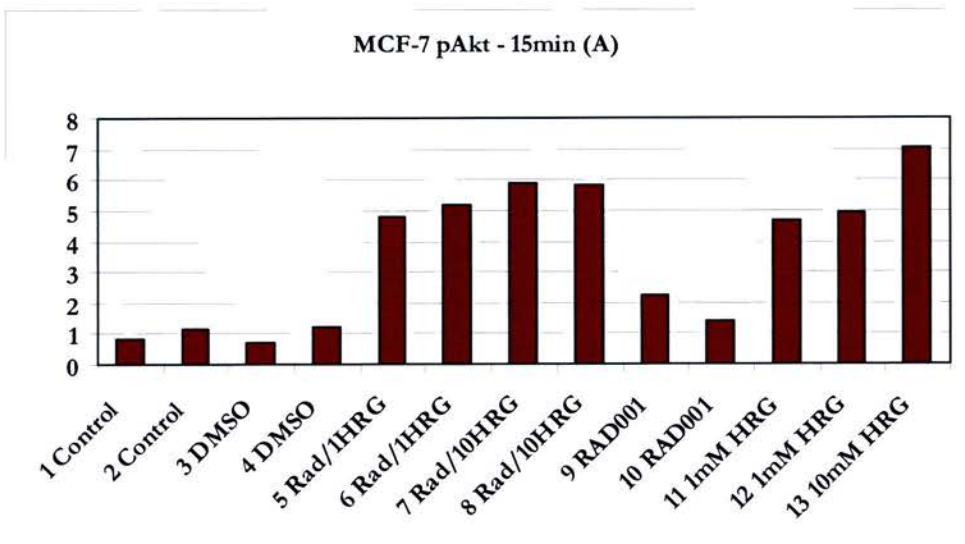


Figure 63 (B)

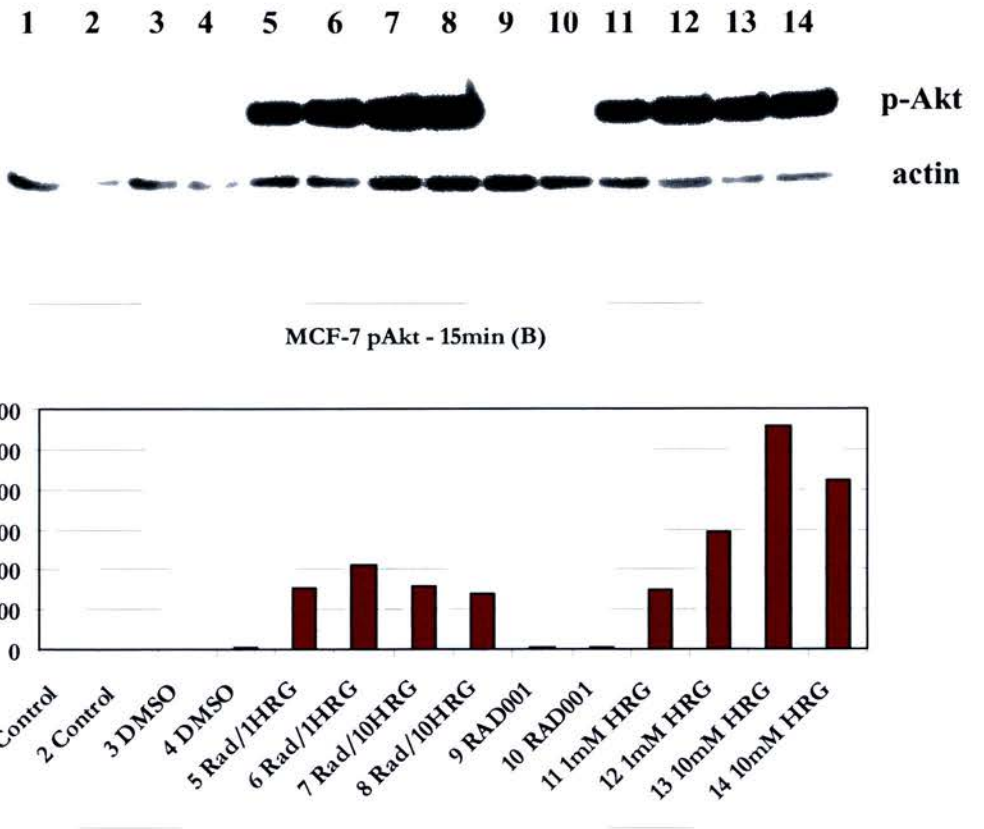
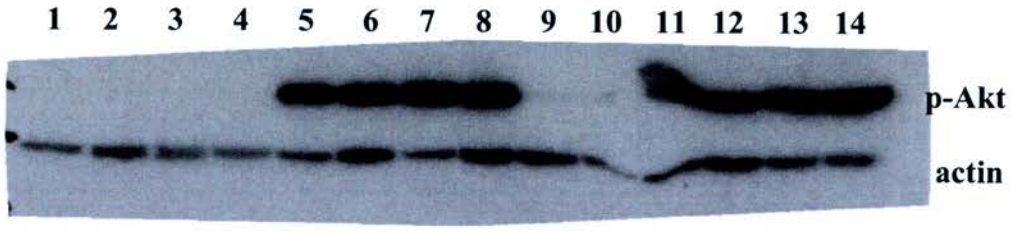


Figure 63
Western blots and Image J quantification for MCF-7 for p-Akt treated for 15min in duplicate (Expt A & B) with quantification normalised to actin, demonstrating marked inhibition of p-Akt with RAD001 alone, but not in the presence of heregulin in duplicate experiments.

Figure 64 (A)


1 2 3 4 5 6 7 8 9 10 11 12 13 14
p-Akt
actin

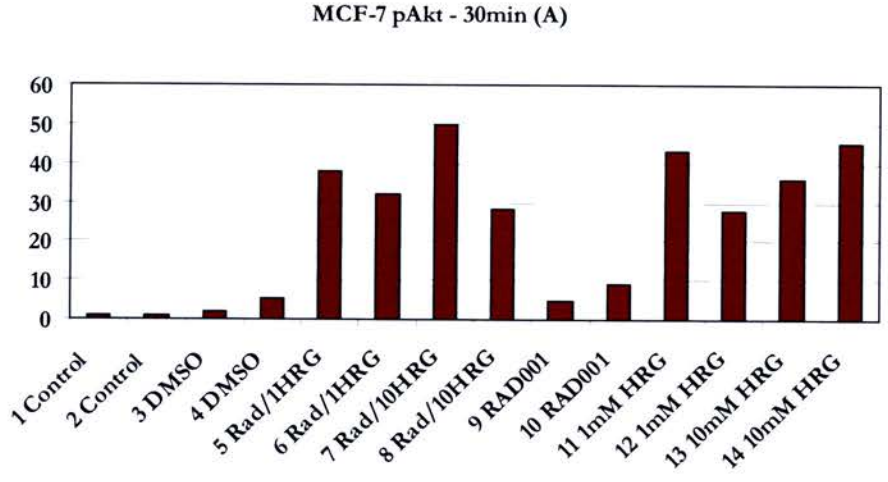

MCF-7 pAkt - 30min (A)
60
50
40
30
20
10
0
1 Control
2 Control
3 DMSO
4 DMSO
5 Rad/1HRG
6 Rad/1HRG
7 Rad/10HRG
8 Rad/10HRG
9 RAD001
10 RAD001
11 1mM HRG
12 1mM HRG
13 10mM HRG
14 10mM HRG

Figure 64 (B) For purposes of analysis the first line (p-Akt) of the top strip and the second line (actin) of the bottom strip were used.

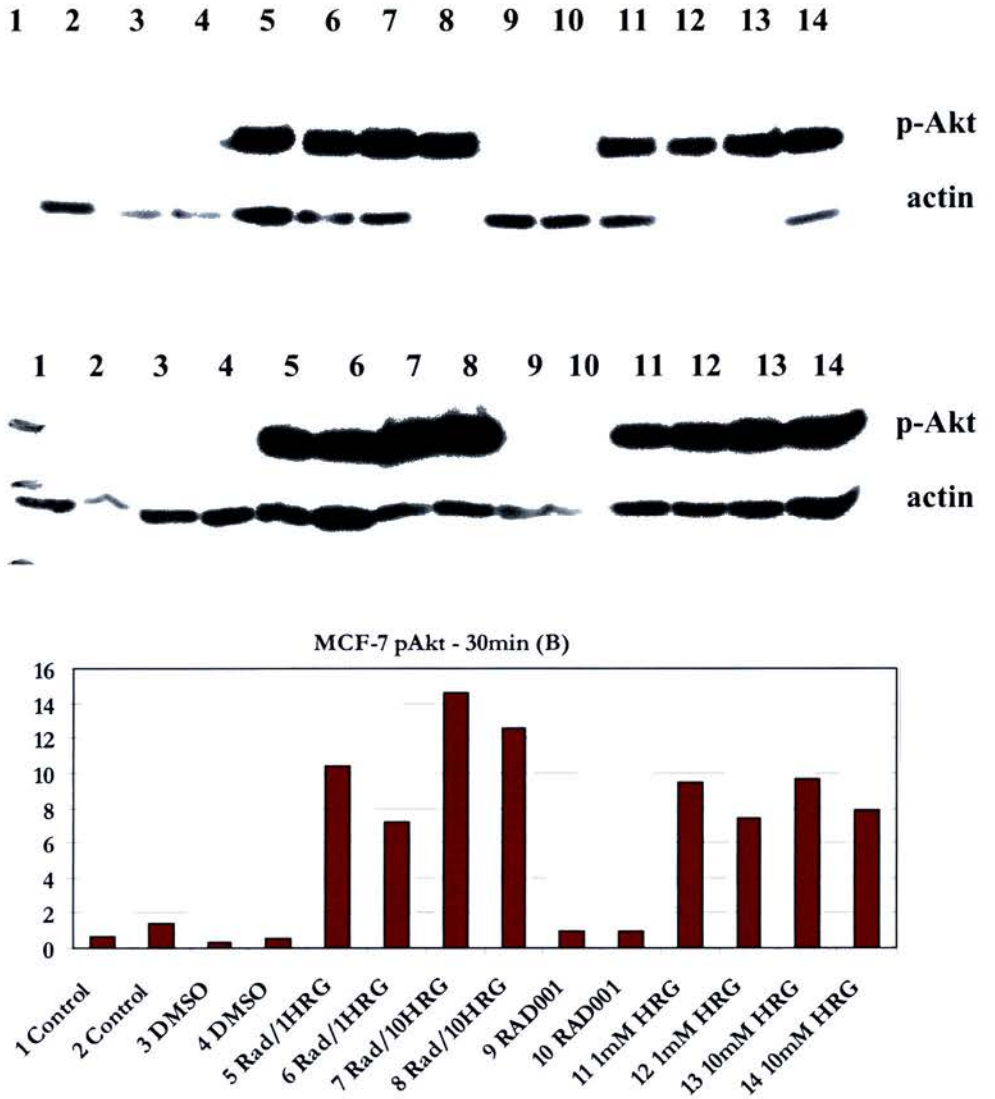
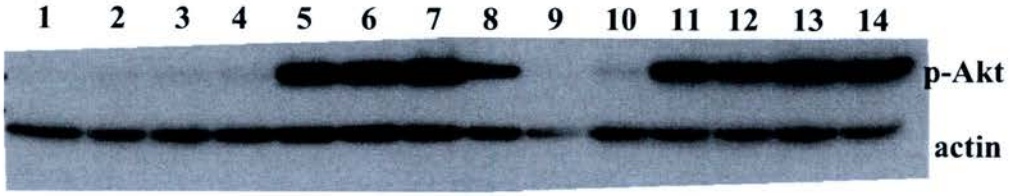


Figure 64
Western blots and Image J quantification for MCF-7 for p-Akt treated for 30 mins in duplicate (Expt A & B) with quantification normalised to actin, demonstrating marked inhibition of p-Akt with RAD001 alone, but not in the presence of heregulin in duplicate experiments.

Figure 65 (A)



MCF-7 pAkt - 60min (A)

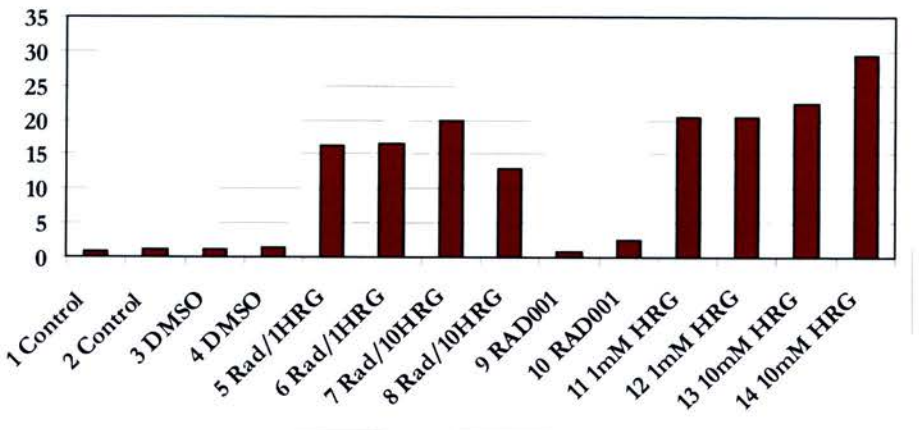


Figure 65 (B) For purposes of analysis the second line (actin) of the top strip and the first line (p-Akt) of the bottom strip were used

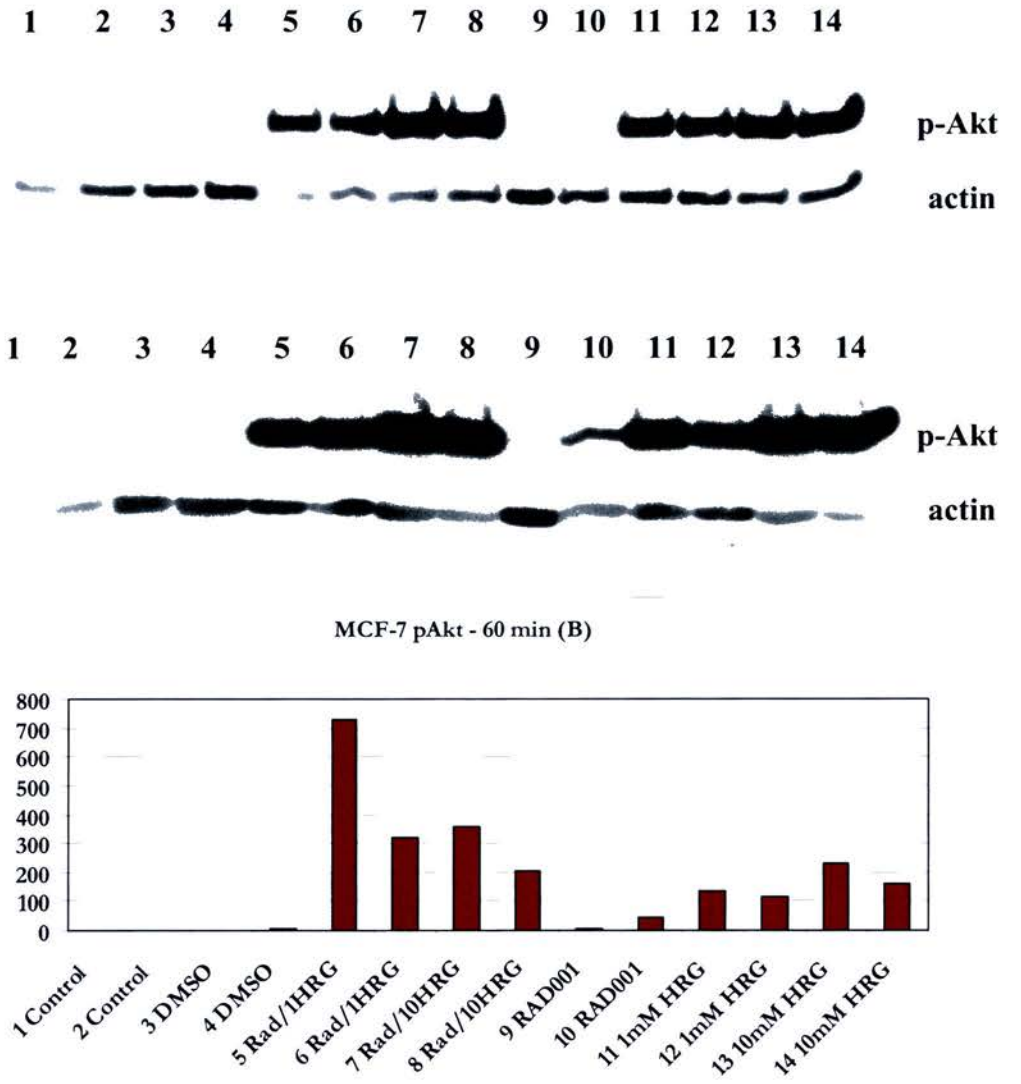
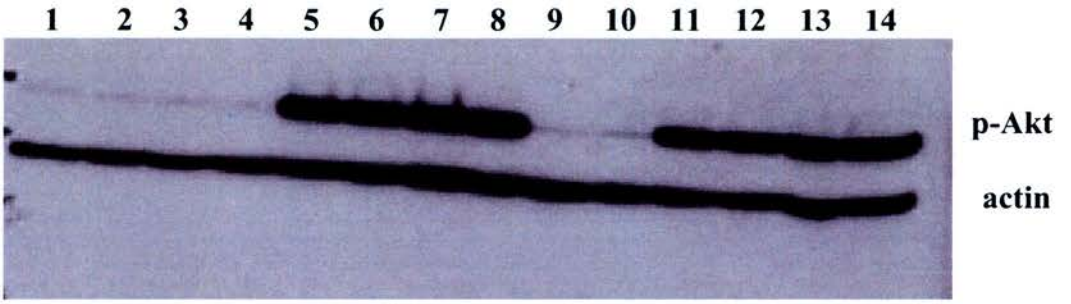


Figure 65
Western blots and Image J quantification for MCF-7 for p-Akt treated for 60 mins in duplicate (Expt A & B) with quantification normalised to actin, demonstrating marked inhibition of p-Akt with RAD001 alone, but not in the presence of heregulin in duplicate experiments.

Figure 66 (A)



MCF-7 pAkt - 24hours (A)

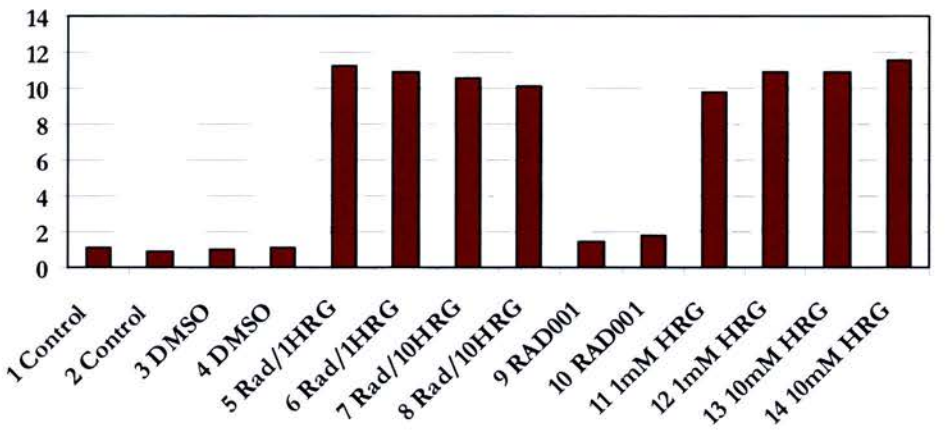


Figure 66 (B) For purposes of analysis the second line (actin) of the top strip and the first line (p-Akt) of the bottom strip were used.

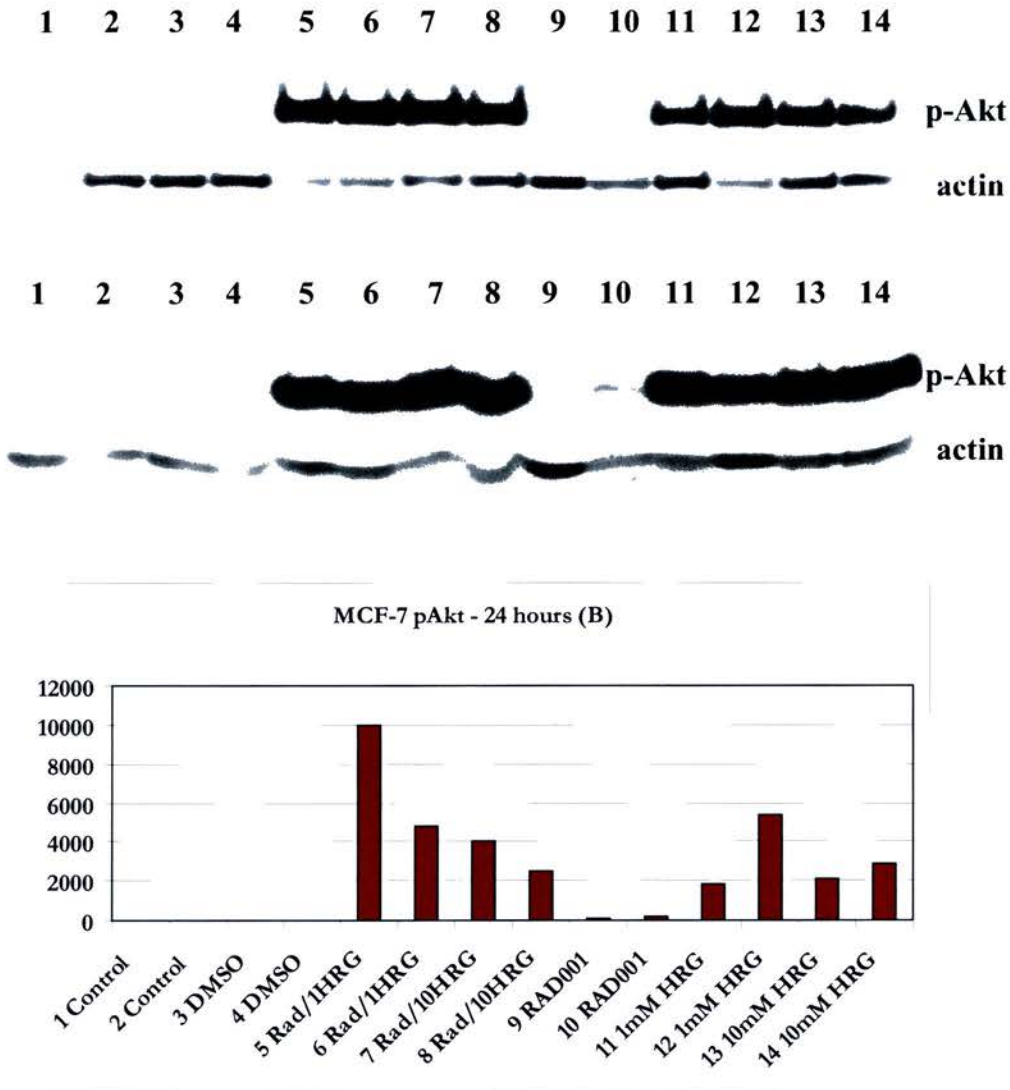
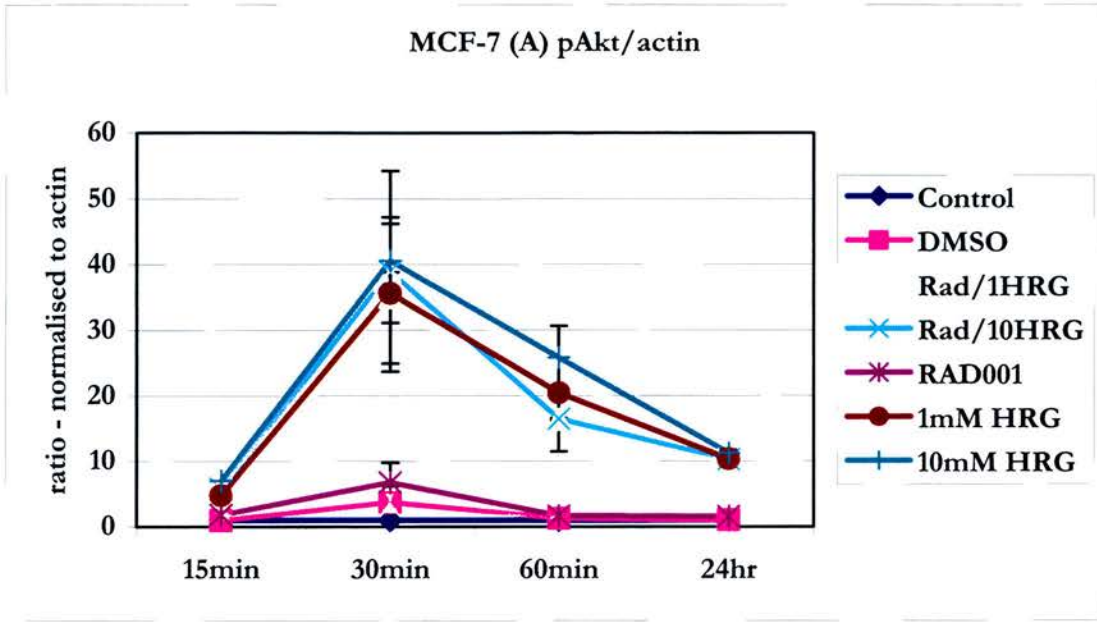


Figure 66
Western blots and Image J quantification for MCF-7 for p-Akt treated for 24 hours in duplicate (Expt A & B) with quantification normalised to actin, demonstrating marked inhibition of p-Akt with RAD001 alone, but not in the presence of heregulin in duplicate experiments.



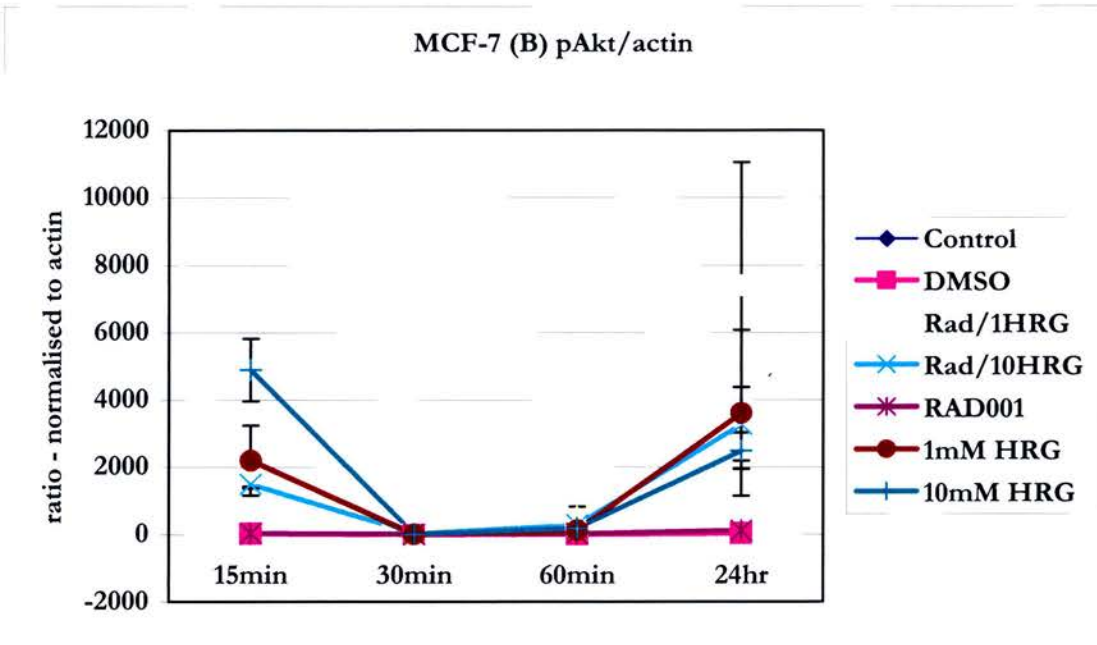
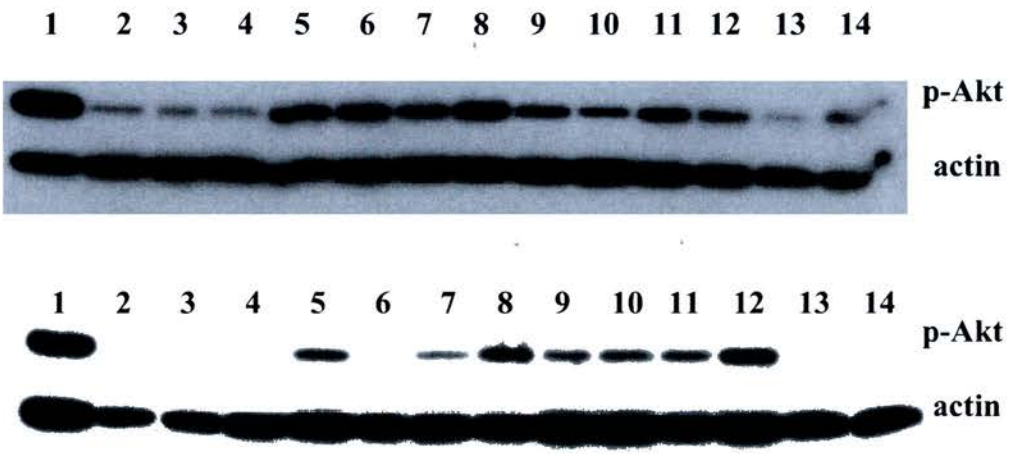


Figure 67
Effect over time of treatment with RAD001 on p-Akt in cell line MCF-7 for duplicate experiments A & B. There is stimulation of p-Akt with heregulin, and inhibition of p-Akt throughout the 24 hour period with RAD001 alone, but not for RAD001 in the presence of heregulin.

MDA-MB-453 cell line

Figure 68 (A) For the purposes of analysis the first line (p-Akt) of the top strip and the second line (actin) of the bottom strip were used.



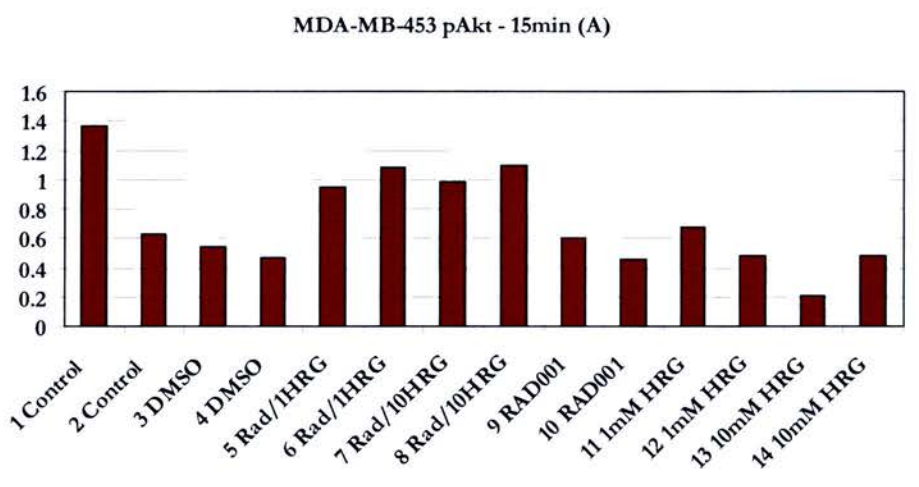


Figure 68 (B) For purposes of analysis the first line (p-Akt) of the top strip and the second line (actin) of the bottom strip were used.

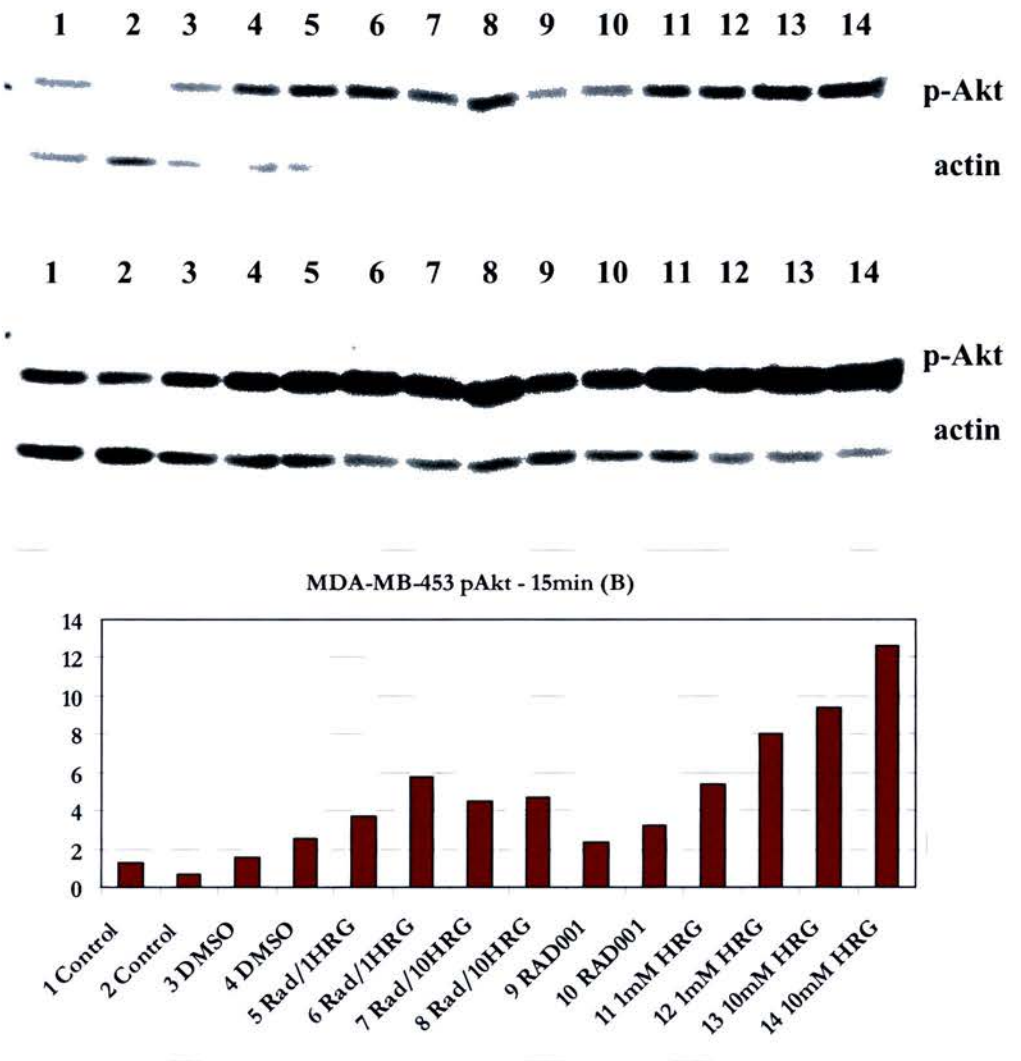
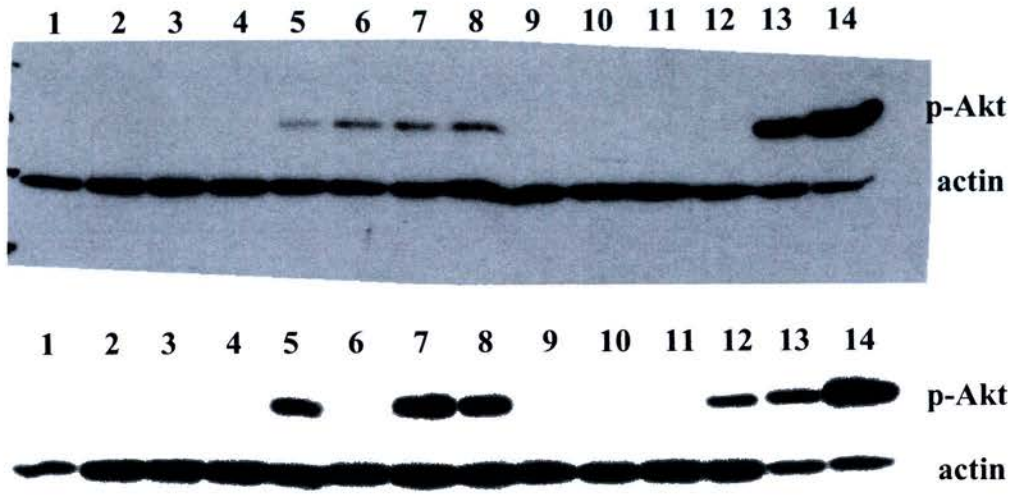


Figure 68
Western blots and Image J quantification for MDA-MB-453 for p-Akt treated for 15 mins in duplicate (Expt A & B) with quantification normalised to actin, demonstrating some inhibition of p-Akt with RAD001 alone, but not in the presence of heregulin in duplicate experiments.

Figure 69(A) For purposes of analysis the second line (actin) of the top strip and the first line (p-Akt) of the bottom strip were used.



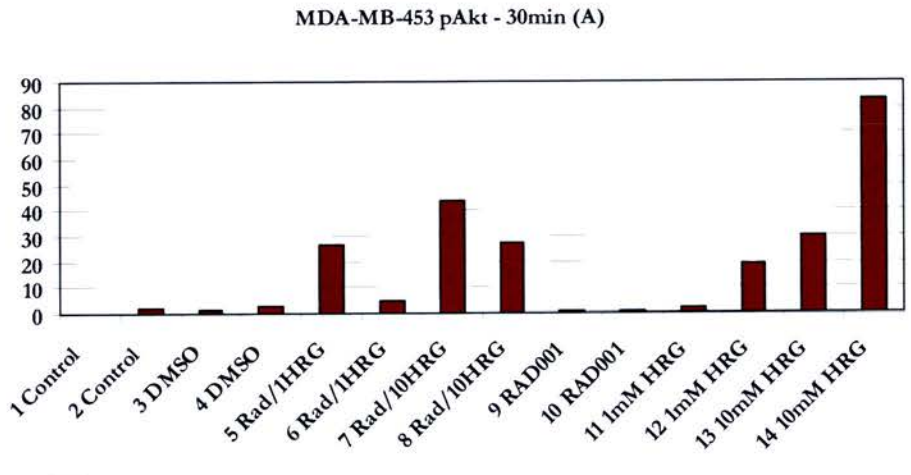
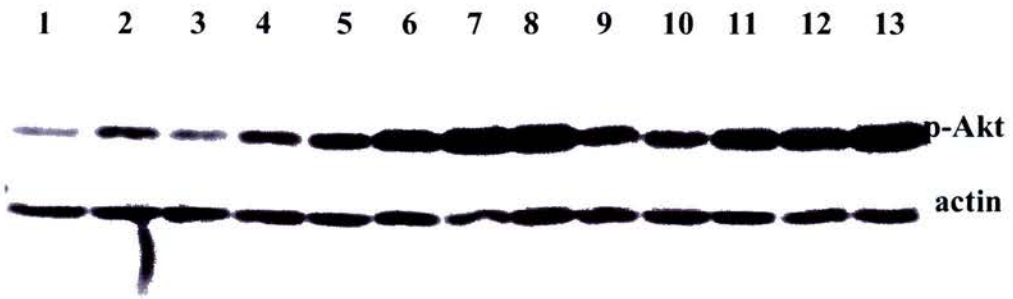


Figure 69 (B)



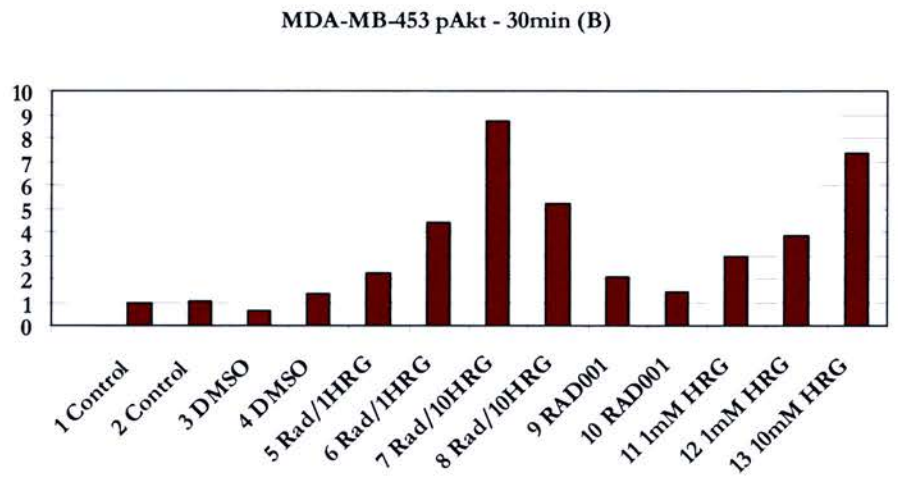
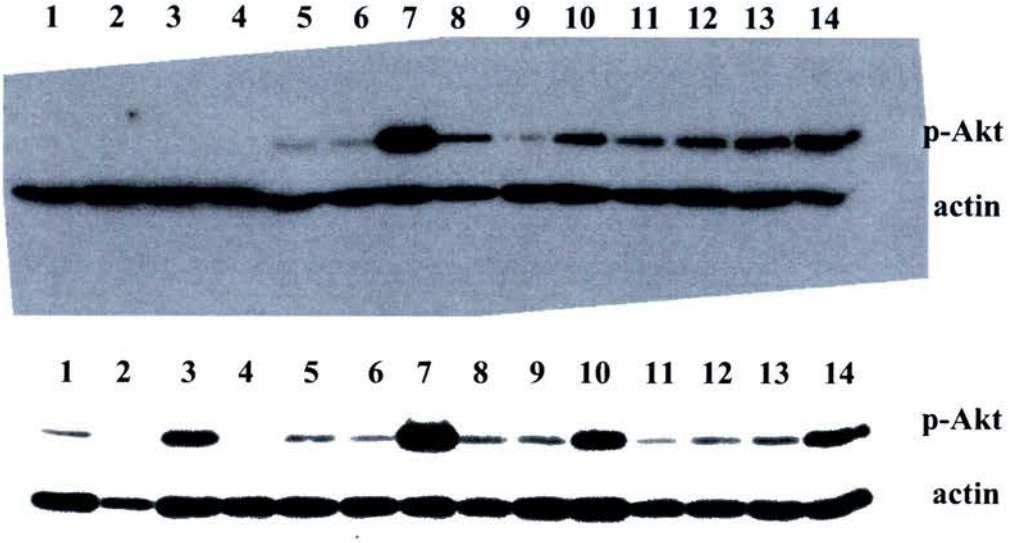


Figure 69
Western blots and Image J quantification for MDA-MB-453 for p-Akt treated for 30 mins in duplicate (Expt A & B) with quantification normalised to actin, demonstrating marked inhibition of p-Akt with RAD001 alone, but not in the presence of heregulin in duplicate experiments.

Figure 70(A) For the purposes of analysis the second line (actin) of the top strip and the first line (p-Akt) of the bottom strip were used.



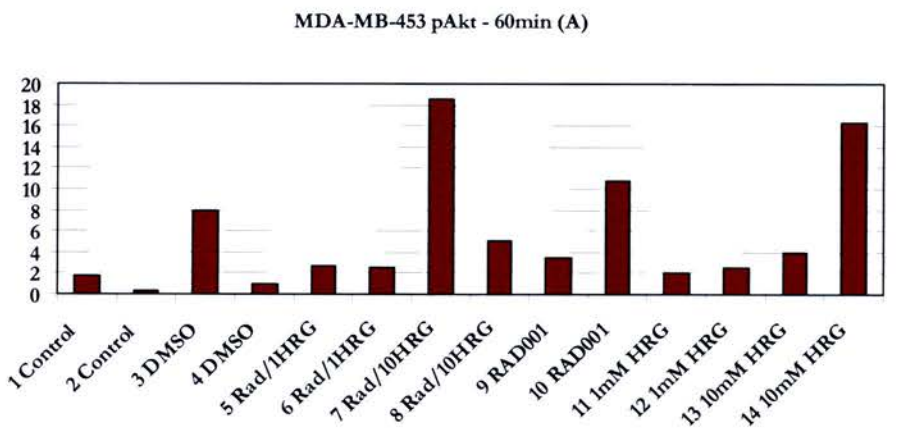
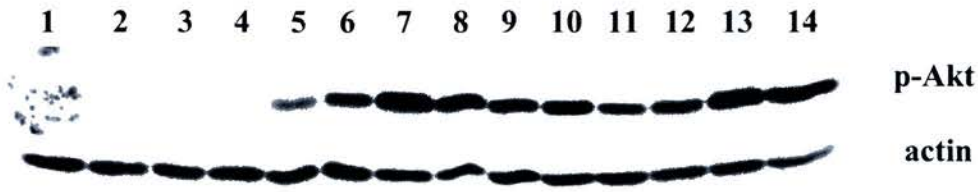


Figure 70 (B)



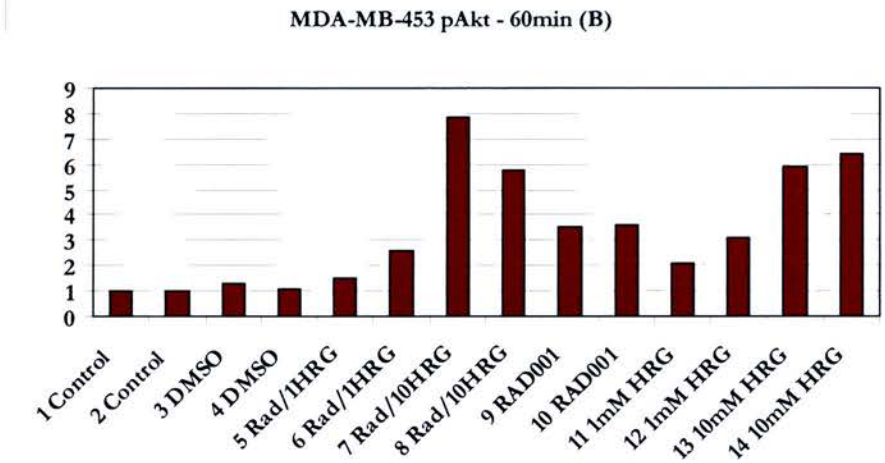
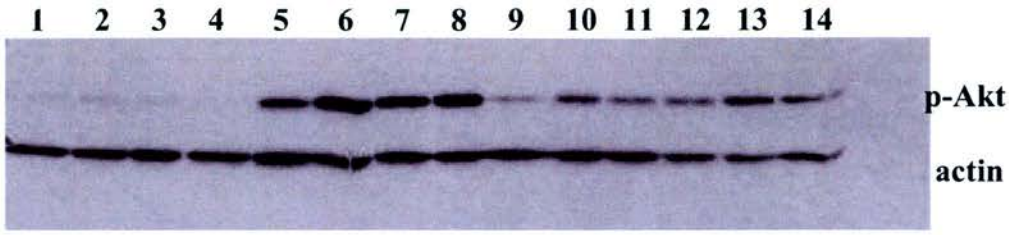


Figure 70
Western blots and Image J quantification for MDA-MB-453 for p-Akt treated for 60 mins in duplicate (Expt A & B) with quantification normalised to actin, demonstrating no sustained inhibition of p-Akt with RAD001 alone or in the presence of heregulin in duplicate experiments.

Figure 71 (A)



MDA-MB-453 pAkt - 24hours (A)

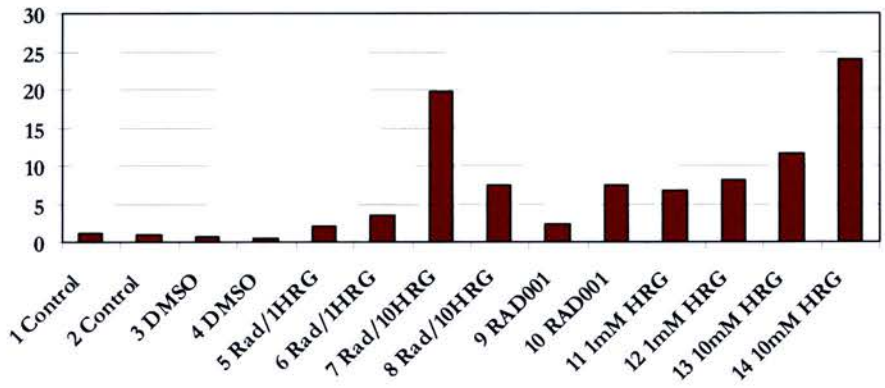


Figure 71 (B)

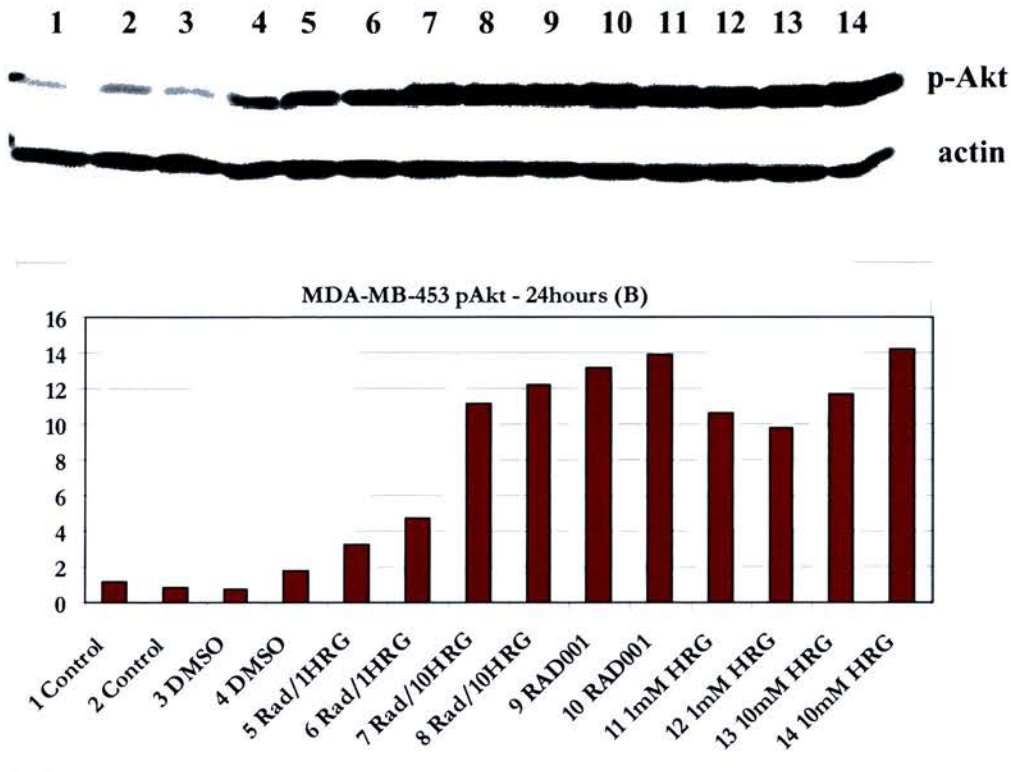


Figure 71
Western blots and Image J quantification for MDA-MB-453 for p-Akt treated for 24 hours in duplicate (Expt A & B) with quantification normalised to actin, demonstrating stimulation of p-Akt with heregulin and in (A) some inhibition of p-Akt with RAD001 alone and in combination with 1nM heregulin, but in (B) no sustained inhibition of p-Akt with RAD001 alone in duplicate experiments.

Figure 72

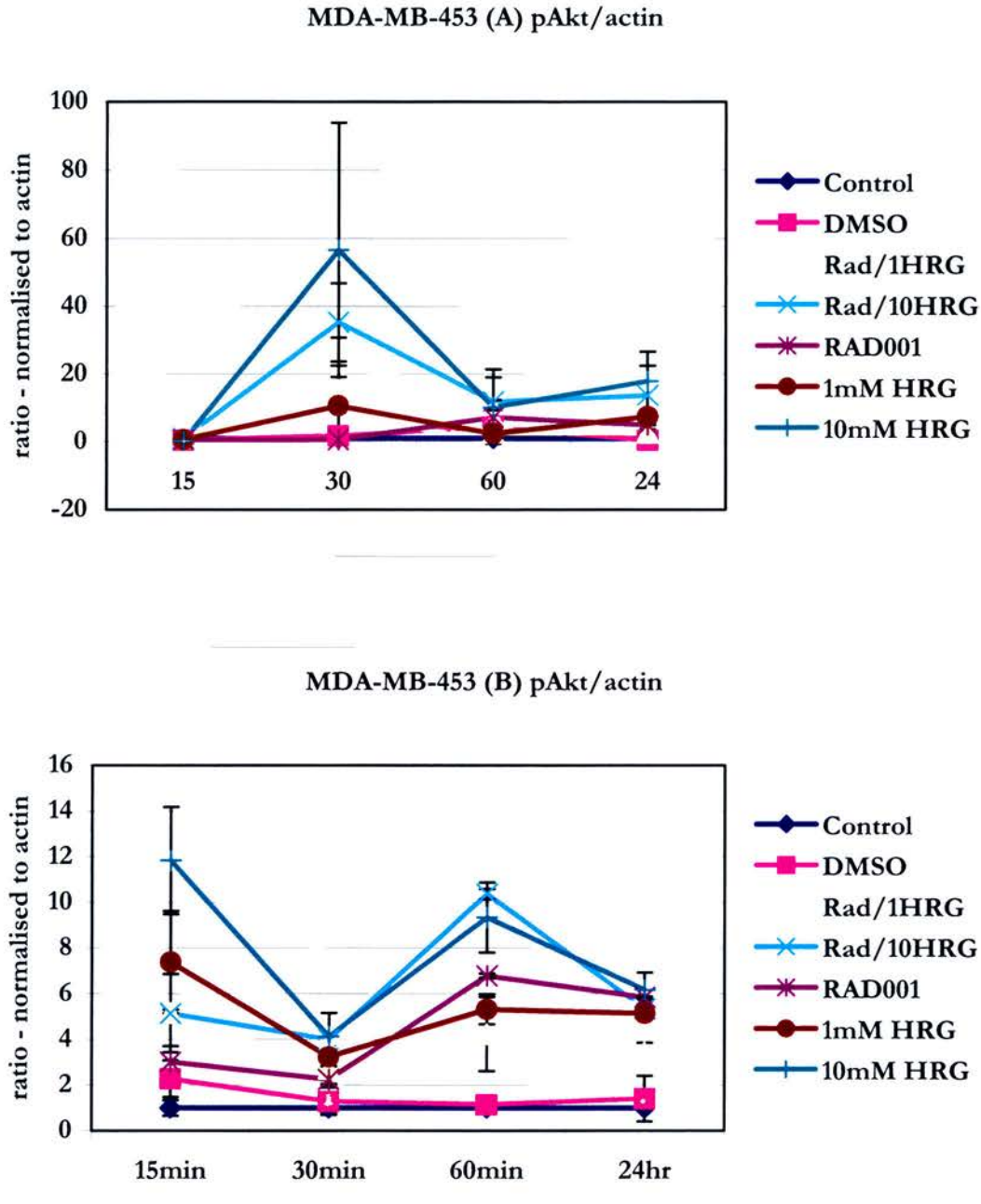


Figure 72
Effect over time of treatment with RAD001 on p-Akt in cell line MDA-MB-453 for duplicate experiments A & B. In (A) there is inhibition of p-Akt sustained throughout the 24 hour period with RAD001 alone, but not for RAD001 in the presence of heregulin, with stimulation of p-Akt with 10nM heregulin. In (B) there is inhibition of p-Akt initially, then less marked for 60 mins and 24 hours, with stimulation of p-Akt in those treated with 10nM heregulin.

Discussion

The primary intention of this cell line study was to further investigate the effects of RAD001 treatment upon p-Akt and the potential significance of p-Akt as a marker of response to RAD001 treatment. In order to achieve this, it was necessary to assess the response to treatment of cell lines expressing low and high levels of p-Akt. To reduce the possibility of results differing between cell lines, the same cell line was analysed both with and without heregulin stimulation. Stimulation of breast cancer cells with heregulin β1 has previously been shown to rapidly phosphorylate Akt with similar effects to transfecting cells with constitutively active Akt. [234]

To determine downregulation of the mTOR pathway p70S6k expression was assessed, and to determine the effects of p-Akt expression this was directly measured. As a result of previous findings that incubation with RAD001 over a short time period downregulates p-Akt, but prolonged treatment (>24 hours) upregulates p-Akt, [231] it was decided to include a 24 hour incubation period for this RAD001 study.

Original pairing of cell lines selected was on the basis of PTEN methylation and ER positivity. This pairing was lost as the results for the cell line MDA-MB-361 were rendered invalid due to contamination of all the stock of that cell line with mycobacteria towards the end of the time of the study. The results from the studies using MDA-MB-361 cell line stock have therefore not been included here.

The two remaining cell lines are therefore MCF7, ER positive and PTEN unmethylated and MDA-MB-453, ER negative and methylated for PTEN. As a result of this lack of pairing, it is difficult to extrapolate from the results any conclusive evidence over the role of PTEN or ER status on the mTOR pathway. From the previous discussion of the role of PTEN in the mTOR pathway it could be expected

that a cell line that is PTEN methylated will have inactivation of the normal PTEN suppressor mechanism of inhibiting the PI3K-Akt pathway. This cell line would thus be expected to display high levels of activation of Akt and be more likely to be sensitive to mTOR inhibition by RAD001 treatment. Contrary to this hypothesis were findings from a study of various tumour cell lines treated with RAD001, which did not show a higher sensitivity of PTEN unmethylated cell lines to RAD001 except for in glioblastoma cell lines. [209] It has however been shown with another mTOR inhibitor CCI-779 (Wyeth Pharmaceuticals) that in wildtype PTEN breast cancer cell line MDA-MB-468 and in glioblastoma studies there is an increase in sensitivity to mTOR inhibition. [182, 235] These MDA-MB-468 cells also had elevated Akt and mTOR signalling.

The results from the MCF7 cell line after treatment with RAD show that there is consistent downregulation of p70S6k, and thus the mTOR pathway at all time points with RAD001 treatment only. RAD001 was less effective at downregulating p70S6k in the presence of heregulin and failed to inhibit p-Akt in the presence of heregulin stimulation. RAD001 reduced the activity of p70S6k with no suggestion of reduction of effect over time with increased length of treatment.

A similar but less pronounced reduction in p70S6k was also seen in the MDA-MB-453 cell line with RAD001 treatment over time. In contrast to the MCF-7 cell line however, the cells that were treated with RAD001 and heregulin also had a reduction in p70S6k, in comparison to those treated only with heregulin. While the duplication of this experiment did not yield results for p70S6k, it could be surmised that the differences between the two cell lines may be related to the PTEN methylation status of the MDA-MB-453 cell line. As stated above, a cell line that is PTEN methylated

will have inactivation of the normal PTEN suppressor mechanism of inhibiting the PI3K-Akt pathway. This cell line may thus be expected to display high levels of activation of Akt. However, it can be seen that in the MDA-MB-453 cell line the expression of p-Akt is less consistent across the samples than that of MCF-7, particularly in the presence of heregulin and at longer treatment time points. It has previously been noted that heregulin β1 failed to induce Akt phosphorylation in an ER negative variant of MCF-7 cells, while the transient transfection of ERα restored the Akt phosphorylation by heregulinβ1, indicating that the mechanism by which heregulin stimulates Akt phosphorylation is mediated by ERα. [234] This would be consistent with our findings of a lack of activation of Akt in the ER negative cell line MDA-MB-453, except at the higher heregulin concentration (10nM). From the same published study there is evidence to suggest that there is a role of HER-2 in the mechanism of heregulin stimulation of Akt phosphorylation. Both cell lines used in the study described herein were HER-2/3 positive.

Treatment with RAD001 in the MCF7 cells produced a significant reduction in p-Akt in cells that were not stimulated with heregulin for each of the time points in the 24 hour treatment period. In MCF7 cells treated with heregulin there was no change in p-Akt in either the control cells or those treated with RAD001 and heregulin, indicating that RAD001 does not reduce p-Akt in the presence of heregulin in this breast cancer cell line.

The limitations of this study were firstly in relation to the lack of pairing for ER and PTEN status, which would have given further information primarily into the heregulin effects upon Akt stimulation but would have given breadth to the study allowing more robust conclusions. The practical limitations of Western blotting and

the subjective nature of the results achieved from this procedure, even with the quantification method allowed by the Image J system, limit the application of the results obtained, and introduce further degree for interpretation bias and error.

It can be concluded from these data that in a PTEN unmethylated and ER positive cell line (MCF-7), RAD001 is effective for up to 24 hours at downregulating the mTOR pathway as assessed by p70S6k, and is also effective at downregulating expression of p-Akt in the absence of heregulin stimulation. In the PTEN methylated and ER negative cell line MDA-MB-453, there was also downregulation of the mTOR pathway as assessed by p70S6k over the time period of 24 hours treatment. However, there was no real pattern of change in expression of p-Akt over the time of treatment with RAD001, and there was a lack of evidence of stimulation of p-Akt by heregulin except at the higher dose (10nM), in keeping with previous studies. These results are in contrast to previous findings of resistance to RAD001 at 24 hours treatment, and are in keeping with the findings of the immunohistochemical results of a reduction in nuclear p-Akt in breast tumours treated with RAD001 and a reduction in cytoplasmic p-Akt in tumours that responded with a reduction in proliferation to RAD001 in this thesis.

Endocrine treated breast tumour study

Aims

In the biological study forming part of this thesis, p-Akt was found to be different between responders and non-responders (as assessed by Ki67) after RAD001, thus identifying p-Akt as a potentially significant marker of RAD001 response and downregulation of mTOR. The aim was to determine baseline expression and change with treatment of p-Akt in tumours with known endocrine responsiveness and endocrine resistance, to further investigate the potential use of RAD001 in combination with endocrine agents to prolong or prevent resistance to treatment.

Patients, Materials and Methods

As part of an ongoing prospective audit in Edinburgh 182 patients with large operable or locally advanced breast cancers that were oestrogen rich (ER 5 or more on Allred score) were treated with neoadjuvant letrozole. [113] All women were postmenopausal, defined as amenorrhea for 1 year or luteinising hormone and follicular stimulating hormone levels in the postmenopausal range. Patients were followed up initially at 2, 6 and 12 weeks to assess tolerance and to measure response with assessment based upon measurement with callipers and imaging (ultrasound at 0, 6 and 12 weeks and mammography at 0 and 12 weeks). Good intra- and inter-observer variability was shown for all three modalities, with the highest accuracy with ultrasound. At 3 months all patients were reviewed by a single surgeon and a treatment decision taken. The decision was either to perform surgery, to continue letrozole, or to switch to another therapy. Diagnostic core biopsies were

taken prior to treatment, and at 3 months after treatment, and were core biopsies if the patient was not proceeding to surgery, or sections from the surgical excision specimen if the patient underwent surgery.

Clinical and ultrasound volumes were calculated using the formula $\pi D^3/6$ where 'D' is the mean diameter of two perpendicular measurements on clinical or mammographic assessment and the mean of two diameters and the depth measurement on ultrasound. A patient with no clinical mass palpable after 3 months was considered as having 100% reduction in clinical volume or a complete clinical response. The % change in clinical volume was calculated using the formula *100 x (initial volume - final volume)/ initial volume*. The change in volume for 3-6 months was thus calculated using the following formula: *100x (volume at 6 months - volume at 3 months)/ volume at 3 months*, and other volumes were calculated similarly.

Modified WHO criteria were used to evaluate tumour response in the neoadjuvant setting as follows [236]:

- *Complete response (CR):* No measurable tumour.
- *Partial response (PR):* Reduction in tumour size $\geq$ 50% from pre-treatment size.
- *Minor response (MR):* Reduction in tumour size $\geq$ 25% and < 50% from pre-treatment size.
- *No change (NC):* < 25% decrease or <25% increase in tumour size from pre-treatment size.
- *Progressive disease (PD):* 25% or more increase in tumour size from pre-treatment size.

There was thus a cohort of patients treated with neoadjuvant letrozole from whom there is both clinical data on response and biopsy tissue available. Results of response to letrozole over time have been published. [113]

For the purposes of this study and based upon the 3 month ultrasound assessments of response, sections were taken from the biopsy samples of 30 patients who had both diagnostic and 3 month tissue available and clear evidence of resistance (no change (NC) or progressive disease (PD)) or a good response (partial (PR) or minor response (MR)). Those patients who had a complete response to treatment had no residual tumour at time of assessment and it was therefore not possible to include this group in the analysis.

From the study of patients treated with RAD001 it was apparent that p-Akt may be an important biomarker for response to mTOR inhibitors. It has previously been shown that patients who develop resistance have high Akt and increased activity of mTOR. [147] To determine whether patients who had resistance to letrozole had an increase in p-Akt, immunohistochemical staining was performed on the samples pre and post letrozole treatment using the same protocols as described earlier (p-Akt, ser 473). Sections were stained in pairs, and double scoring was performed by the same two scorers as for the RAD001 sections. Staining and counting for Ki67 had previously been reported using the same method and protocol for staining and scoring as describer earlier for the RAD001 biological study.

Statistical analysis

Rank correlation was performed to determine correlation between variables in a non-parametric distribution. Wilcoxon test was carried out for paired samples and Mann-Witney test for independent samples.

Results

Sections of tissue were available from 21 patients who had a partial or minor response to letrozole and from 8 patients who had progressive disease or no change in tumour size after 3 months treatment with letrozole. Of those who had a minor response on USS (n=4), one patient had tumour volume reduction of 36%; in another case this response was confirmed on bidimensional calliper measurement to be a minor response with 48% reduction in tumour volume, and in three other cases was found to be greater with callipers with partial response (>50% tumour volume). Proliferation was measured by Ki67 (%) staining by the same protocol described earlier. For the 29 tumour pairs examined median Ki67 before letrozole treatment was 10.3% (95%CI 5.5, 12.1), reduced to median 1.1% (95%CI 0.8, 2.8) after letrozole treatment. There was a significant reduction in Ki67% for the whole group after letrozole treatment ($p<0.0001$), sustained in subgroup analysis by clinical response. **Figure 73** There was no significant difference in pre-treatment proliferation by Ki67% or change in Ki67% between those that had a clinical response to letrozole and those that did not respond.

There was no significant change in scores for p-Akt in either cytoplasm or nucleus with letrozole treatment for the whole group. No significant difference was found in p-Akt in cytoplasm or nucleus in subgroup analysis for clinical responders or non-responders, although there were numerical differences between responders and non-responders. Of those that responded to letrozole clinically (n=21), 71.4% (n=15) had a reduction or zero histoscore for nuclear p-Akt, and 62.5% of those that did not respond (n=5) had an increase or no change in histoscore after letrozole. **Figure 74 and 75; Tables 17 and 18**

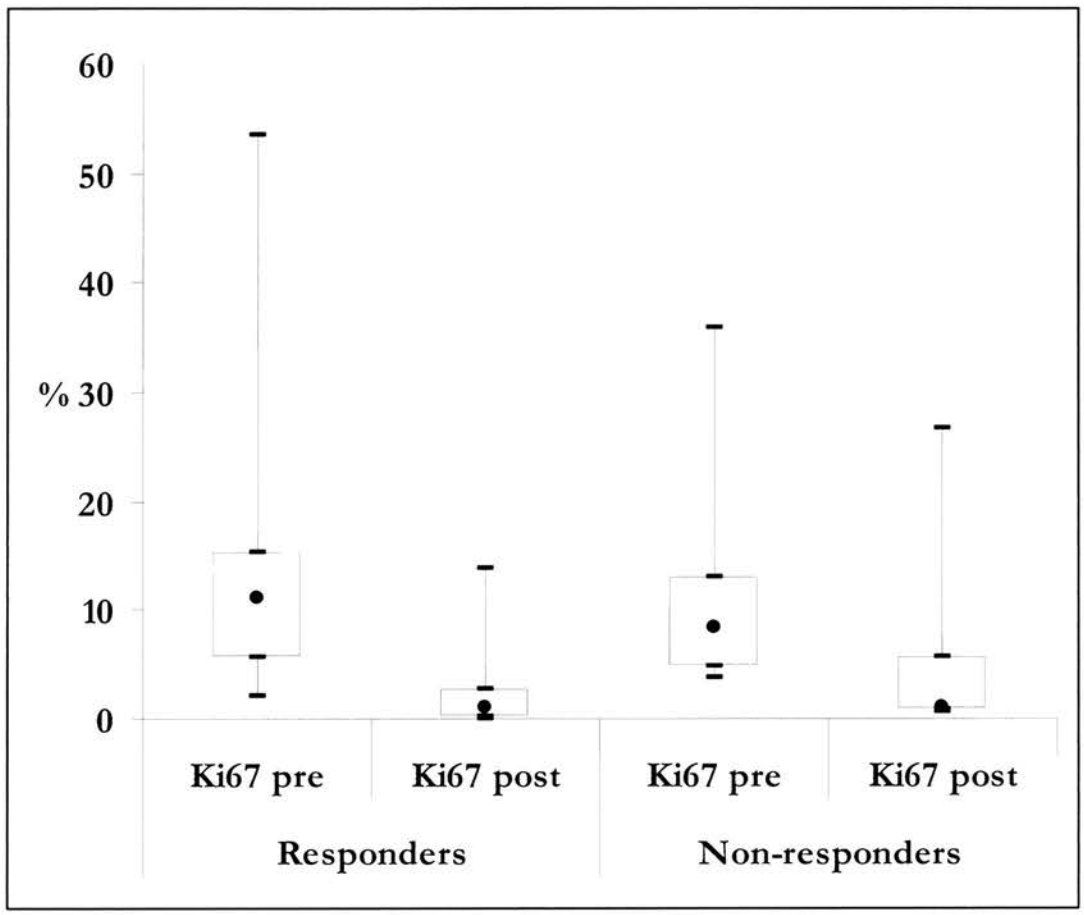


Figure 73
Proliferation by Ki67 (%) pre and post 3 months of letrozole treatment, demonstrating significant reduction in proliferation in both responders (as per clinical assessment, $p<0.0001$) and non-responders ($p=0.0078$), but no significant difference between the two groups.

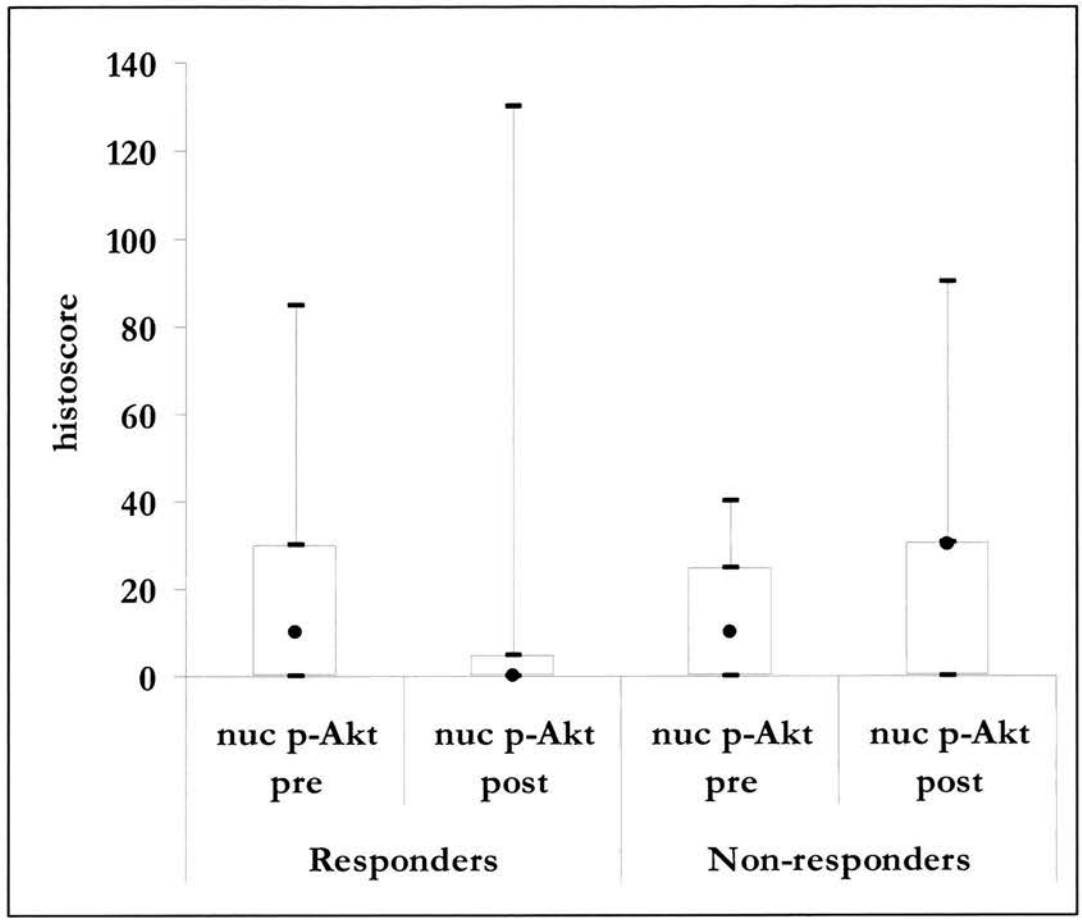


Figure 74
Histoscores for nuclear p-Akt (nuc p-Akt) pre and post letrozole treatment for 3 months, showing no significant change in either clinical responders (R) or non-responders (NR) (R, median histoscore pre 10, 95%CI 0, 31.0; post 0, 95%CI 0, 7.6; p=0.6257; NR, median histoscore pre 10, 95%CI 0, 40.0; post 30, 95%CI 0, 74.0; p=0.2187)

Response to letrozole	No nuclear p-Akt n (%)	Increase in p-Akt n (%)	Decrease in p-Akt n (%)	No change n (%)
Responders	7 (33.3)	4 (19.0)	8 (38.1)	2 (9.5)
Non-responders	1 (12.5)	4 (50)	2 (25.0)	1 (12.5)

Table 17
Summary of changes seen in nuclear p-Akt after letrozole treatment, showing that 50% of non-responders had an increase in nuclear p-Akt after treatment, compared with 19% of responders.

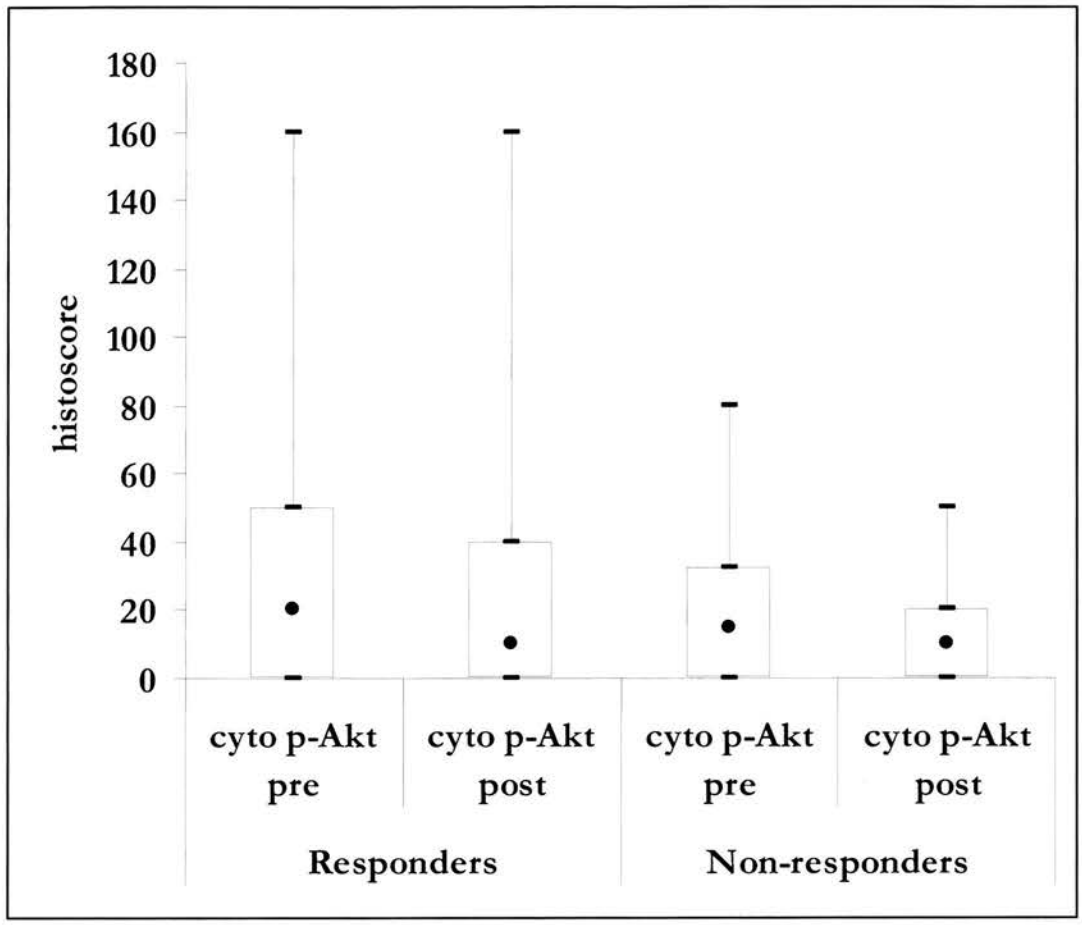


Figure 75
Histoscores for cytoplasmic p-Akt (cyto p-Akt) pre and post letrozole treatment for 3 months, showing no significant change in either clinical responders (R) or non-responders (NR) (R, median histoscore pre 20, 95%CI 0, 51.7; post 10, 95%CI 0, 40.9; p=0.8536; NR, median histoscore pre 15, 95%CI 0, 68.8; post 10, 95%CI 0, 41.6; p=0.6250)

Response to letrozole	No cyto p-Akt n (%)	Increase in p-Akt n (%)	Decrease in p-Akt n (%)	No change n (%)
Responders	4 (19.0)	7 (33.3)	9 (42.9)	1 (4.8)
Non-responders	2 (25.0)	2 (25.0)	3 (37.5)	1 (12.5)

Table 18
Summary of changes seen in cytoplasmic p-Akt after letrozole treatment, showing no difference in cytoplasmic p-Akt between responders and non-responders.

No correlation was found between pre-treatment levels of p-Akt (either nuclear or cytoplasmic) expression and pre-treatment Ki67. There was a significant positive correlation between Ki67 % pre-letrozole and the change in Ki67 with treatment (Spearmans (rho) 0.916, $p<0.0001$) for the whole group and for subgroup analysis of clinical responders (rho 0.947, $p<0.0001$) and non-responders (rho 0.905, $p=0.0167$). There was no correlation between pre-letrozole scores for (either nuclear or cytoplasmic) p-Akt and change in Ki67 with treatment (nuclear staining $p=0.98$; cytoplasmic staining $p=0.41$) for the whole group or the subgroup analysis of responders or non-responders.

Discussion

As can be seen from the above data, the nuclear and cytoplasmic scores for p-Akt prior to treatment are similar in tumours that respond and those that do not respond to letrozole. It is not possible from these data to determine any predictive value of initial levels of p-Akt for response to endocrine treatment. There was a trend toward an increase in nuclear p-Akt in those tumours that are unresponsive to letrozole over 3 months treatment (median histoscore 30 after treatment) compared with no change or a decrease in those tumours that responded (median histoscore 0 after treatment), but this did not reach statistical significance. Despite the small sample size, 50% of those that were resistant to letrozole had an increase in nuclear p-Akt. There was no difference in nuclear or cytoplasmic scores for p-Akt with 3 months treatment, nor between the group who responded to treatment and those who were resistance to treatment. One other study has reported results of a retrospective analysis of tumours from patients treated with endocrine therapy for expression of p-Akt (ser 473). [237] Clinical efficacy data was available for 36 patients with metastatic breast cancer who had received endocrine therapy (median follow-up period not stated). 12 (33%) tumours were positive for p-Akt (defined as >10% cytoplasmic staining), with a positive correlation between HER-2 positivity and p-Akt staining ($p<0.01$). Clinical response rate was poorer in those with HER-2 positive tumours ($p<0.05$) and those with Akt phosphorylation ($p<0.01$), with the smallest clinical benefit in patients with both HER-2 and p-Akt positivity ($p<0.01$), although this subgroup was of small size (n=3). In this study there were a number of flaws including no description of the method of assessment of clinical response, no follow-up time period stated and multiple endocrine therapies used (anastrozole or exemestane n=23, tamoxifen or

tremifen n=15, LHRH agonist +- tamoxifen n=7, medroxyprogesterone acetate n=1). The authors attempted to assess the response rates according to therapy used, but the small numbers make any conclusions drawn questionable. The results are promising in view of the results of the biological study suggestive of a greater response to RAD001 in tumours that are HER-2 positive and high in p-Akt, thus the potential value of RAD001 in tumours that respond less well to endocrine agents.

One of the drawbacks of the pilot study described in this thesis was that tissue samples were utilised retrospectively and there were limitations with the volume of tissue available in what is a valuable tumour series. The other main flaw in design of this study that reflects the difficulty with pre-operative trials is that those patients who had the best sensitivity to letrozole reflected in a complete response were automatically self-selected out of analysis as no tumour was available after treatment due to complete pathological response. This highlights the importance of studies assessing the predictive value of 2 week biopsy for response, as suggested for Ki67 in previous letrozole studies. [117]

One other study has reported results analysing the mTOR pathway in patients treated with letrozole. [238] The original design of the study was a comparative study of letrozole alone versus letrozole with metronomic oral cyclophosphamide in patients aged over 70 years with T2-4, N0-1, ER positive and/or PR positive breast cancer randomised to treatment for 6 months. From the biological samples from 114 patients collected at the time of entry into the study and at surgery after 6 months treatment tissue microarrays were constructed and a retrospective analysis of the mTOR pathway was performed using immunohistochemistry to assess PI3k, p-Akt (ser 473), p-mTOR (ser2448), ERα (ser 118), Ki67, HER-2, p53 and bcl-2. Scoring of

immunohistochemistry was performed by one observer scoring on intensity of staining only: (0) no staining, (1) weak staining, (2) moderate staining, (3) strong staining. A high baseline positive expression of p-Akt (91%), PI3k (97%) and p-mTOR (88%) was noted. Baseline expression of p-Akt had no correlation with tumour variables such as grade, stage, histology, ER, PR or HER-2 status. There was a positive correlation between baseline p-Akt expression and baseline Ki67, but without an increasing trend of Ki67 making the significance doubtful. With both treatment arms of the study combined there was a significant reduction in expression of PI3k ($p=0.002$), p-Akt ($p<0.05$) and p-mTOR ($p=0.00001$) with treatment over 6 months. In the letrozole alone arm of the study the reduction in p-Akt expression did not reach significance ($p=0.84$). Disease response was obtained in 41 of 57 patients in the letrozole arm, and in 50 of 57 in the letrozole and cyclophosphamide arm. For all patients, a reduction in p-Akt was associated with better disease response ($p=0.05$), with no significant relationship to response for PI3k and p-mTOR. Ki67 also decreased after treatment in both arms, and this reduction significantly correlated with p-Akt reduction ($p<0.05$), but not with PI3k or p-mTOR. Baseline expression of PI3k, p-mTOR and p-Akt did not show a predictive role for disease-free survival at median follow-up of 46 months. These findings of an association between reduction in p-Akt and better disease response support the growing body of evidence for the use of mTOR inhibition, and for combined use with endocrine agents for greater response.

Studies are underway using letrozole and RAD001 to trial the evidence of the combination approach to prevent resistance to letrozole through the mTOR pathway.

In a Phase I study of RAD001 18 patients who had metastatic or locally recurrent advanced breast cancer who had received letrozole for ≥4months without objective response (stable or progressive disease) were entered sequentially to continue on letrozole and to receive 5mg RAD001 (first 6 patients) or 10mg RAD001 (next 12 patients). [239] Primary outcomes were for safety, pharmacokinetics and efficacy by tumour response. The most commonly observed adverse events (AEs) were mouth ulcers (n=3/6 with 5mg RAD001, n=6/12 with 10mg RAD001), anorexia (n=0/6 with 5mg, n=8/12 with 10mg) and fatigue (2/6 with 5mg, 6/12 with 10mg). The combination of letrozole and RAD001 did not appear to alter the pharmacokinetics of RAD001. One complete response was seen in the 10mg RAD001 arm in a patient with skin lesions who had a sustained response for 22 months who had progressive disease on letrozole alone. One patient had a volume reduction in liver metastases by 28% and had stable disease for 13 months, having been on letrozole therapy for 2 years prior to the study.

There has been one randomised study of letrozole alone or in combination with RAD001. [240] In this study 270 patients with newly diagnosed ER positive breast cancer >2cm were randomised to receive either letrozole 2.5mg and RAD001 10mg once daily or letrozole 2.5mg and placebo for 16 weeks prior to surgery. The primary outcome was response rate by palpation at 4 months, with response rate by mammography and ultrasound, rate of breast conservation and rate of pathological complete response as secondary outcomes. Tumour biopsies were taken at 0 and 15 days. The two randomised groups had similar baseline age, tumour stage, proposed breast conservation or mastectomy. There was a trend toward a higher response rate in the group who received letrozole and RAD001 with an overall response rate of

68.1% versus 59.1% in the letrozole and placebo group (p=0.062). This trend reached significance when the response was assessed by ultrasound scan (58% with RAD001 and letrozole and 47% with letrozole alone, p=0.035) and on clinical examination (66.7% RAD001 and letrozole vs 54.5% letrozole alone, p=0.021). There were significantly more adverse events in the RAD001 and letrozole group (89.8%) compared with letrozole alone (63.6%), with the rate of grade 3/4 adverse events (AE) 22.6% in the RAD001 + letrozole arm versus 3.8% in the placebo + letrozole arm. The most frequently occurring AEs were mouth ulcers (36.5% with RAD001 and letrozole versus 6.1% with letrozole alone), rash (20.4% versus 7.6%) and fatigue (17.5% versus 9.8%). The most frequent grade 3/4 AEs with RAD001 + letrozole were hyperglycaemia (n=7), mouth ulcers (3), interstitial lung disease/ pneumonitis (3) and infections (3). In the RAD001 and letrozole group 46.4% discontinued treatment before 112 days, compared to 18.9% discontinuing letrozole alone. This rate of discontinuation is higher than results for the single agent study performed as part of this thesis and this may be due to the higher 10mg dose of RAD001 used within the combination study, as well as a longer study time period. This combination study was designed to also incorporate testing a number of candidate predictive response markers with the aim of determining potential biomarkers of response. [241] Those biomarkers analysed by immunohistochemistry were ER, PR, Ki67, S6 total and p-S6 (235/236 and 240/244), total Akt and p-Akt (s473), PTEN, cyclin D, AIB1; HER-2 by FISH; PI3kinase and p53 were analysed by mutational analysis. PTEN and cyclin D in this study were analysed using a prototype assay developed in collaboration with Dako. Biomarker evaluation was possible on 181 patients who had sufficient treatment duration. Of the 181 biomarker

evaluable cases there were 79 in the combination arm and 102 in the letrozole alone arm. Marked downregulation in progesterone receptor and cyclin D1 were seen in response to letrozole. Phospho-S6 levels showed dramatic down-regulation only in response to RAD001. Cell cycle response, as defined by either % reduction in Ki67 at day 15 or by proportion of patients with Ki67$\leq$2 at day 15, was also significantly higher in the RAD001 + letrozole arm (57% vs 30% for Ki67$\leq$2 at day 15, $p<0.01$). The increased cell cycle response rate in the RAD001 arm was found in all subsets of tumours, including PTEN-positive, PIK3CA wild-type tumours.

Of the 79 cases in the letrozole and RAD001 arm, 44 had high pre-treatment expression of p-S6 (240/244) with a response rate in this subgroup of 81.8%, compared with a response rate of 60% in those with low pre-treatment expression of p-S6 ($p=0.216$). This only reached significance when those patients who did not have sufficient treatment duration were included (n=26, 25 of which were from the RAD001 and letrozole treatment arm) which improved the significance to $p=0.028$. For the letrozole alone arm, the expression of p-S6 was high in 53/101 cases, with response rate of 67.9% in the high p-S6 pre-treatment subgroup versus 58.3% in the low p-S6 pre-treatment subgroup (p=NS). In our biological study of RAD001 5mg as a single agent there was a trend toward a greater reduction in p-S6 scores in those tumours that responded to treatment (as measured by Ki67) compared with those that did not respond. No other biomarkers analysed in this study were identified as potential predictors of response to RAD001. There were only small numbers of patients with HER-2 positive tumours in the study, and no data is available for the response rates in the HER-2 positive versus negative groups. In the RAD001 and letrozole arm 50/79 (63.3%) had high p-Akt expression, with response rates of 74%

versus 69% for high and low pre-treatment p-Akt (p=0.999). The rates were similar for the letrozole alone arm. High Ki67 was not found to predict for response to RAD and letrozole or letrozole alone, but a significantly higher proportion of patients in the RAD001 arm had an antiproliferative response defined as logarithm of percentage positive Ki67 of less than 1 at day 15. These improved response rates with combination of letrozole and RAD001 are suggestive of an increased response to letrozole in the neoadjuvant setting. There is a need to assess the lower dose of RAD001 as used in this thesis in order to determine if the high rate of discontinuation due to side effects can be reduced with lowering the dose. If this is the case then longer follow-up studies would be required to assess the potential benefits of RAD001 in combination with endocrine agents to prevent or delay the development of resistance to these agents.

While the scope of this study is of the potential role of RAD001 in the prevention or delay of endocrine resistance, there are now emerging studies investigating RAD001 in combination with signal transduction agents, thus far only in cell lines. RAD001 in combination with the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor gefinitib was found in non-small cell lung cancer cells to suppress both S6 activation and MAPK signaling, and had an increased growth inhibitory effect compared with either agent alone. [242] In another study in renal cell cancer cell lines, RAD001 in combination with a duel EGFR and vascular endothelial growth factor (VEGF) receptor inhibitor, AEE778, inhibited cell proliferation. [243] These combinations of mTOR inhibition with other therapeutic agents targeted at specific signalling pathways are encouraging, and warrant further investigation in the clinical setting.

Conclusions

Breast cancer is a common cancer in the UK, with 100 new cases diagnosed every day. [1] Management of breast cancer has traditionally been local treatment with surgery followed by systemic treatment with chemotherapy or hormonal agents. Neoadjuvant treatment has been employed for inoperable or large tumours, and more recently has been recognised as useful for determining response to agents in the longer term, rather than awaiting recurrence. This in turn has led to the concept of tumour profiling to determine response to treatment. The best example of this is ER status which has been shown to be important in determining response to aromatase inhibitors and tamoxifen. [36, 59, 70-73] Despite ER positivity, however, a significant problem with the use of these hormonal agents is the development of resistance, either de novo or acquired over time with treatment. Signal transduction pathways have been implicated in this resistance mechanism, such as the PI3K/ Akt/ mTOR pathway. [119-121, 124] Breast tumours with high levels of p-Akt and mTOR expression are associated with resistance to treatment, increased local recurrence and poorer overall survival. [147] Preclinical studies of mTOR inhibitors have shown restoration of response to tamoxifen and that the combination of tamoxifen and mTOR inhibitors has synergistic antitumoural effect. [149]

This is the first clinical study to report results of RAD001 5mg daily in early breast cancer, a dose that was tolerated by most patients in the study, with the exception of the most commonly reported symptom of mouth ulcers. Of the eight patients who experienced this side effect, four withdrew from the study with Grade I or II (mild or moderate) level symptoms. Patients were aware that the drug was on a trial basis and not for treatment purposes, and this may have influenced the tolerability of

symptoms for patients. It is reasonable to expect that the level of tolerability of a drug in the early stage of treatment in a pre-operative setting will be different than that in an adjuvant or advanced study due to different levels of expectation and previous experience of patients. The significant reductions in white cells and neutrophils with RAD001 treatment are of concern, particularly in the pre-operative setting. There was no increase in infective complications in the patients participating in this study, and in particular among those who had reduction in neutrophils counts, and all those who had reduced neutrophils had an appropriate response to surgery with a raise in neutrophil counts post-operatively. Had information regarding the nadir of 14 days in these blood parameters been available prior to commencement of the study, it may have been necessary to reconsider the duration of treatment to avoid coinciding with this nadir. Similarly, the reduction in platelets could have resulted in significant peri-operative bleeding. There were 4 patients in the group who developed haematoma, but in only one of these patients was the platelet count reduced. The timing of this study coincided with the introduction of the use of low molecular weight heparin different to that used previously in the Edinburgh Breast Unit, and it was noted that there was an increase in bleeding complications during that time period, resulting in a subsequent switch back to the original prophylactic therapy, with resolution of this problem. [186]

The primary outcome measured in this pre-operative study was the baseline and change in proliferation with treatment with RAD001 as measured by Ki67. The significant reduction in proliferation with 5mg RAD001 confirms the antitumoural activity of RAD001 at this dose. Given the problems of tolerability of RAD001 at 10mg experienced in the letrozole-RAD001 combination trial, [240] it is encouraging to

see mTOR inhibition confirmed by reduction in p-S6 and proliferation at a dose of 5mg daily. These data would suggest this dose will be effective and tolerable in patients with early breast cancer.

Tumours that had the greatest reduction in proliferation were those that had high levels of proliferation and were of higher tumour grade before treatment. These highly proliferative tumours had high levels of p-Akt before treatment. Neither p-Akt, p-mTOR nor p-S6 were shown to be predictive for response to RAD001 as measured by reduction in proliferation.

The significant reduction in nuclear phosphorylation of Akt with RAD001 treatment indicates inhibition of both mTOR raptor and mTOR rictor in the clinical setting. It was originally thought that the mTOR rictor complex was rapamycin insensitive, but recent cell line work has suggested that prolonged treatment with rapamycin results in inhibition of rictor. [231] The clinical study described herein shows that with RAD001 nuclear p-Akt was reduced in almost all cases, and cytoplasmic p-Akt reduced in those tumours with high scores pre-treatment, suggesting tumours with high p-Akt are more sensitive to RAD001. Further investigation of this in a clinical setting by selecting tumours on the basis of p-Akt score is recommended to further assess this.

We were unable to confirm in cell line studies the influence of varying p-Akt expression on the activity of RAD001. In the PTEN unmethylated, ER positive MCF7 cell line there was reduction of mTOR activity as assessed by p70S6k with RAD001 in the absence of heregulin stimulation. The heregulin treated cells had increased p-Akt compared with controls but RAD001 treatment did not have a demonstrable effect upon p70S6k in the presence of heregulin. In the MDA-MB-453

cells the results are more difficult to interpret due to the mechanism by which heregulin upregulates Akt and a lack of p-Akt stimulation in response to heregulin, particularly over time. It can be concluded that in a PTEN unmethylated, ER positive cell line RAD001 is effective up to 24 hours at downregulating the mTOR pathway as assessed by p70S6k in the absence of heregulin stimulation, but due to incomplete cell line pairing the conclusivity of these results is limited to this interpretation.

The clinical significance of this potential use of p-Akt as a predictive marker lies in previous findings of elevated p-Akt and mTOR activity in tumours resistant to endocrine therapy, suggesting a promising role for the combination of mTOR inhibitor and endocrine inhibition. From our study of tumours before and after treatment with letrozole there was a trend towards a reduction in nuclear phosphorylation of Akt in those who responded to letrozole, and an increase in those that were resistant, but with no difference in baseline values between responders and non-responders to suggest a predictive value. This may be due to small sample size but similar lack of predictive value of p-Akt was found in the combination study of RAD001 and letrozole. [241]

Tumours were not selected for this study on the basis of tumour type, ER, PR or HER-2 status, although all tumours had some level of ER positivity. There appeared to be a greater response in tumours that were HER-2 positive. Combining this broad range of baseline tumour characteristics may have affected the results of this study, as these represent biologically different tumours with potentially different mechanisms and drivers for proliferation, and different clinical outcomes. [219] Further studies should focus on more defined populations to avoid this potential dilution of

results, in addition to investigation more specifically on tumours with high proliferation, high p-Akt and HER-2 positivity.

The findings of the biological study demonstrate the effectiveness of RAD001 at a dose of 5mg daily at reducing proliferation in early breast cancer. Further study is warranted to investigate the potential role of p-Akt as a predictive tool for RAD001 response, and to investigate combination therapy at a dose of 5mg to reduce the rate of adverse events and thus completion of treatment. This would allow analysis of the role of mTOR inhibition in preventing or delaying endocrine resistance.

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Appendices

1. **Known inhibitors or inducers of isoenzyme CYP3A**
2. **Patient Information Sheet**
3. **Consent form**
4. **Patient Diary**
5. **GP Information sheet**
6. **Ethics approval letter**
7. **Hospital Research and Development approval letter**
8. **Medicines and Health Regulatory Authority approval letter**

Appendix 1
Known inducers and inhibitors of isoenzyme CYP3A (those in common use have been italicised)

Inducers

Carbamazepine
Dexamethasone
Ethosuximide
Glucocorticoids
Griseofulvin
Nafcillin
Nelfanivar
Oxcarbazepine
Phenobarbital
Phenylbutazone
Phenytoin
Primidone
Progesterone
Rifabutin
Rifampicin
Rofecoxib (mild)
St John's wort
Sulfadimidine
Sulfinpyrazone
Troglitazone

Inhibitors

Amiodarone
Anastrozole
Azithromycin
Cannabinoids
Cimetidine
Clarithromycin
Clotrimazole
Cyclosporin
Danazol
Delavirdine
Dexamethasone
Diethydithiocarbamate
Diltiazem
Dirithromycin
Disulfiram
Entacapone

Inhibitors (cont)

Erythromycin
Ethinyl estradiol
Fluconazole (weak)
Fluoxetine
Fluvoxamine
Gestodene
Grapefruit juice
Indinavir
Isoniazid
Itraconazole
Ketoconazole
Metronidazole
Mibefradil
Miconazole (moderate)
Nefazodone
Nelfinavir
Nevirapine
Norfloxacin
Norfluoxetine
Omeprazole (weak)
Oxiconazole
Paroxetine (weak)
Propoxyphene
Quinidine
Quinine
Quinupristin and dalfopristin
Ranitidine
Ritonavir
Saquniavir
Sertindole
Sertraline
Troglitazone
Troleandomycin
Valproic acid (weak)
Verapamil
Zafirlukast
Zileuton

Please Reply To: **Edinburgh Breast Unit, Western General Hospital, Edinburgh EH4 2XU.**
Tel: 0131 537 2907 Fax: 0131 537 2653 email: jmd@ed.ac.uk

A STUDY TO EVALUATE THE BIOLOGICAL EFFECTS OF RAD 001 ON INVASIVE BREAST CANCER

Patient Information Leaflet

You are being invited to take part in a research study. Before deciding, it is important that you understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear, or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the study?

The Edinburgh Breast Unit is involved in a range of studies trying to identify new and effective drugs for breast cancer. RAD001 is a drug that is already used in patients who have had a kidney or liver transplant. There is some evidence that is may be an effective treatment for breast cancer.

RAD 001 is a drug which works against growth factors produced by tumours. Normal cells are under strict control. One of the mechanisms by which growth is controlled is through agents called growth factors. If you cut your finger growth factors are produced and this stimulates the skin to produce more cells to heal the cut. As soon as the cut has healed, the flow of growth factors is reduced to stop the skin overgrowing. As part of the cancer forming process, abnormal cells including cancer cells produce increased amounts of growth factors and this causes the cancer cells to grow in an uncontrolled manner. Increased amounts of growth factors continue to be produced even though the cancer cells are growing faster than normal cells.

RAD 001 is a growth factor inhibitor, and studies in cell lines and in animal models have shown that RAD 001 is effective at reducing the rate at which breast tumours grow. The aim of this study is to investigate whether RAD001 is effective at slowing down or stopping the growth of breast cancers by looking at the number of cells growing. Specimens of the cancer taken before and after the treatment will be stained to see the growing cells so that they can be counted under the microscope. We will also look at which parts of the cancer cells are switched off by the drug by further staining of the cancer and by looking at the levels of activity of genes in the cancer cells themselves.

Please Reply To: **Edinburgh Breast Unit, Western General Hospital,**
Edinburgh EH4 2XU.
Tel: 0131 537 2907 Fax: 0131 537 2653 email: jmd@ed.ac.uk

Why Have I Been Chosen?

You have been invited to take part in this study because we know from your core biopsy that you have an early breast cancer, and it has been decided that the best treatment for you will be surgery. If you agree to take part in this study you will receive RAD001 for 14 days before your operation. This is long enough for the drug to have the effects on the cancer. There is no evidence to suggest that this will be of long term benefit to you, but the information we get from this study may help us to better treat future patients with breast cancer.
Initially 30 patients will be invited to take part in this study.

What Will Be Involved?

Your participation in this study is entirely voluntary and if you decide not to take part it will not affect your medical care in any way. If you decide to take part you will take one tablet a day for 14 days before your operation. The day before surgery you will have blood removed to check that the RAD 001 has not had any effects on your body. When you have surgery and your cancer is removed, a portion of the cancer will be taken to investigate whether there has been any changes between the initial biopsy you had performed at diagnosis and the sample removed at the time of surgery.
It will involve one extra visit to the clinic and two extra blood tests if you take part in this study. You will be reimbursed for any extra expenses related to this study including travel expenses.
You should continue to take all your usual medications. There are no dietary or lifestyle restrictions when taking this drug.

What Are The Benefits of This Study?

The benefit of the new drug RAD 001 is not known at this time. It is hoped that this new drug will prove useful in the treatment of women with breast cancer. There is evidence that RAD 001 should reduce the number of cancer cells growing in the tumour so and the aim of performing the study is to investigate whether RAD 001 is a drug that is likely to be useful in the treatment of breast cancer.

Please Reply To: **Edinburgh Breast Unit, Western General Hospital,**
Edinburgh EH4 2XU.
Tel: 0131 537 2907 Fax: 0131 537 2653 email: jmd@ed.ac.uk

Are There Any Risks?

All medicines may cause side effects and some patients may have unforeseen risks. In studies with RAD001, there have been no major toxicities noted at the dose of 5mg – the dose which will be used in this trial. The most common side effects reported at the 5mg dose have been skin rash (acne type or red non-specific rash), fatigue and mouth ulcers. One patient had a lowering of the platelet blood count (platelets are important for blood clotting) after 15 days of treatment, but this lowering was not to serious levels and resolved without any treatment. More serious side effects have been reported at higher doses of RAD001, but few patients have had to stop treatment. The serious side effects reported at higher doses have included tiredness, mouth ulcers, nausea and breathlessness. Other side effects noted to mild or moderate degree at higher doses included lowering of platelet count, raised cholesterol in the blood, diarrhoea, constipation, vomiting, headache, cough and skin rashes – either acne or reddening of the skin on the neck, face, body, or arms and legs.
It is possible that you may develop some of the side effects listed above, although it is a low dose of RAD001 being used for a short period of time. Please report all side effects to us. You will be carefully monitored and we will take a sample of blood the day before your operation to check that there have been no major changes as a result of the drug.

If you do develop problems, report these immediately to our research team at the Edinburgh Breast Unit by contacting Lorna Renshaw the study nurse, or Jane Macaskill the study research fellow (contact numbers on next page).

If you do become ill or injured as a result of participation in this clinical trial, medical treatment will be provided by the hospital. Novartis, the company who produce this drug, will abide by the code of practice recommended by the Association of British Pharmaceutical Industry.

Suppose I Refuse to Participate or Change My Mind?

Your doctor's main concern is your medical care and either you or he/she may stop your study treatment at any time if it is not suitable for you. You may withdraw from the study at any time, without giving a reason, and it will not affect your future treatment in any way. If you stop taking the drug you will not receive any other treatment before surgery – this being the current standard of care. Should you decide to withdraw from the study, any tissue taken specifically for the study will be destroyed.

Please Reply To: **Edinburgh Breast Unit, Western General Hospital, Edinburgh EH4 2XU.**
Tel: 0131 537 2907 Fax: 0131 537 2653 email: jmd@ed.ac.uk

Will My Identity Remain Confidential?

Yes. With your permission, your GP will be informed that you are taking part in the study. Your doctor may allow a "responsible person" from the authorized medicines regulatory body to check the results relating to your treatment. Your name will not be disclosed and you will only be identified by your initials and your study number. All results will be coded and cannot be identified as your own except by the research team here at Edinburgh Breast Unit. All research documentation will be retained for 15 years here at the Western General Hospital.

Who Can I Contact if I Have Any Problems or Questions?

If you have any concerns or questions about this study at any time, please contact:
Mr J Michael Dixon (Consultant Surgeon), Edinburgh Breast Unit, Western General Hospital, Edinburgh Tel: 0131 537 2907

You can also contact the research nurse for the study:
Lorna Renshaw, Research Nurse, Edinburgh Breast Unit, Western General Hospital, Edinburgh Tel: 0131 537 1615 or 0131 537 1000 + ask for bleep 5559

If you want independent advice on this study, please contact
Dr Ian H Kunkler, Consultant in Clinical Oncology, Department of Clinical Oncology, Western General Hospital, Edinburgh Tel: 0131 537 2214

Please Reply To: **Edinburgh Breast Unit, Western General Hospital, Edinburgh EH4 2XU.**
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A STUDY TO EVALUATE THE BIOLOGICAL EFFECTS OF RAD 001 ON INVASIVE BREAST CANCER

Informed Consent

Study Number: Centre Number: .....Patient Identification Number:.....

Name of Patient: .....

Name of Clinician: .....

Please Initial Boxes

1. I confirm that I have read and understand the information sheet for the above study and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3. I agree to my General Practitioner being informed of my participation in this study. ☐

.....	.....	.....
Name of Patient	Date	Signature
.....	.....	.....
Name of Person Taking Consent (if different from researcher)	Date	Signature
.....	.....	.....
Researcher	Date	Signature

Copies: 1 for patient, 1 for researcher, 1 to be kept with hospital notes

Dear Patient,

We would be grateful if you would record when you take your tablets for the course of your treatment. If you miss any tablets, please would you record the reason why.

Also if you have any side effects during the drug treatment, please record the details of the symptom(s) next to the days that the symptom(s) occur.

Thankyou for your co-operation.

Day	Date tablets taken	Reason for any missed tablets	Side effects (if any)
1			
2			
3			
4			
5			

Day	Date tablets taken	Reason for any missed tablets	Side effects (if any)
6			
7			
8			
9			
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Please Reply To: **Edinburgh Breast Unit, Western General Hospital,
Edinburgh EH4 2XU.
Tel: 0131 537 2907 Fax: 0131 537 2653 email: monicakmcgill@hotmail.com**

A STUDY TO EVALUATE THE BIOLOGICAL EFFECTS OF RAD 001 ON INVASIVE BREAST CANCER

Letter to General Practitioner

Dear Doctor

Your patient has agreed to take part in the above trial and to take RAD 001 which is a new drug which is being developed by Novartis Pharmaceuticals. It is a growth factor inhibitor to be taken for 14 days before surgery. Although it has not been used in many patients with cancer, it has been widely used in patients having renal transplants. The only problems experienced in these studies have been a reduced platelet count, although no patient as yet developed bleeding problems. A reduction in neutrophil count with some increase in non-specific infections has also occasionally been reported.

The aim of this study is to determine whether RAD 001 is a potent growth factor inhibitor and produces a reduction in proliferation in the cancer and an increase in cancer cell death.

We do not anticipate any problems with such a short term period of treatment but if your patient develops any side effects which could be related to the drug treatment, then they have been informed that they should stop the treatment immediately and let us know. Your patient has also been informed that she may withdraw from the study at any time without giving a reason and that this would not affect her treatment in anyway.

Yours sincerely,

Mr J Michael Dixon,
Consultant Surgeon and Senior Lecturer

Appendix 6

NHS

Northern and Yorkshire Multi-Centre Research Ethics Committee

Direct Dial: 0191 374 4151
Facsimile: 0191 374 4102
e-mail: sandy.brunton-shiels@durhamclspct.nhs.uk

Northern & Yorkshire MREC
John Snow House
Durham University Science Park
Durham DH1 3YG

27 January 2005

Mr JM Dixon
Consultant Surgeon and Senior Lecturer in Surgery
Edinburgh Breast Unit
Western General Hospital
Edinburgh
EH4 2XU

Dear Mr Dixon

Full title of study:	*A study to evaluate the biological effects of RAD001 on invasive breast cancer*
REC reference number:	*04/MRE03/89*
Protocol number:	*1*
EudractCT number:	*2004-001712-31*

Thank you for your letter of 26 January 2005, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document Type:	Version:	Dated:	Date Received:
Application	amended	17/11/2005	12/01/2005
Investigator CV John Michael Joseph Dixon	11	01/11/2004	19/11/2004
Protocol	1	01/11/2004	19/11/2004

Covering Letter	N/A	17/11/2004	19/11/2004
Summary/Synopsis Study Flow-Chart	1	01/11/2004	19/11/2004
Letter from Sponsor	N/A	28/09/2004	19/11/2004
GP/Consultant Information Sheets	1	01/11/2004	19/11/2004
Participant Information Sheet	4	26/01/2005	26/01/2005
Participant Consent Form	1	01/12/2004	12/01/2005
Investigator's Brochure	Edition 3	30/08/2004	19/11/2004
Response to Request for Further Information		07/01/2005	12/01/2005
Response to Request for Further Information		25/01/2005	25/01/2005
Response to Request for Further Information		26/01/2005	26/01/2005
Part B Application form amended		25/01/2005	25/01/2005

Management approval

The study should not commence at any NHS site until the local Principal Investigator has obtained final management approval from the R&D Department for the relevant NHS care organisation.

Notification of other bodies

The Committee Administrator will notify the research sponsor and the Medicines and Health-Care Products Regulatory Agency that the study has a favourable ethical opinion.

Statement of compliance

This Committee is recognised by the United Kingdom Ethics Committee Authority under the Medicines for Human Use (Clinical Trials) Regulations 2004, and is authorised to carry out the ethical review of clinical trials of investigational medicinal products.

The Committee is fully compliant with the Regulations as they relate to ethics committees and the conditions and principles of good clinical practice.

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

04/MRE03/89	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project,

Dr Simon Thomas
Chair

Enclosures *Standard approval conditions*

Appendix 7

University Hospitals Division

ROYAL INFIRMARY OF EDINBURGH

51 Little France Crescent, Edinburgh, EH16 4SA

NHS Lothian

HAC/R&D/app1a/patnoncomm

22/12/2004

Mr J Mike Dixon
Edinburgh Breast Unit
Western General Hospital
Crewe Road
EDINBURGH
EH4 2XU

RESEARCH & DEVELOPMENT OFFICE

Tel: 0131 242 3330
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R&DOffice@luht.scot.nhs.uk

R&D Director
Dr Heather Cubie
FRCPath

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PhD, MBA

Accountant
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BAcc

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Alison Clark
BSc(Hons)

Information Officer
Patricia Murray
BEng(Hons)

Commerical Research Officer
Sara Lindsay
BSc(Hons), PGDip

Assistant Accountant
Neil McLean

PA/Office Manager
Mrs Glynis Ormond

Administrative Assistant
Emma Lewis

Dear ~~Mr Dixon~~ Mike

R&D Project ID: **2004/W/BU/01**
REC Ref: **MREC: 04/MRE01/89**
Title: **A study to evaluate the biological effects of RAD001 on invasive breast cancer**

The above project has undergone a review of resource and financial implications by the R&D Office and I am satisfied that all the necessary arrangements have been set in place.

On behalf of the Chief Executive and Medical Director, I am happy to give Divisional management approval to allow the project to commence, subject to the approval of the appropriate Research Ethics Sub-Committee having also been obtained.

We would ask you to note that under Section A, question 35 of the COREC application form, NHS Lothian provides indemnity for negligence for NHS and honorary clinical staff wherever research involves patients attending the hospitals. It is not empowered to provide non negligent indemnity for patients or volunteers.

Yours sincerely,

Dr Heather Cubie
R&D Director

cc Secretary, Research Ethics Sub-Committee

Fine /

Thank Heather
Let Jutta know /

Appendix 8

MHRA

Medicines and Healthcare products Regulatory Agency

[illegible] Tower
[illegible] Lane, London SW8 3NQ

Direct Line: 0207 084-2327
Facsimile: 0207 084-2443
Room 12- 242

15/11/04

[illegible]

Dr H. Cubie
Lothian University Hospitals NHS Trust
R & D Department
Royal Infirmary of Edinburgh
Little France Crescent
EDINBURGH
EH4 2XU

CTA Number:
Eudract Number: 2004-001712-31

11 November 2004

Dear Dr Cubie

THE MEDICINES FOR HUMAN USE (CLINICAL TRIALS) REGULATIONS 2004 S.I. 1031
Product Type: Medicinal Product with Special Characteristics
Product: RAD001
Protocol number: 2004/W/BU/01, August 2004

NOTICE OF ACCEPTANCE

I am writing to confirm that the Licensing Authority, acting under regulation 18(2)(b) or (c), or 19(2)(a) or (8), or 20(2)(a) or (5), of the Regulations and according to the type of medicinal product involved[1], accepts your request to carry out a clinical trial in accordance with your application received on 06 October 2004 which we acknowledged in our letter dated 11 November 2004 subject to you receiving a favourable opinion from the relevant ethics committee in accordance with regulation 15(1). You may therefore carry out the trial as notified, but I must remind you of the Authority's powers under regulation 31 to suspend or terminate a clinical trial if the conditions set out in regulation 31(1)(a) and (b) are satisfied.

Remarks:
* Certican tablets are licensed in an EU Member State (MA nos 18690-3). No further information on the marketed product is required. The applicant should confirm whether the product to be used is the marketed product or not. The marketed product is available in strengths of 0.25, 0.5, 0.75 and 1mg. The application form makes reference to a 5mg strength being used. The applicant should also confirm the maximum daily dose since this is given in the application form as 30mg but as 5mg in the protocol. If the product being used is Certican tablets then the application form should be revised to show the use of a product which has a MA in another EU Member State. The relavant Member Statae and the MA number should be provided.

[1] The Licensing Authority's authorisation powers for clinical trials are regulation 18 for those involving general medicinal products, regulation 19 for those involving medicinal products for gene therapy etc., and regulation 20 for those involving medicinal products with special characteristics.

[illegible]

* A sample of the labelling should be provided.

The authorisation is effective from the date of this letter and may continue under this authorisation. In accordance with regulation 27, you must notify the Licensing Authority within 90 days of the conclusion of the trial, that it has ended.

Yours sincerely

Mrs Salma Syed
Clinical Trial Unit