DELAY OF BREAST CARCINOGENESIS BY GREEN TEA CATECHINS AND BLACK TEA THEAFLAVINS IN THE C3 (1) SV40 T/t ANTIGEN (TAG) TRANSGENIC MOUSE MODEL

Thesis submitted in fulfillment for the degree of Doctor of Philosophy at the University of Leicester

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Delay of breast carcinogenesis by green tea catechins and black tea theaflavins in the C3 (1) SV40 T/t antigen (TAg) transgenic mouse model

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Polyphenolic constituents of tea have been shown to impair proliferation and survival of neoplastic cells in vitro and prevent breast cancer in rodents. Epidemiological studies suggest that high consumption of green tea is associated with a delay in breast cancer recurrence. Much less is known about the potential cancer chemopreventive properties of black tea constituents. The aim of this study was to compare the efficacies of an extract of black tea (BTT) consisting predominantly of theaflavins with that of a green tea extract (GTC), containing mainly catechins. BTT/GTC reduced proliferation, increased apoptosis and decreased malondialdehyde DNA adduct levels (M1dG) in breast cancer cells in vitro. Pharmacological properties of BTT and GTC were explored in the C3 (1)/SV40 T/t-antigen transgenic (TAg) mouse model of mammary carcinogenesis. Female TAg mice develop mammary tumours characterised by inactivated tumour suppressor genes p53 and Rb, thus mimicking an insidious hormone-insensitive form of human breast cancer. Mice received drinking water (controls) or drinking water containing BTT or GTC (0.05%/0.01%). Intervention with either BTT or GTC (0.05%) increased TAg mouse survival accompanied with a decrease in tumour volume and tumour burden. Microscopic evaluation revealed that GTC or BTT consumption decreased the size of the largest lesion compared to controls. Increased survival was accompanied by a reduction in proliferation and an increase in apoptosis, as reflected by a decrease in PCNA levels and an increase in cleaved caspase 3 levels. Both tea extracts reduced tumour M1dG adduct levels. The results intimate that both GTC and BTT delay mammary tumour development in the TAg mouse model and M1dG and apoptotic signalling molecules may perhaps constitute suitable biomarkers of efficacy.
This thesis is dedicated to my mum and dad
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CHAPTER 1

INTRODUCTION
Chapter 1: Introduction

1.1 Breast cancer

1.1.1 Carcinogenesis

Cancer is a class of diseases or disorders characterised by uncontrolled division of cells. These cells invade other tissues, either by direct growth into adjacent tissue through invasion or by implantation into distant sites by metastasis in which cancer cells are transported through the blood or lymphatic systems (King, 2000). Cancer is one of the leading causes of death in developed countries and may affect people at all ages, with risk increasing with age (Parkin et al., 1992). Clinically, cancer is defined as a large group of diseases that vary in age of onset, state of cellular differentiation, rate of growth, invasiveness, metastatic potential and response to treatment and prognosis. A molecular and cell biology definition of cancer is a small number of diseases caused by molecular defects in cell function due to alterations to the genome (Ruddon, 1995). Ultimately cancer is a disease resulting from an accumulation of genetic modifications that presents the cell with survival advantage. The evolution of an invasive cancer cell from a normal cell is termed carcinogenesis. Carcinogenesis is a complex, multistage process. The sequence of events involved in carcinogenesis has been derived from animal studies, through cellular and molecular biology and by direct evaluation (King, 2000).

![Diagram of the multistage process of carcinogenesis](adapted from King, 2000)

The process of carcinogenesis (see fig 1.1) commences with the initiation step. It can involve the application of a cancer-inducing agent (carcinogen). The nature of carcinogens
is diverse and includes chemical agents such as benzo[a]pyrene, a polycyclic aromatic hydrocarbon found in cigarette smoke, or ethylene oxide, a gaseous product of the petrochemical industry also produced naturally by ripening fruit, or physical agents such as UV light from the sun, radiation and x-rays (Boffetta, 2004).

**Initiation**

Initiation involves a change in the target cell and is due to alterations in the genetic makeup of the cell. This change may occur randomly during DNA replication or when a carcinogen interacts with DNA, causing damage to the DNA and resulting in mutations to genes. This initial damage rarely results in cancer because the cell has in place many mechanisms to repair damaged DNA. However, if repair does not occur and damage to DNA is in a gene that regulates cell growth, proliferation, DNA repair or a function of the immune system, then the cell is more prone to becoming cancerous. The process of initiation is permanent and irreversible. The initiation step is very rapid. Initiated cells may grow very slowly or not grow at all unless induced by promoting agents (Franks and Teich, 2003).

**Promotion**

During promotion, agents such as hormones, cigarette smoke, diet or bile acids increase cell division by interfering with the process of differentiation. Cell differentiation is the transformation of identical progenitor/stem cells to specific cell type's e.g. hepatic stem cells to hepatocytes etc. Promoting agents interfere with the transformation of these cells by inducing proliferation of initiated cells. During promotion, cells may still be sensitive to normal growth-inhibiting factors in the body. The outcome for neoplastic cells depends on the balance between multiple factors such as promoting agents and the severity of the changes in the initiated cells. This explains why some preneoplastic or even fully transformed tumours are identified but do not appear to be growing and sometimes even regress. Many promoting agents appear to act, partly through their ability to cause inflammation and irritation. Different cell types may respond differently to the same agent. Promoting agents can transform a single potential cancer cell into a multicellular tumour, but may not be considered carcinogenic. The promotion stage is reversible as demonstrated by the fact that lung cancer risk reduces after smoking cessation (Franks and Teich, 2003).
**Progression**

Progression is the term applied to changes that occur once the tumour has formed which lead to tumour invasion and metastasis. During progression, there is further growth and expansion of tumour cells. Mutations that occur are in genes that regulate growth and cell function such as oncogenes, tumour suppressor genes and DNA mismatch repair genes. This stage is characterised by further cell differentiation and autonomous growth. Autonomous cell growth is the ability of cells to grow outside their normal environment and to metastasise to other parts of the body. The transformations that occur in cells such as altered sensitivity to adjacent cells, growth factor production, changes in receptors that initiate signal transduction and alterations to downstream transduction pathways are events leading to autonomous cell growth.

To summarise, exposure of cells to mutational events (DNA damage) combined with proliferative pressure (promotion) generates cellular changes required for carcinogenesis. Progression of tumours to a more differentiated state would be a continuation of these events (King, 2000).

### 1.1.2 Breast cancer incidence

Breast cancer is the third most common cancer in the world, with one in nine women developing the disease in the UK and USA (Adami et al., 1995; Ferlay et al., 2004). The incidence of breast cancer still continues to increase, despite the introduction of national screening programmes and awareness campaigns (Parkin et al., 1992). Breast cancer incidence rates worldwide vary greatly with a lower incidence observed in Japan and China (King, 2000) compared with western countries which demonstrate a 4 fold increased incidence compared to less industrialised nations (Colditz, 2005; Ferlay et al., 2004). In addition, the risk of breast cancer increases when migrants from less industrialised areas relocate to highly industrialised areas for example, Asian migrants in the US who adapt to a western lifestyle experience a "western" incidence of breast cancer (Ziegler et al., 2005). The rate of breast cancer incidence in migrants does not mimic that of the host population until the second generation; nevertheless some increase can occur quite rapidly. An eighty
percent increase in breast cancer incidence has been observed in Asian migrants after 10 or more years residence in the U.S (Hanby et al., 2000; Ziegler et al., 2005).

1.1.3 Aetiology of breast cancer

Breast cancer aetiology is complex and involves multiple genetic changes (mutations) that can cause abnormal cell behaviour (Bowcock, 1999). Mutations can be classified as either sporadic or hereditary. Hereditary mutations (5-10%) are due to germline mutations in breast cancer susceptibility genes. Genes including BRCA1, BRCA2, p53, pTEN and ATM have been associated with hereditary mutations (Hanby et al., 2000). The majority of mutations are sporadic (90-95%) and cannot be passed onto the next generation because they occur in a single somatic cell. Sporadic mutations occur as a result of lifestyle and environmental factors. For example exposure to chemical carcinogens, smoking, use of the contraceptive pill, inactivity, and poor diet can contribute to development of cancer (Barnard, 2004). Geographic differences in breast cancer incidence rates may be explained by variations in dietary, socioeconomic and reproductive factors (Yuan et al., 1995). Reproductive risk factors associated with breast cancer include early menarche, late first birth, late menopause and post menopausal obesity (Kelsey et al., 1993; Wu et al., 2005). Reproductive risk factors do not differ significantly between Japanese and American populations (Wu et al., 2003a). Age at first birth and age at menopause between the two populations are similar. An obvious difference between Eastern and Western societies is diet. Among the dietary differences are the lower consumption of high fat foods, lower caloric intake and the higher consumption of dietary fibre and soy based food products in the East (Hirose et al., 2003; Ziegler, 2004). In addition, it appears that serum oestrogen levels are lower in women from the East, compared to women from the United States or Western Europe (Shimizu et al., 1990). Increased serum oestrogen levels are associated with obesity, which may increase the risk of breast cancer in postmenopausal women (Bowcock, 1999). It has been proposed that a low fat and high fibre diet results in a decrease in serum oestrogen levels in Japanese women (Barnard, 2004).

Oestrogen’s are steroid hormones that play a significant role in the growth and development of the mammary gland (Thomas, 1984). Oestrogens appear to not only be associated with normal cell growth but also breast cancer cell growth. Oestrogens
significantly contribute to breast carcinogenesis leading to the development and/or progression of this disease (Wu et al., 2005). Oestrogen expression is mediated through an intracellular receptor, the oestrogen receptor (ER), which can stimulate proliferation. An ER is a receptor for oestrogens such as oestradiol (the main endogenous human estrogen). ER’s are intracellular proteins and are present on the cell surface membrane and within the cell nucleus. The two different ER proteins produced from the ESR1 and ESR2 genes are known as the α and β receptors. The two different receptor subtypes can form mixed dimers in the presence of ligands. Hence, there are three combinations: ERα (αα), ERβ (ββ) and ERαβ (αβ). Different tissues express the subtypes in different proportions, and therefore have different responses to stimulation. Oestrogen’s enter cells through the cell membrane and bind to ER, changing the receptor into an active transcription factor which binds to DNA as a dimer at specific oestrogen responsive elements (EREs). Binding to EREs regulates the expression of a variety of genes (Sanchez et al., 2002). Since oestrogen levels are mediated by the ER, the role of ER has been extensively examined in clinical breast cancer (Wu and Yu, 2006). ER status is an important factor in the management of breast cancer as it is useful in predicting patient outcome. Breast tumours can be classified as either oestrogen receptor positive tumours (ER+ve) or oestrogen receptor negative tumours (ER-ve). ER+ve tumours are more common in older women and are generally more differentiated, slower growing, and carry a better prognosis. In contrast, ER-ve tumours are associated with early recurrence and poor patient survival. Subsequent to the menopause, when oestrogen is no longer produced by the ovaries, peripheral tissues, such as adipose and muscle are the next highest sources of oestrogen (Wu and Yu, 2006).

1.1.4 Stages and treatment of breast cancer

Staging is a process used to assess the size and location of breast cancer, reflecting the extent to which cancer has spread in the body. Staging is used to determine optimal treatment strategies. Several tests are performed to help stage breast cancer including clinical breast examination, biopsy, chest x-ray, mammography, bone scan, CT scan and MRI scan (Bowcock, 1999). Early detection of breast cancer is performed by mammography. This diagnostic method is aimed at early detection of invasive cancer and is limited by the fact that this may still be too late to affect survival (Franks and Teich, 2003).
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Treatment of breast cancer entails resection and using one or more therapies to prevent recurrence. Localised breast cancer can often be treated by lumpectomy i.e. removing cancerous tissue whilst leaving the remaining breast tissue intact. Once the cancer has metastasised to distant sites, chemotherapy and radiotherapy are prescribed. The stages of breast cancer can be defined as stage 0, I, IIA, IIB, IIIA, IIIB and stage IV (advanced metastatic breast cancer) (Donegan, 1992). Stage 0 is a contained cancer that has not spread beyond the breast ductal system. These breast cancers (15-20%) are detected by clinical examination. Two stage 0 diagnoses exist, lobular carcinoma in situ (LCIS) and ductal carcinoma in situ (DCIS). LCIS does not behave as a cancer, although the microscopic features of LCIS are abnormal and are similar to malignancy. LCIS is a predictive marker for a significantly increased risk of breast cancer. LCIS patients undergo clinical breast examinations every four months, in addition to yearly mammography. Some patients may be given tamoxifen (which will be discussed further on in this section) to prevent development of breast cancer. DCIS, which is confined to the milk ducts in the breast, does not spread into fatty breast tissue or to any other part of the body (such as lymph nodes). DCIS can be detected by mammography and breast biopsy. Standard DCIS treatment is breast-conserving therapy (BCT), lumpectomy followed by radiation treatment or mastectomy. Stage I breast cancer is characterised by a tumour that is 2cm or less in diameter, which has not spread to lymph nodes. Stage IV breast cancer is diagnosed when the primary cancer has metastasised to other parts of the body (such as bone, lung, liver and brain). Treatment of stage I breast cancer consists of BCT, lumpectomy and axillary node dissection (removal of underarm lymph nodes) followed by radiation whereas in stage IV, the cancer is so advanced that treatment usually involves extending survival time and relieving symptoms. Systemic treatment (treatment that affects entire body) such as chemotherapy, hormonal therapy or both is often recommended. Mastectomy or use of tamoxifen may provide symptom relief in some cases (Ruddon, 1995).

1.1.5 Chemoprevention

Cancer chemoprevention involves delaying or reversing the onset, progression or recurrence of cancer by the use of naturally occurring or synthetic agents. Cancer chemopreventive agents can be classified as antimutagens/carcinogenic blocking agents,
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antiproliferatives or antioxidants, though many have more than one mechanism of action (Ruddon, 1995). Many chemopreventive interventions are aimed at preventing the induction or progression of tumours at a comparatively early stage, in contrast to chemotherapeutic agents which are directed towards established tumours (Hudson et al., 2006). Prevention of carcinogenesis by chemopreventive agents can involve blocking the initiation step or preventing or delaying promotion and/or progression. Blockade of initiation events could be brought about by agents that decrease the metabolic activation or increase detoxification of chemical carcinogens (Chen and Kong, 2005).

1.1.5.1 Chemoprevention of breast cancer

Evidence for the notion that breast cancer is preventable comes from epidemiological data (Colditz, 2005). Chemopreventive agents investigated for the prevention of breast cancer, include selective oestrogen receptor modulators (Fabian and Kimler, 2005; Gasco et al., 2005), aromatase inhibitors (Kendall and Dowsett, 2006) and monoclonal antibodies (Zwierzina, 2003). The contribution of diet in chemopreventive studies has also been investigated (Colditz, 2005).

**Selective oestrogen receptor modulators**

**Tamoxifen**

Tamoxifen is an oral, non steroidal, competitive oestrogen receptor antagonist. Tamoxifen can block the binding of oestrogen to ER and therefore inhibits oestrogen-stimulated growth (Nayfield, 1995). Tamoxifen has been used in chemoprevention studies to reduce risk of ER+ve breast cancer (Gasco et al., 2005). A reduction in the risk of invasive breast cancer was reduced in women receiving tamoxifen (20mg/day) compared with a placebo. No significant reduction was observed in ER-ve breast cancers (Lippman et al., 1998). Although tamoxifen is effective as a chemopreventive agent in women with high risk of breast cancer, it is also associated with an increased risk of venous thromboembolic events (VTE) and endometrial cancer (Kendall and Dowsett, 2006).
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**Raloxifene**

Similar to tamoxifen, raloxifene is a selective oestrogen receptor modulator and has been investigated in chemoprevention trials against breast cancer. Raloxifene has an advantage over tamoxifen in that it is not associated with an increase risk of endometrial cancer (Gasco et al., 2005; Lippman et al., 1998).

**Aromatase inhibitors**

Aromatase inhibitors reduce the amount of available oestrogen by blocking its synthesis. These are inhibitors of cytochrome P-450 aromatase enzyme, (CYP 19) which are responsible for the peripheral conversion of androgens into oestrogens in post-menopausal women. Studies show that incidence of breast cancer is significantly lower after intervention with anastrozole (1mg/day) compared with tamoxifen (20mg/day), and with fewer side effects (Baum, 2002). Aromatase inhibitors are beginning to show important benefits in terms of objective response rates, stabilising the disease and longer survival. Additional considerations include excellent tolerability that may alter quality of life for the patient. Both tamoxifen and aromatase inhibitors are directed towards preventing and/or delaying ER+ve breast cancers. However, ER+ve precancerous lesions may be resistant to tamoxifen interventions (Chung et al., 2002). Potential mechanisms of resistance include the loss of ER, or mutations in the ER. Altered production of autocrine growth factors and expression of anti-oestrogen binding sites has been studied as mechanisms of anti-oestrogen resistance (Zwierzina, 2003). Women with a family history of breast and ovarian cancer have a higher risk of developing ER-ve breast cancer compared with the general population and approximately 20-30% of all invasive breast cancers are ER-ve. This high risk of developing ER-ve breast cancer accompanied with resistance to anti-oestrogens and poor prognosis associated with ER-ve breast cancers, contribute to the fact that novel chemopreventive agents are required for ER-ve breast cancers.

**Monoclonal antibodies**

**Herceptin**

The tyrosine kinase inhibitor, Herceptin, was the first monoclonal antibody registered for metastatic breast cancer (Zwierzina, 2003). Epidermal growth factor receptors EGFR
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(ERBb1) and EGFR2 (ERBb2/Her2) are over-expressed in at least 30% of breast cancers and over-expression of these receptors has been associated with poor prognosis (Menard et al., 2000).

Diet

Specific chemopreventive drugs are aimed towards preventing either ER+ve or ER-ve breast cancers, whereas dietary agents are useful in preventing both types of breast cancer (Adebamowo et al., 2005). It has been estimated that 30-40% of all cancers can be prevented by appropriate diets, physical activity and maintenance of body weight (Donaldson, 2004). Colditz (2005) examined the high school diet among young nurses and the proliferative benign disease was used as a marker of breast cancer risk and breast cancer incidence. Women free from benign breast disease completed a questionnaire in 1998, which had some 131 food items, asking them to recall their dietary intake during high school. Incident cases of proliferative benign breast disease were examined. A positive relation between animal fat and incidence of proliferative disease and an inverse relation between vegetable fat and benign breast disease were observed. A similar positive association between breast cancer and fat and red meat consumption and an inverse association between breast cancer and dietary fibre, fruit and vegetables intake has been observed in a separate study (Aggarwal and Shishodia, 2006). Potential of diet-derived chemopreventive agents in reducing the risk of cancers has been investigated in several epidemiological studies (D'Incalci et al., 2005). These studies have isolated and identified chemopreventive agents such as indole-3-carbanol and sulforaphane from cruciferous vegetables, epigallocatechin gallate (EGCG) from green tea, resveratrol from red grape skin and curcumin from the spice turmeric and studied their effects on different types of cancers (Borek, 2004). For example, a significant inverse association with intake of beans and lentils was observed with breast cancer risk in a study conducted by Adebamowo et al (2005). There are many dietary constituents that have been investigated in different types of cancer, including tea. The ability of the chemopreventive agents present in green and black teas to prevent breast carcinogenesis is the main focus of the research presented in this thesis.
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1.2 Preclinical models of breast carcinogenesis

Animal models for breast cancer research have been developed for over 100 years. Several mouse strains were initially produced by selective inbreeding. These mouse strains developed spontaneous mammary tumours with characteristic incidence frequencies (Sukumar et al., 1995; Wagner, 2004). Subsequent to this, rat breast tumour models were developed, utilising exposure to chemical carcinogens. These rodent models are useful in identifying risk factors (chemicals, diet) that may play a role in breast cancer progression and more significantly to the development of prevention and treatment strategies (Sukumar et al., 1995) Immunodeficient nude mouse models were developed during the 1960s, which opened the door to xenograft models of human breast cancer. More recently, transgenic and knockout rodent models with specific genetic alterations or deficiencies have been used for studying breast cancer development (Heppner et al., 2000).

1.2.1 Chemical, viral induced rodent models

In chemical-induced rodent models, highly potent carcinogens such as methylating agents (N-nitroso-N-methylurea, NMU) and polycyclic hydrocarbons (7, 12-dimethylbenz[a]anthracene, DMBA) are used to initiate mammary cancer. DMBA is an indirect acting carcinogen and is transformed into an active form when metabolised (Wagner, 2004). Initiation of mammary carcinogenesis occurs at the genetic level. DMBA forms adducts with both deoxyadenosine and deoxyguanosine nucleotides. The formation of these adducts generates mutations (Sukumar et al., 1995). Approximately 90% of female Sprague-Dawley rats developed mammary tumours within 7-20 weeks following exposure to DMBA (15mg/kg). The tumours analysed were generally malignant and well differentiated papillary adenocarcinomas (Guo et al., 2005; Kavanagh et al., 2001). Virally induced breast cancer models employ mouse mammary tumour virus (MMTV) (Sukumar et al., 1995). MMTV is a biological carcinogen which when incorporated into the host cellular genome, induces somatic mutations. The clonal outgrowth of cells with integrated MMTV genome, induces mammary tumourigenesis (Huang et al., 2005). Carcinogen-induced mammary tumours and virally-induced mammary tumours have been significant in understanding mammary gland tumourigenesis (Kim et al., 2003).
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Rat mammary gland cancers tend to be invasive and can spread into surrounding stroma and muscles, but rarely metastasise, unlike most human breast cancers. Among rat carcinomas, a range of differentiation phenotypes are observed but still tend to be generally regarded as well differentiated in comparison to human breast cancers (Shan et al., 2004).

1.2.2 Xenograft models

Xenograft mouse models are easy to generate, relatively inexpensive and tumour formation tends to have a relatively short latency and therefore are popular models for preclinical evaluation of potential chemotherapeutic and chemopreventive agents (Kavanaugh and Green, 2003; Zhou et al., 2004). Xenograft mouse models are created by inoculation of breast cancer cell lines subcutaneously into immunodeficient mice. Xenograft experiments employ immunodeficient nude or severe combined immunodeficiency (SCID) mice. Nude mice are characterised phenotypically by the lack of hair growth and lack of a thymus gland. Since T lymphocytes involved in cell-mediated immunity are produced by the thymus gland, tumour grafts from individual species can grow in these animals and immune rejection will not occur. SCID mice lack functional T and B cells due to a spontaneous mutation, which inactivates DNA protein kinase (Bosma et al., 1983). There are currently many xenograft models available for use in human breast cancer research but the most commonly used is the MCF-7, ER+ve xenograft model (Kim et al., 2003).

The oestrogen dependent MCF-7 breast cancer cell line shares similar features to oestrogen-dependent human breast cancer in that both depend on oestrogen for growth and both express oestrogen receptors ERα and ERβ. When BALB/c nude mice or SCID mice were injected with MCF7 cells, the tumours formed consist of poorly differentiated tumour cells (Liao et al., 1995; Zhou et al., 2004). For oestrogen-independent and ER-negative breast cancers, the MDA-MB-231 cell line, derived from a patient with poorly differentiated adenocarcinoma of the breast is subcutaneously transplanted into SCID mice (Sartippour et al., 2001).

There are a number of human breast cancer cell lines that will metastasise in xenograft models, but these xenograft models can be limiting in their use for preclinical trials for a number of reasons. The animals lack a normal immune response (Wagner, 2004) and therefore do not reflect the complexity of tumour progression in humans. All breast cancer
cell lines have been adapted to grow in culture and therefore are likely to have different environmental requirements compared to primary breast tumour cells. Cell lines that have been cultivated for too long, are subject to variation (genetic drift) usually due to viral or mycoplasma infection (Kim et al., 2003). Inaccuracies in xenograft models can also exist when transplanting cells directly into experimental mice e.g. different site of implantation and age and strain of mice used. In addition, xenograft models have low oestrogen levels compared to humans, therefore hormonal supplementation is required (Kim et al., 2003). An animal model with maintained oestrogen levels, adequate to support tumour growth would have greater clinical relevance than one with depleted oestrogen levels (Wagner, 2004).

1.2.3 Transgenic animal models

Transgenic animals can be employed in studies to identify genes that are expressed during development, and to investigate how mutation or deletion of these genes affects growth and development (Gusterson et al., 1999; Maroulakou et al., 1994). In transgenic animals, exogenous genes or partial gene sequences are introduced into early embryonic cells of the developing organism. The DNA then becomes fully integrated into the genome. Animals of different genotypes are mated and the function of the genetic manipulation can be studied in the offspring (Cardiff, 2001). The first method for developing transgenic animals involved infection of mouse embryos with retroviruses, e.g. SV40 early promoter and MMTV LTR (long terminal repeat) (Ruddon, 1995). The commonly used technique employed now is the direct microinjection of recombinant DNA into the pronucleus of a fertilized egg or the introduction of DNA into embryonic stem cells (Ruddon, 1995). Transgenic animal models may be favoured for use in preclinical investigations over other breast cancer animal models because they do not use carcinogens, they have a functional immune system and possess a short latency period. Transgenic animals are generated to mimic human diseases through over-expression or activation of genes associated with human cancer development (oncogenes) or downregulation and functional deactivation of anti-oncogenes (suppressor genes) (Andrechek and Muller, 2000). Transgenic animals may also include mechanisms involving elimination of target (suppressor genes) via gene
knockout e.g., p53, STAT 5a. Cancer progression is delayed in mice with reduced levels of STAT 5a expression (Ren et al., 2002).

Although transgenic mouse models for breast cancer are valuable to study molecular pathways of neoplastic transformation and tumourigenesis in vivo, all animal models of breast carcinogenesis differ from human breast cancer. For example, the biology of rodents and their tumours differs from that of humans and human cancer, e.g. size, number of tumours, the degree of maturation and differentiation in mouse tissues are dissimilar to humans. The shorter lifespan of rodents means that observable tumours must have a rapid programme of progression as mice can develop very malignant tumours within a short time period. Breast cancer in humans usually spreads via the lymphatic system, starting with local lymph nodes, followed by distant metastasis predominantly to the bone, the brain, the adrenal glands, liver and lung. In contrast, mouse mammary cancers metastasise primarily to lung (Kim et al., 2003). Nevertheless, taking these differences into account, transgenic animal cancer models help researchers to understand the natural history of disease progression and therefore are useful preclinical tools (Kavanaugh and Green, 2003).

In general, breast cancer is not genetically or phenotypically uniform and therefore one model can never be sufficient to replicate various forms of disease. The advantage of one model over another depends on the scientific hypothesis, experimental design and on the type of study (Wagner, 2004). Consequently transgenic animal models that closely emulate human diseases and possess endpoint biomarkers should be used in chemopreventive studies (Gupta et al., 2001).
1.3 The C3(1) SV40 T,t antigen (TAg) transgenic mouse model

The C3(1) SV40 T,t antigen (TAg) transgenic mouse model is a unique model system for studying formation of breast tumours. This is an attractive model because it allows researchers to study the multistage progression of tumour development. The C3 (1) TAg mouse model develops proliferative mammary lesions that progress over time to adenocarcinoma. The latency period is relatively short and all female mice develop adenocarcinomas that progress into invasive carcinomas. The mammary lesions develop over a predictable time course with histological similarities to human breast cancer (Yoshidome et al., 2000).

In this model, the 5' flanking region of the C3(1) component of rat prostatic binding protein gene has been used to direct the expression of the early region of the Simian Virus 40 (SV40) large tumour antigen, small t antigen (TAg) to the epithelium of the mammary gland. The SV40 TAg binds to and functionally inactivates p53 and Rb (retinoblastoma) proteins, which are required for cell homeostasis. Inactivation of p53 and Rb appears to interfere with cell cycle regulation, as often occurs in human cancer (Yokoyama et al., 2000). In human breast cancer, p53 and Rb are often deleted or mutated (Green et al., 2000). The expression of TAg is necessary for tumour development in these animals. However not all TAg expressing cells are hyperplastic or develop carcinoma. Therefore TAg expression is not sufficient by itself for complete transformation. Evidently, other genetic events are required for complete transformation to occur (Maroulakou et al., 1994).

Histological evaluation shows that in this transgenic model, mammary tumours share similarities with their human counterparts. At about 8 weeks of age, atypia of mammary ductal epithelium develops, progressing to mammary intraepithelial neoplasia (MIN) at 12 weeks of age. MIN resembles human ductal carcinoma in situ (DCIS). At about 16 weeks of age, invasive carcinomas develop in female mice. The carcinomas share similar features to human breast cancer as infiltrating ductal carcinomas. All female mice carrying the transgene develop mammary cancer (Green et al., 2000).

Human mammary tumours can progress from an ER+ve to an ER-ve state (Chung et al., 2002). Thus, it is useful to investigate the efficacy of chemopreventive agents in ER+ve and ER-ve breast cancer models. Normal mammary ductal cells and low-grade MIN lesions
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in which TAg is activated, express ER. However in high grade MIN and invasive carcinomas, expression of ER mRNA and protein is reduced. In this model, expression of the transgene may be stimulated by oestrogen. Following initiation by TAg, cellular factors are up-regulated promoting tumourigenesis. During the MIN stage, neither oestrogen nor expression of ER are required to promote tumourigenesis or for further progression to invasive carcinoma. It is possible that ER has been downregulated in order to provide a growth advantage for invasive tumour development. Cellular factors that may be induced in this model include over-expression of Ki-ras or loss of p53, Rb and p21 functions. Expression of Her2/neu mRNA was reported to be slightly elevated (Yoshidome et al., 2000). TGF-α and EGFR are upregulated at MIN stage, an early event in this model, whereas c-myc expression was only upregulated in carcinomas and not in MIN lesions suggesting this upregulation may be a late event (Yoshidome et al., 2000).

The predominant site for metastasis in this model is the lungs, although metastasis to liver, adrenal glands and heart has been observed. Metastasis to brain, bone and regional lymph nodes have not been observed demonstrating a major biological difference between behaviour of TAg mouse tumours and human breast cancer (Green et al., 2000).

Holzer et al (2003) characterised and developed a series of cell lines from the C3(1) TAg transgenic mammary carcinomas, representing the progressive stages of tumour development. M28N2 and M27H4 derived from hyperplastic lesions were weakly tumourigenic. M6 was derived from carcinoma and M6c from metastatic lesions. M6 and M6c cells expressed lower levels of ERα compared to the other cell lines and to normal mammary tissue. These cell lines provide researchers with a convenient in vitro model for conducting either preliminary experiments or experiments that compliment an in vivo study, such as the C3(1) TAg transgenic mouse model. The C3(1) TAg transgenic mouse model shares similar characteristics to other cell lines, in particular MDA-MB-468 breast cancer cells. The late-stage TAg mouse model and MDA-MB-468 cells are ER-ve, Rb negative and p53 negative. MDA-MB-468 cells also share similar genetic properties to the TAg model such as over-expression of EGFR (Lev et al., 2004; Xu et al., 2005). A study by Gupta et al (2005) into the effects of Mullerian inhibiting substance on the C3 (1) TAg model and in a breast xenograft model using MDA-MB-468 cells demonstrated similar results. Mullerian inhibiting substance reduced the number of palpable tumours and mean
mammary tumours in both models. Consequently the MDA-MB-468 xenograft model may complement the TAg model effectively. This particular cell line can be utilised to identify end point biomarkers to evaluate chemopreventive agents, which can then be applied to an \textit{in vivo} study using the C3(1) TAg model.
1.4 Tea

Fruit and beverages such as tea and red wine constitute the main sources of polyphenols (Manach et al., 2004). Polyphenols are natural compounds that are widely distributed in plant foods. Several hundred polyphenolic structures have been identified in edible plants. These molecules are generally involved in defence against ultraviolet radiation or invasion by pathogens and are secondary metabolites in plants. Polyphenols can be classified into distinct groups, based on structural elements. The most abundantly occurring polyphenols in plants are phenolic acids, flavonoids, stilbenes and lignans. Flavonoids and phenolic acids are an important part of the human diet, accounting for 60% and 30% respectively of total dietary polyphenols (Lambert et al., 2005; Nichenametla et al., 2006). Flavonoids can be further divided into 6 subclasses: flavonols, flavones, isoflavones, flavanones, anthocyanidins and flavanols. The actual content of polyphenols in plants can be affected by environmental factors. These factors may be agronomic (culture in greenhouses or fields, biological culture, fruit yield per tree) or pedoclimatic (soil type, sun exposure, rainfall). Exposure to light has a considerable effect on most flavonoids (Manach et al., 2004).

Tea is prepared from the dried leaves of a plant, *Camellia sinensis*. *Camellia sinensis* is native to South East Asia but is cultivated in more than 30 countries around the world. The amount produced and consumed varies in individual countries (Saleem et al., 2003). A variety of factors effect the chemical composition of the tea leaves, including, season, climate, horticultural practices, and the type and age of the plant (Mukhtar and Ahmad, 2000). Tea is the highest consumed beverage in the world, next to water, with per capita consumption of approximately 120ml/d. Tea comprises phenolic acids, mainly caffeic, quinic, and gallic acids, catechins (flavanol) and other polyphenols such as quercetin, myricitin and kaempferol (flavonols). The polyphenolic components present in green and black tea are shown in table 1.1. An amino acid found only in tea leaves, theanine, imparts a pleasantly sweet taste to tea. Theanine is degraded to glutamic acid and has been shown to have relaxation effects in humans. Theanine is also known to decrease blood pressure and increase anti-tumour activity of chemotherapeutic drugs such as doxorubicin and
idarubicin and to ameliorate some of the side effects of these agents (Sugiyama and Sadzuka, 2004). These drugs are used for treatment of breast, ovarian and other types of cancer. Theanine is considered the main component responsible for the taste of green tea (Sugiyama and Sadzuka, 2003; Sugiyama and Sadzuka, 2004). Tea can be classified into three types, black tea, green tea and oolong tea. Black tea is consumed primarily in western countries and in some Asian countries, whereas green tea is consumed primarily in China, Japan and countries in North Africa and the Middle East. Oolong tea production and consumption is confined to South East China and Taiwan. The total amount of tea produced and consumed in the world consists of approximately 78% black, 20% green and <2% oolong tea.

1.4.1 Green and black tea

Green and black tea is produced by different manufacturing process. Figure 1.2 summarises the stages of tea production.

![Diagram of tea production stages](http://www.teahealth.co.uk/th/facts/l.htm)

**Figure 1.2: Stages of green and black tea production**

(http://www.teahealth.co.uk/th/facts/1.htm)
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For green tea production, freshly harvested leaves are rapidly steamed to inactivate enzymes (polyphenol oxidase). The inactivation of enzymes protects the leaves from oxidation (termed fermentation in the tea industry) and allows production of a dry, stable product (Mukhtar and Ahmad, 2000). Green tea composition consequently resembles that of fresh tea leaves. The main polyphenols in green tea are catechins (GTC) and they account for the characteristic colour and contribute to the flavour of green tea. The major catechins in green tea are (−)-epicatechin (EC), (−)-epicatechin-3- gallate (ECG), (−)-epigallocatechin (EGC), and (−)-epigallocatechin-3-gallate (EGCG). EGCG has received the most attention in respect to the health benefits of tea, as it is the most abundant polyphenol in green tea. A brewed cup of green tea can contain up to 300mg of EGCG (Saleem et al., 2003). The structures of the major polyphenolic compounds present in green tea are illustrated below.

![Image of catechins in green tea](image)

Figure 1.3: Structure of catechins in green tea (Yang et al., 2000)

Black tea is produced by allowing fresh leaves to wither until their moisture content is reduced to approximately 55% of the original weight. The withered leaves are then rolled and crushed, initiating fermentation of the polyphenols. During the fermentation process, simple polyphenols, such as catechins, are oxidised to complex condensed polyphenols, known as theaflavins and thearubigins. These polyphenols give rise to the characteristic colour and flavour of black tea. Theaflavins comprise theaflavin, theaflavin-3-gallate,
theaflavin-3'-gallate and theaflavin-3, 3'-digallate and account for 2-6% of the dry weight in brewed black tea. The major fractions of black tea polyphenols, generally known as thearubigins, have higher molecular weights and are poorly characterised chemically, but account for >20% of the solid weight of brewed black tea (Yang et al., 2002).

\[ R = \text{galloyl group} \]

**Figure 1.4: Structure of theaflavins and thearubigins (Mukhtar and Ahmad, 2000).**

Tea is usually prepared by infusing tea leaves in hot water. A typical tea beverage, brewed for 3 min and prepared in a proportion of 1g leaf to 100ml water, usually contains 250-
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350mg tea solids. In general the caffeine content in green and black tea ranges from 3 to 6% (Saleem et al., 2003)

Table 1.1: Polyphenolic composition of green and black tea (%wt/wt) (Siddiqui et al., 2006)

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Green tea</th>
<th>Black tea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechins</td>
<td>30-42</td>
<td>3-10</td>
</tr>
<tr>
<td>Flavonols</td>
<td>5-10</td>
<td>6-8</td>
</tr>
<tr>
<td>Other flavanoids</td>
<td>2-4</td>
<td>-</td>
</tr>
<tr>
<td>Theogallin</td>
<td>2-3</td>
<td>-</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>Quinic acid</td>
<td>2.0</td>
<td>-</td>
</tr>
<tr>
<td>Theanine</td>
<td>4-6</td>
<td>-</td>
</tr>
<tr>
<td>Methylxanthines</td>
<td>7-9</td>
<td>8-11</td>
</tr>
<tr>
<td>Theaflavins</td>
<td>-</td>
<td>3-6</td>
</tr>
<tr>
<td>Thearubigins</td>
<td>-</td>
<td>12-18</td>
</tr>
</tbody>
</table>

Table 1.2: Constituents of the polyphenols in crude extracts of green tea and black tea (Kuroda and Hara, 1999)

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Green tea (µg/ml)</th>
<th>Black tea (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(-)-Epicatechin (EC)</td>
<td>98</td>
<td>37</td>
</tr>
<tr>
<td>(-)-Epicatechin-3-gallate (ECG)</td>
<td>90</td>
<td>73</td>
</tr>
<tr>
<td>(-)-Epigallocatechin (EGC)</td>
<td>411</td>
<td>42</td>
</tr>
<tr>
<td>(-)-Epigallocatechin-3-gallate (EGCG)</td>
<td>444</td>
<td>128</td>
</tr>
<tr>
<td>Theaflavins</td>
<td>0</td>
<td>64</td>
</tr>
<tr>
<td>Theaflavin (TF)</td>
<td>0</td>
<td>22</td>
</tr>
<tr>
<td>Theaflavin -3-gallate</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>Theaflavin-3',gallate</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>Theaflavin-3,3' digallate</td>
<td>0</td>
<td>9</td>
</tr>
</tbody>
</table>
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The stability of green tea catechins and black tea theaflavins is pH-dependent. In alkaline solution, both green tea catechins and black tea theaflavins are unstable and tend to decompose, whereas in acidic solution green tea and black tea are stable. Individual catechins and theaflavins demonstrate different stability conditions. EGCG and EGC are less stable compared to EC and ECG and among the theaflavins, theaflavin-3-gallate is the most stable compound. Overall, green tea catechins tend to be more stable than theaflavins (Su et al., 2003).

1.4.2 Bioavailability of tea polyphenols

Tea polyphenols have low bioavailability (Chow et al., 2005) because they are poorly absorbed (Lambert and Yang, 2003) and rapidly eliminated (Manach et al., 2004). During absorption, polyphenols are conjugated in the small intestine and in the liver. This process involves methylation, sulphation, and glucuronidation and decreases the efficacy of the polyphenols and facilitates their biliary and urinary elimination by increasing their hydrophilicity. Polyphenols and their derivatives are eliminated in urine and bile. Although polyphenols are able to penetrate tissues, specifically those in which they are metabolised, they do not tend to accumulate within specific target tissues (Manach et al., 2004). In humans, EGCG can be detected in the plasma in a free form. The other catechins tend to be highly conjugated with glucuronic acid and/or sulphate (Manach et al., 2005). EGC can be detected as the glucuronidated and sulphated forms with a small amount present as the free form (Lambert and Yang, 2003). Most of the ingested EGCG apparently does not get into the peripheral circulation, and the absorbed EGCG is excreted through the bile (Lee et al., 2002). Results suggest that EGCG is mainly excreted through the bile, and that EGC and EC are excreted through both the bile and urine (Chen et al., 1997). The limited bioavailability of EGCG is due to its rapid conversion to methylated, glucuronidated, and sulphated metabolites (Yang et al., 2006b). Little is known about the bioavailability of theaflavins
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1.5 Cancer chemoprevention and tea

Consumption of tea is associated with a variety of health benefits such as improving blood flow (Vita, 2003), eliminating toxins, improving resistance to various diseases (Demeule et al., 2002) and combating cancer (Mukhtar and Ahmad, 2000) and cardiovascular diseases (Tokudome et al., 2005). Tea polyphenols have antimutagenic activity, which has been extensively investigated, mostly in microbial systems (Apostolides et al., 1997) and in mammalian in vitro and in vivo systems (Kuroda and Hara, 1999). Several epidemiological studies as well as studies in animal models have shown that tea has a potentially preventive effect on various cancers such as those of the skin (Lu et al., 2002), prostate (Bettuzzi et al., 2006), lung (Baker et al., 2005) and breast (Sun et al., 2006).

1.5.1 Chemoprevention of human breast cancer by green tea

A number of epidemiological studies have demonstrated an association between green tea consumption and breast cancer risk (Seely et al., 2005). Nakachi et al. (1998) examined the association between consumption of green tea and clinical characteristics of breast cancer. Consumption of green tea, ≥ 5 cups a day, decreased the number of metastatic axillary nodes among premenopausal patients and reduced risk of stage I and II breast cancer. In addition, increased expression of progesterone receptor (PgR) was observed among postmenopausal women. Increased expression of PgR and metastasis to axillary lymph nodes are considered reliable predictors of recurrence (Donegan, 1992). Inoue et al. (2001) also found that long-term consumption of green tea, prior to clinical cancer onset was associated with significantly reduced recurrence of stage I and stage II breast cancer. However no association between green tea and stage III breast cancer was observed, suggesting that green tea has no effect on advanced stages of breast cancer (Inoue et al., 2001). Similar effects of green tea consumption on breast cancer risk were observed in women in Hiroshima and Nagasaki in Japan (Key et al., 1999). The risk of breast cancer was highest between the ages of 55-59 and this risk was reduced by increased consumption of green tea. The relative risk of breast cancer in patients consuming 2-4 cups of green tea/day was 0.86. This was reduced to 0.78 in patients consuming ≥ 5 cups/day (Key et al., 1999). The three studies discussed assess the effects of green tea consumption in Japanese women with risk...
of breast cancer. Japan has high per capita green tea consumption. Therefore studies using a population with low green tea consumption would confirm the significant effects presently observed between green tea and low breast cancer risk. A study conducted in Chinese, Japanese and Filipino women in Los Angeles County showed that green tea consumption significantly reduced risk of breast cancer compared to non-green tea drinkers (Wu et al., 2003b). The inverse association with green tea was observed in each Asian ethnic group both US born and migrants, in smokers and non-smokers and alcohol and non-alcohol drinkers and daily coffee or non-daily coffee drinkers. Confounding dietary factors such as alcohol intake and lifestyle factors did not have an influence on consumption of green tea (Wu et al., 2003a). Although these studies show a significant association between green tea and breast cancer prevention, a study by Suzuki et al (2004) found no association between green tea intake and risk of breast cancer. Despite the fact that green tea intake had no effect on risk of breast cancer occurrence, consumption of 5 or more cups of green tea a day shows a statistically significant trend towards the prevention of breast cancer development. Evidence suggests that green tea consumption may prevent stage I and II breast cancer recurrence. Most of the studies did not exhibit a dose-response relationship; nevertheless optimal effect was achieved at the highest dose in all cases (Seely et al., 2005).

1.5.2 Chemoprevention of human breast cancer by black tea

Black tea is only consumed at a low level in Eastern populations and as a result, studies conducted in the Eastern populations have not found a significant association between black tea and risk of breast cancer. Epidemiological studies have shown that the risk of breast cancer was highest among those who drank black tea compared to those who drank both green tea and black tea or green tea only. The risk of breast cancer did not differ significantly between black tea drinkers and non-black tea drinkers (Wu et al., 2003a). Studies conducted by Goldbohm et al (1996) and Wu et al (2005) showed that black tea consumption had no effect on breast cancer risk. However (Sun et al., 2006) reviewed epidemiological studies on black tea and breast cancer risk and found a modest decrease in breast cancer risk with black tea consumption in case controlled studies (Ewertz and Gill, 1990; McLaughlin et al., 1992) while a modest increase in breast cancer risk with black tea consumption was observed in cohort studies (Baum and Spittle, 1999; Goldbohm et al.,
1996; Key et al., 1999). Wu et al (2005) also observed a slight effect of black tea consumption on risk of breast cancer, however this reduced risk of breast cancer was not significant and was only associated with high black tea consumption. These contradicting results from epidemiological studies render an association between black tea consumption and breast cancer inconclusive.

1.5.3 The effects of green tea on animal models of breast cancer

The inhibitory action of tea and constituents of tea have been demonstrated in various animal models (Yang et al., 2000). In chemoprevention studies, in vivo animal experiments are considered as a standard because the pharmacological effects of chemopreventive agents in these animal models can be measured and then used in clinical trials (Baliga et al., 2005). Tea, mainly green tea, has been evaluated in laboratory animal models. Both green tea and EGCG have demonstrated chemopreventive activity in both ER+ve and ER-ve xenograft models and in chemically induced breast cancer models (Kavanagh et al., 2001; Sartippour et al., 2001).

Liao et al (1995) examined the effect of EGCG on the growth of mammary tumours in athymic (nude) mice. BALB/c mice were inoculated with oestrogen dependant, ER+ve MCF-7 cells. EGCG (1mg in 0.1ml water) was injected intraperitoneally every day for 14 days. The initial tumour size was reduced by about 40% within 14 days, while control tumours grew by 40% during this same period. Sartippour et al (2001) analysed the effects of various doses of green tea in SCID mice, subcutaneously injected with ER-ve MDA-MB-231 cells. SCID mice received green tea (0.62g/l, 1.25g/l and 2.5g/l) in the drinking water. Green tea significantly inhibited the growth of tumours in a dose-dependent manner compared to control. This reduction in tumour volume by green tea was also observed in a study by Zhou et al (2004) in which MCF-7 cells were orthotopically implanted in the mammary fat pads of female SCID mice supplemented with oestrogen. Green tea (1.5% in drinking water) reduced tumour size and significantly reduced final tumour weight by 56% (p<0.05). The concentrations of green tea and EGCG employed in the studies discussed were relatively high. A reduced concentration of green tea was employed in a recent study by Baliga et al (2005). Different cell numbers (1x10^6 and 1x10^4) were inoculated subcutaneously into BALB/c mice. These groups were referred to as high and low risk.
groups respectively. Green tea polyphenols (GTP) delayed occurrence of tumours by 3 days and 7 days in the high and low risk group, respectively. In the high risk group, tumour volume (42% and 60%) was reduced in both 0.2% and 0.5% GTP groups, respectively. In addition GTP (0.2%, 0.5%) significantly reduced the number of metastatic tumour nodules, size of metastatic lung nodules and total primary weight of mouse skin after 16 days and 30 days treatment.

The dose-dependent effect of green tea catechins (GTC) was examined in a study by Tanaka et al (1997) in which female Sprague Dawley rats were induced with an intragastric dose of DMBA. Rats were either induced with DMBA and treated with green tea or treated with green tea and then induced with DMBA. Rats were fed 1, 0.1 or 0.01% of GTC before or after induction with DMBA for 2 weeks and then received basal diet alone for 35 weeks. GTC decreased development of mammary tumours with both initiation and post-initiation phase administration. In the initiation phase groups, tumour burden and total tumour number were reduced by GTC treatment but reduction was not statistically significant. In contrast, in the post-initiation group, tumour burden and total tumour number were significantly reduced in the 1% GTC (p<0.05) and 0.01% GTC group (p<0.05) compared to controls. The mean size of tumours was not significantly altered in treated rats compared to control rats, suggesting that GTC showed a weak chemopreventive action on DMBA-induced rat mammary carcinogenesis. This chemopreventive effect of GTC was only observed in the post-initiation stage in a non-dose-dependent manner. The weak activity of GTC may be due to the fact that GTC was administered for only two weeks. Hirose et al (1994) examined the effects of GTC on DMBA-induced rat tumours. Rats were either placed on a diet containing 1.0% GTC or basal diet for 35 weeks. Although final incidence and tumour burden were not significantly reduced compared to the control group, the number of survivors in the treated groups (93.8%) at the end of the experiment was significantly greater than in the basal diet group (33.3%). Mean size of tumours when analysed at week 18 was significantly smaller in the GTC group compared to the control group. The effects of green tea given in drinking water (0.3%), on mammary carcinogenesis induced by a low dose (15mg/kg) of DMBA were investigated in rat (Kavanagh et al., 2001). The mean latency to first tumour was significantly longer in the green tea fed groups (84 days) than in the water fed groups (66 days, P=0.001). The mean
number of tumours in animals receiving green tea was significantly reduced compared to water-fed rats.

1.5.4 The effects of black tea on animal models of breast cancer

There is evidence of chemoprevention by black tea in animal models of breast carcinogenesis. A study by Weisburger et al (1997) examined the effects of black tea [1.25% (w/v)] and black tea with milk [1.85% (v/v)] on female Sprague-Dawley rats. Rats received tea from 42 days of age. Rats were either gavaged with DMBA (5mg) or received 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) as the initial carcinogen. Black tea with milk demonstrated a slightly more pronounced effect than black tea alone. Rats receiving tea or tea with milk showed a slightly lower incidence of tumours and a definite decrease in multiplicity and tumour volume compared to rats on water. Rate of tumour appearance were similar in all DMBA-treated groups, in rats receiving tea, tea with milk and water. However, in groups receiving IQ there was a considerable delay in tumour appearance in groups given tea compared to control rats (drinking water alone). IQ-induced mammary gland tumours were seen by week 18, whereas in animals receiving tea, most tumours appeared after wk 34. Although this study supports the use of tea and tea with milk as chemopreventive agents, it does not show statistical significance and tumour multiplicity and incidence of tumours in the tea groups were influenced by the initial carcinogen used. Green tea reduced the multiplicity of tumours when rats were gavaged with DMBA, however green tea increased the number of tumours when administered with IQ. However tumour volumes for the tea groups were similar in the IQ and DMBA groups. In addition, a dose of 5mg of DMBA was used in this study, whereas in studies with green tea a higher concentration of DMBA has been used (15mg-50mg/kg of body weight,(Kavanagh et al., 2001). Rogers et al (1998) examined the influence of black tea on DMBA-induced mammary gland tumours in female Sprague-Dawley rats. Rats were either fed control or high fat diet (HF) and were induced with DMBA (15mg/kg). Rats received either black tea (2%) in the drinking water or water alone. Rats on a HF diet that received black tea showed a significant reduction (P<0.01) in tumour burden and total tumour weight per tumour bearing animal compared with rats on a HF diet and water. Therefore this suggests that black tea may partially block the promotion of tumourigenesis in rats on a HF diet.
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1.6 Mechanisms by which tea may prevent breast cancer

As discussed in section 1.1, carcinogenesis comprises three different stages, initiation, promotion and progression. Many potential cancer protective agents, including tea polyphenols, can impede the initiation stage of carcinogenesis by acting as blocking agents e.g., by altering expression of carcinogen metabolising enzymes (Chen and Kong, 2005; Hakim et al., 2004) or can reverse or arrest the promotion and progression of cancer thus acting as suppressing agents e.g., by altering crucial factors that control cell differentiation, senescence, proliferation, or apoptosis (Fujiki, 1999). Several studies on the mechanisms of action of tea polyphenols have been conducted in cell lines (Yang et al., 2006a) and have gained considerable attention in cancer research (Khan et al., 2006). For example, studies have revealed that tea inhibits the proliferation of breast cancer cells by inducing apoptosis (Agostini et al., 2005), or by interfering with cellular signalling markers (Masuda et al., 2003). Other proposed mechanisms involve the antioxidative activity of tea (Rietveld and Wiseman, 2003)

Biomarkers reflect biological events that occur between exposure to external or endogenous carcinogens and the development of cancer (Sharma and Farmer, 2004). A biomarker may show a quantitative change, such as an increase in cell proliferation or a specific event such as a genetic mutation. Biomarkers of efficacy are markers that may be modified by chemopreventive agents and are measured to help explain experimental results, for example, by relating the effects of interventions on molecular and cellular pathways (Downing, 2001). Development of markers of efficacy and surrogate end-point biomarkers is important in chemoprevention studies (Downing, 2001). The use of biomarkers of efficacy, along with valid surrogate end-point biomarkers (to show drug activity), would greatly improve the development of chemopreventive agents by allowing shorter, smaller, and less expensive chemopreventive trials (Lippman et al., 1998). Reduction in cell proliferation markers, induction of apoptosis and measuring oxidative damage (Sharma and Farmer, 2004; Sharma et al., 2001) could serve as biomarkers of efficacy in
chemoprevention trials. These biomarkers of efficacy are mechanistically associated with the disease process.

1.6.1 Effect of tea on proliferation of mammary cancer cells in vitro

1.6.1.1 Green tea and proliferation

The effects of various catechins, in particular EGCG on proliferation have been extensively studied in breast cancer cell lines (Guo et al., 2005; Kim et al., 2006; Masuda et al., 2003). Green tea extract (GTE), EGCG, EC, EGC and ECG exerted an inhibitory effect on proliferation of MDA-MB-231 cells (Kavanagh et al., 2001; Vergote et al., 2002). The inhibitory effects of GTC and EGCG appeared equivalent and were dose-dependent. Other results demonstrate a slightly higher potency for GTE than EGCG (Sartippour et al., 2001). EGC inhibited the growth of MCF-7 breast cancer cells in a dose-dependent manner (Kavanagh et al., 2001). In contrast, the growth of normal breast cells was not modified by EGC (200μM) under similar experimental conditions. Mittal et al (2004) found that EGCG and EGC (30-50μg/ml) inhibited proliferation of MCF-7 cells dose-dependently and the growth inhibitory effect of EGCG was significantly greater (P<0.001) than that of EGC. However, exposure to EC or ECG was ineffective in inhibiting the proliferation of MCF-7 cells. This is consistent with a study by Vergote et al (2002) in which EC did not inhibit the growth of MDA-MB-231 and MCF-7 cells. Several other breast cancer cell lines have been investigated. For example, Hs578t, an epithelial breast cancer cell line, which is ER -ve and derived from a mammary carcinosarcoma, and D3-1 cells, derived by DMBA-mediated transformation of ER-ve MCF-10F cells, which were established from a patient with fibrocystic disease and do not display characteristics of a malignant phenotype. Doses of green tea polyphenols and EGCG between 40μg/ml and 80μg/ml slowed the growth of these cell lines (Kavanagh et al., 2001). EGCG also exerted inhibitory effects on the MMTV-Her2/neu cells, NF639 and SMF cells, derived from mammary gland tumours (Pianetti et al., 2002). EGCG (20-80μg/ml) decreased the rate of proliferation of NF639 cells and inhibited colony formation in soft agar in a dose dependent fashion. A study by Masuda et al (2003) showed that EGCG inhibited the growth of BT-747 breast cancer cells and significantly reduced cell viability of 4T1 cells in a dose-dependent manner. This
reduction was only observed at concentrations of 20-100μg/ml. A lower dose of EGCG (10μg/ml) did not produce any significant reduction in cell viability (Baliga et al., 2005)

1.6.1.2 Black tea and proliferation
The effect of black tea on breast cancer cell lines was examined by Way et al (2004). Black tea polyphenols (10μM), theaflavin (TF1), theaflavin 3 gallate (TF2) and theaflavin 3-3’ -digallate (TF3) inhibited the proliferation of MCF-7 cells, induced by 100nM of dehydroepiandrosterone (DHEA). DHEA acts as a precursor for the biosynthesis of oestrogenic and androgenic compounds such as 17β-oestradiol, in peripheral tissues. Aromatase converts DHEA to oestradiol stimulating cell proliferation. To date, this is the only study conducted on black tea polyphenols and breast cancer cell lines. Black tea polyphenols inhibited cell proliferation in a dose-dependent manner in several other cancer cell lines, derived from tissues other than the breast (Bhattacharyya et al., 2005).

Little is known about the biological effects of individual black tea polyphenols, in particular theaflavins.

1.6.2 Effect of tea on cell the proliferation marker PCNA
Proliferating cell nuclear antigen (PCNA) is a 36-kDa acidic nuclear polypeptide involved in nucleic acid metabolism (Chu et al., 1998). It plays a fundamental role in the initiation of cell proliferation. PCNA is a cell cycle regulated protein that occurs in dividing cells and is undetectable or present in small amounts in resting cells. Elevated expression of PCNA appears in the nucleus during the late G1 phase immediately before onset of DNA synthesis, which approaches a maximum during S phase and declines again during G2 and M phase. Therefore the level of PCNA is directly associated with the rate of cellular proliferation and DNA synthesis (Zheng et al., 2005). Tea polyphenols, in particular green tea, inhibited proliferation of breast cancer cells, determined by a reduction in PCNA levels (Thangapazham et al., 2006).

1.6.3 Induction of apoptosis by tea
Tea polyphenols can act as suppressing agents by inducing apoptosis or cell cycle arrest and therefore contributing to the inhibition of the promotion and progression stage of
carcinogenesis (Ahmad et al., 1997). Many studies have demonstrated induction of apoptosis or cell cycle arrest with tea polyphenols (Khan et al., 2006). Apoptosis is defined as programmed cell death (Martin, 2006). In apoptosis, unwanted or genetically damaged cells are eliminated from the living system. This elimination is considered as a highly ordered protective mechanism and is essential for normal development, turnover and replacement of cells (Agostini et al., 2005). Apoptosis can be triggered through either the intrinsic or the extrinsic pathway (see figure 1.5). The extrinsic pathway is initiated by stimulating transmembrane death receptors such as Fas receptors, where as the intrinsic pathway is initiated through the release of signalling factors by mitochondria (Martin, 2006). The intrinsic apoptotic pathway involves nuclear condensation, activation of caspase 3 and cleavage of poly(ADP)ribose polymerase (PARP) (Baliga et al., 2005). Green tea polyphenols induced apoptosis in breast cancer cells (Zhao et al., 2006). In addition, EGCG induced Bax oligomerisation and depolarization of mitochondrial membranes to facilitate cytochrome c release into the cytosol (Khan et al., 2006). Cytochrome c is found in cells attached to the outer surface of the inner mitochondrial membrane and is largely localized in the cristae, where it plays a role in the electron transport system. During apoptosis, cytochrome c is released from the cristae into the cytosol, a crucial step in apoptosis induction. The release of cytochrome c can trigger caspase activation and ultimately induce apoptosis (Qanungo et al., 2005). Chemopreventive compounds can target apoptotic regulatory pathways. Such targets include the Bcl-2 family of proteins. These proteins are molecular integrators of both simultaneous cellular pro-death and pro-survival signals. Therefore the balance between Bcl-2, Bcl-xl, anti-apoptotic proteins and pro-apoptotic proteins Bax and Bak can regulate the release of cytochrome c from the mitochondria. Once released to cytoplasm, cytochrome c binds and activates apoptotic protease activating factor (APAF-1), which activates pro-caspase 9, an initiator of caspase cascade. This process is suppressed by molecules that prevent cytochrome c release including the anti-apoptotic protein Bcl-2. Caspases are a family of 12 cysteinyl aspartate-specific proteases involved in apoptosis and are subdivided into initiator (caspases 8, 9, 10) and executioner (caspases 3, 6, 7) caspases. Modulating the mechanisms of caspase activation and suppression is a critical molecular target in chemoprevention, since these processes lead to apoptosis. The intrinsic and extrinsic pathways for apoptosis converge at
caspase 3. Active caspase 9 and caspase 8 respectively have been shown to directly cleave and activate caspase 3 (Katunuma et al., 2006). Caspase 3 cleaves and activates directly or indirectly other effector caspases such as caspase 6 or 7. EGCG induced the release of cytochrome c in \textit{in vivo} and \textit{in vitro} metastatic mouse mammary carcinoma cells (Baliga et al., 2005). Apoptosis was induced by changes in levels of Bcl-2/Bax protein. The increase of Bax levels and the concomitant decrease in Bcl-2 levels increased APAF formation and increased the cleavage of caspase 3 and PARP proteins. Green tea catechins and black tea theaflavins are very potent inhibitors of the anti-apoptotic Bcl-2 family proteins Bcl-x\textsubscript{L} and Bcl-2 (Leone et al., 2003).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1_5.png}
\caption{The intrinsic and extrinsic pathways of apoptosis. \url{http://www.bioteach.ubc.ca/CellBiology/Apoptosis/index.htm}}
\end{figure}

\section*{1.6.4 Effect of tea on cellular signalling}

Information from a stimulus outside the cell is transmitted from the cell membrane, into the cell and along an intracellular chain of signalling molecules to stimulate a response. This process is known as signal transduction. Signal transduction molecules induced by tumour
promoters are targets for chemopreventive agents, including tea compounds (Bode and Dong, 2004).

Table 1.3: Mechanisms by which tea polyphenols interfere with cellular signalling events germane to carcinogenesis

<table>
<thead>
<tr>
<th>Tea polyphenol</th>
<th>Concentration</th>
<th>Host/cell line</th>
<th>Biochemical event</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGCG</td>
<td>0.3μM</td>
<td>MCF-10A</td>
<td>↓ Met phosphorylation</td>
<td>(Bigelow and Cardelli, 2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MDA-MB-231</td>
<td>↓ AKT phosphorylation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↓ ERK phosphorylation</td>
<td></td>
</tr>
<tr>
<td>ECG</td>
<td>0.6μM</td>
<td>MC7-10A</td>
<td>↓ Met phosphorylation</td>
<td>(Bigelow and Cardelli, 2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↓ AKT phosphorylation</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↓ ERK phosphorylation</td>
<td></td>
</tr>
<tr>
<td>EC</td>
<td>No effect</td>
<td>MC7-10A</td>
<td>No effect</td>
<td>(Bigelow and Cardelli, 2006)</td>
</tr>
<tr>
<td>EGC</td>
<td>10-20μM</td>
<td>MCF10A</td>
<td>↓ AKT phosphorylation</td>
<td>(Bigelow and Cardelli, 2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↓ ERK phosphorylation</td>
<td></td>
</tr>
<tr>
<td>EGCG</td>
<td>25-100μM</td>
<td>MDA-MB-231, NIH 3T3, HCT 116, HEK 293T</td>
<td>Blocks Wnt signalling</td>
<td>(Kim et al., 2006)</td>
</tr>
<tr>
<td>EGCG</td>
<td>20-80μg/ml</td>
<td>4T1 cells</td>
<td>↑ apoptotic cells</td>
<td>(Baliga et al., 2005)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↓ antiapoptotic protein Bcl2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>↑ proapoptotic Bax release cytochrome c, induction of Apaf 1 and cleavage of caspase 3 + poly (ADP ribose) polymerase.</td>
<td></td>
</tr>
<tr>
<td>Green tea</td>
<td>0.5% in drinking water</td>
<td>Xenograft model</td>
<td>↓ Bcl2 expression, ↑ Bax expression ↓ PCNA ↑ cleaved caspase 3</td>
<td>(Baliga et al., 2005)</td>
</tr>
<tr>
<td>-----------</td>
<td>-----------------------</td>
<td>-----------------</td>
<td>---------------------------------------------------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>EGCG</td>
<td>40-60µg/ml</td>
<td>MCF</td>
<td>↓ apoptotic cells ↓ telomerase activity ↓ expression of hTERT, subunit of the telomerase enzyme known to correlate with telomerase activity</td>
<td>(Mittal et al., 2004)</td>
</tr>
<tr>
<td>EGCG</td>
<td>30µg/ml</td>
<td>BT-474</td>
<td>↓ pHer2, ↓ pSTAT3 ↓ cyclin D1 and Bcl-xl</td>
<td>(Masuda et al., 2003)</td>
</tr>
<tr>
<td>EGCG</td>
<td>10-30µg/ml</td>
<td>BT-474</td>
<td>Inhibition of transcriptional activity of c-fos and cyclin D1 promoters</td>
<td>(Masuda et al., 2003)</td>
</tr>
<tr>
<td>EGC</td>
<td>100µM</td>
<td>MCF-7</td>
<td>↑ Apoptosis, ↓ Bcl2, ↑ Bax Caspase inhibitors reduced</td>
<td>(Vergote et al., 2002)</td>
</tr>
<tr>
<td>EGC</td>
<td>100µM</td>
<td>MDA-MB-231</td>
<td>↑ apoptosis</td>
<td>(Vergote et al., 2002)</td>
</tr>
<tr>
<td>EGCG</td>
<td>50µg/ml</td>
<td>MMTV-Her 2/neu NF639 cells</td>
<td>↓ NF-kB activity</td>
<td>(Pianetti et al., 2002)</td>
</tr>
<tr>
<td>EGCG</td>
<td>40µg/ml</td>
<td>NF639 cells</td>
<td>↓ pAKT</td>
<td>(Pianetti et al., 2002)</td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th>EGCG</th>
<th>20-80μg/ml</th>
<th>NF639 cells</th>
<th>↓ pHer/neu</th>
<th>(Pianetti et al., 2002)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGCG</td>
<td>40μg/ml</td>
<td>SMF cells derived from MMTV-Her2/neu mouse mammary tumour</td>
<td>↓ pHer/neu</td>
<td>(Pianetti et al., 2002)</td>
</tr>
<tr>
<td>EGCG</td>
<td>40μg/ml</td>
<td>Pro-B cell line cloned stably expressing Her2/neu + EGFR-4</td>
<td>↓ pHer/neu</td>
<td>(Pianetti et al., 2002)</td>
</tr>
<tr>
<td>EGCG</td>
<td>40 or 80μg/ml</td>
<td>Hs578T</td>
<td>↑ p27kip1 cyclin dependent kinase inhibitor</td>
<td>(Kavanagh et al., 2001)</td>
</tr>
<tr>
<td>EGCG</td>
<td>80-160μg/ml</td>
<td>Hs578T</td>
<td>Increased G1 arrest, ↑ apoptosis</td>
<td>(Kavanagh et al., 2001)</td>
</tr>
</tbody>
</table>

Growth factor receptors link information from outside the cell to the intracellular environment. One of the most important families of growth factor receptors are the tyrosine kinase receptors. An example of a tyrosine kinase receptor is the epidermal growth factor receptor (EGFR) (Navolanic et al., 2003). Many human epithelial cancers including breast cancer over-express EGFR (Fernandez Val et al., 2002; Thiery-Vuillemin et al., 2005). EGFR belongs to a family consisting of 4 receptors, erbB1 to erbB4 (erythroblastosis virus protein B) each demonstrating a distinct ligand specificity (King, 2000). In order to activate a receptor tyrosine kinase, the ligand usually has to bind to two adjacent receptor chains simultaneously.

Her-2/neu (or c-erbB-2) oncogene, which is the second member of the EGFR family (EGFR-2), is over-expressed in approximately 30% of breast cancers and is associated with poor overall survival. In particular, it has been found associated with increased metastatic potential and resistance to chemotherapeutic agents (Chung et al., 2002; Hamilton and Piccart, 2000; Massod and Bui, 2002). An EGFR consists of an extracellular ligand binding
domain, a transmembrane region and a cytoplasmic tail. The tail has tyrosine kinase and ATP binding site activities and so is capable of autophosphorylation. EGFR is a plasma membrane glycoprotein that dimerises when a ligand is bound to it resulting in activation of intrinsic tyrosine kinase activity and tyrosine phosphorylation. Autophosphorylation of the cytoplasmic face of the receptor generates a site for intracellular proteins. Once proteins are bound, a cascade of biochemical and physiological responses occur that constitutes mitogenic signal transduction. These signalling cascades regulate multiple cellular processes such as proliferation, differentiation, survival and transformation (Lev et al., 2004; Pianetti et al., 2002).

Figure 1.6: EGFR signaling pathway

http://en.wikipedia.org/wiki/MAPK/ERK_pathway

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Signal transduction is induced when activated receptors then phosphorylate several molecules downstream. The first stage of EGFR activation involves the recruitment of the adaptor protein (Shc). This promotes the binding of Grb2/Sos, which causes the activation of Ras/Raf. Activation of Ras and Raf stimulates several intracellular processes (see figure 1.6). In this cascade, Ras interacts with and activates Raf, which in turn phosphorylates and activates MAP/ERK kinase 1/2 (MEK1/2). Activated MEK 1/2 then phosphorylates ERK 1/2. The JNK 1/2/3 and p38 α/β/γ pathways are parallel to the MAPK cascades in mammalian cells. Once activated MAPKinases (ERK, JNK, p38) activate ELK and cJUN. Phosphatidylinositol-3-kinase (PI3K) is activated by receptor tyrosine kinase and synthesises the second messenger, phosphatidyl inositol-3,4,5-triphosphate which is necessary for phosphorylation of AKT. AKT directly phosphorylates the pro-apoptotic protein Bad, thus enhancing the anti-apoptotic function of Bcl-Xl. Recently EGCG (10-20μg/ml) has been shown to inhibit the MAPK pathway in human colon cancer cells (Khan et al., 2006; Shimizu et al., 2005). EGFR tyrosine kinase activation is believed to initiate multiple cellular responses associated with mitogenesis and cell proliferation. EGCG inhibits phosphorylation of EGFR and this may be a result of blocking the binding of EGF to its receptor (Doss et al., 2005). EGCG was found to inhibit activation of EGFR and also Her2 and multiple downstream signalling pathways in human breast cancer cell lines (Doss et al., 2005; Masuda et al., 2003) (see table 1.3). EGFR deregulation can occur frequently in human breast tumours and extensive research efforts have been focused on finding a correlation between EGFR expression and clinical outcome (Lo et al., 2005). Over-expression of EGFR and subsequent activation of the tyrosine kinase downstream of EGFR induces cell proliferation.

1.6.5 Modulation of carcinogen-metabolising enzymes

The drug-metabolising enzyme system comprises phase I (oxidation, reduction and hydrolysis) and phase II (glucuronidation, sulphation, acetylation, methylation and conjugation with glutathione) enzymes. These enzymes can assist in the production or detoxification of chemical carcinogens. The risk of an individual exposed to carcinogens is attributed to the physiological balances between potential genetic mutations and drug metabolising enzymes, including their levels of expression. Induction of detoxifying
enzymes can promote protective mechanisms against carcinogens, reactive oxygen species (ROS), superoxide anions, hydrogen peroxide and hydroxyl radicals. Thus, inducers of detoxifying enzymes can potentially prevent tumour initiation (Khan et al., 2006). Tea polyphenols can be considered as blocking agents because they induce detoxifying enzymes (Chen and Kong, 2005). The antioxidant activity of tea polyphenols promotes the expression of genes that encode detoxifying defence proteins, such as glutathione peroxidase, γ glutamylcysteine synthetase, NAD(P)H: quinine reductase and heme oxygenase (HO-1) (Na and Surh, 2006).

1.6.6 Antioxidation

A large number of studies support the notion that oxidative damage to DNA, lipids and proteins may contribute to the development of cancer (Aust and Eveleigh, 1999). Many in vitro studies show that tea polyphenols have strong antioxidant properties and therefore may protect cells and tissues against free oxygen radicals. Mechanisms of action may include antioxidant and free-radical scavenging activity, and stimulation of detoxification enzymes through induction or modification of phase I and phase II metabolic enzymes. (Rietveld and Wiseman, 2003) A variety of environmental chemical and physical agents can cause oxidative DNA damage. Endogeneous generation of oxidants can also occur, such as hydroxyl radical and peroxynitrite, which can lead to oxidation of DNA, and ultimately cause cancer (Aust and Eveleigh, 1999; Caporaso, 2003). Oxygen radicals generated by endogenous and exogenous agents can attack DNA bases or deoxyribose residues to produce damaged bases or strand breaks or alternatively, oxygen radicals can attack lipid or protein molecules and generate intermediates that will react with DNA to form adducts. The production of free-radicals such as reactive oxygen species (ROS), oxidized bases, bulky DNA adducts and DNA strand breaks may cause cytogenetic alterations such as deletions, amplifications and/or mutations in critical oncogenes and tumour suppressor genes, leading to cellular transformation and neoplasia (Smith et al., 2003). Lipid hydroperoxides formed by lipid peroxidation are relatively short-lived and are either reduced by glutathione peroxidases to unreactive fatty acid alcohols or they react with metals to produce a variety of products (e.g. epoxides, aldehydes) which are reactive.
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The major aldehyde products of lipid peroxidation are malondialdehyde (MDA) and 4-hydroxynonenal (HNE) (Marnett, 2002; Marnett, 2000).

**Malondialdehyde adduct levels**

MDA is a highly mutagenic and carcinogenic product of lipid peroxidation. MDA reacts with nucleic acid bases at physiological pH to form adducts with deoxyguanosine (dG), deoxyadenosine (dA) and deoxycytosine (dC). It does this by reacting with N2 and N1 of dG with the subsequent loss of a water molecule to form a pyrimidopurinose, (deoxyguanosine pyrimido[1,2-a]purin10(3H)one), abbreviated as M1dG. The amount of M1dG produced from the reaction of MDA and DNA has been reported to be roughly 5 times the amount compared to M1dA. M1dC is formed in trace amounts (Marnett, 2002).

![Figure 1.7: MDA reaction with DNA generates adducts (Marnett, 2002).](image)

The formation of M1dG by MDA is not the only method reported to generate M1dG; base propenals can react with DNA to form M1dG (and presumably other DNA-derived adducts) and tend to be more reactive and mutagenic than MDA (see fig 1.8) (Marnett, 2002). MDA can also arise via the cyclooxygenase pathway of arachidonic acid metabolism. M1dG has been detected in human breast, liver and white blood cells. The correct background levels of M1dG in different human tissues can be analysed and any increase in adduct levels in relation to increased oxidative stress and lipid peroxidation can be monitored (Leuratti et al., 1998).
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![Diagram of M1dG formation]

**Figure 1.8: Schematic diagram for formation of M1dG.** ROS = Reactive oxygen species; PUFA = polyunsaturated fatty acid; COX-2 = cyclooxygenase 2 and PGH2 = prostaglandin H2 (Sharma et al., 2001; Sun et al., 2004).

The levels of M1dG in human white blood cells and breast tissue have been determined. M1dG and putative M1dA adduct levels were higher in normal breast tissue of women with breast cancer compared to the normal tissue of women without breast cancer (Marnett, 2000; Wang et al., 1996). A study by Akbulut et al (2003) investigated the variations of MDA levels in patients with early breast cancer. Increased levels of MDA in patients with
early stage breast cancer were observed when compared with patients without breast
cancer. MDA has been reported as an important biomarker for chemoprevention using
experimental rodent models of carcinogenesis (Sharma and Farmer, 2004). The relationship
between M1dG levels in colorectal tumours and diet have been investigated (Leuratti et al.,
2002). The MDA levels in plasma were found to be reduced in two studies after
consumption of green tea (Freese et al., 1999; Nakagawa et al., 1999; Rietveld and
Wiseman, 2003). Effect of BTT on MDA-DNA adduct levels has not been studied
previously.

MDA-DNA adduct levels may be promising biomarkers of efficacy for quantifying
oxidative DNA damage in the early stages of the carcinogenic process. For this reason,
MDA-DNA adducts may be useful in verifying efficacy of chemopreventive agents
(Bartsch and Nair, 2004). M1dG is a reliable biomarker to assess oxidative damage because
the number of M1dG adducts are significantly affected by the amount of ROS production
(Jeong et al., 2005). M1dG is a relatively stable product which does not vary according to
the extraction procedure used.
Chapter 1: Introduction

1.7 Aims

Polyphenolic constituents contained in green tea impair proliferation and survival of neoplastic cells in vitro and prevent cancer in rodents including that of the mammary gland (see section 1.5 and 1.6 in introduction). Epidemiological studies suggest that high consumption of green tea is associated with a delay in breast cancer recurrence. Much less is known about the potential cancer chemopreventive properties of black tea constituents. The epidemiological evidence for breast cancer prevention in populations consuming black tea is inconclusive. The biological effects of black tea polyphenols, in particular theaflavins, have not been compared or studied in detail at the molecular level. As a result there is insufficient evidence to support the notion that black tea polyphenols prevent breast carcinogenesis. The aim of this study was to determine whether it is appropriate to evaluate green and/or black tea extracts for the chemoprevention of mammary cancer in humans. In order to achieve this aim, the work had the following objectives:

* To explore the efficacy of an extract of black tea, BTT (consisting predominantly of theaflavins) and a green tea extract, GTC (comprising of mainly catechins) on an animal model of breast carcinogenesis; C3(1) SV40 TAg transgenic mouse model.

* To find a suitable dose of BTT and GTC for development in human clinical trials.

* To investigate potential biomarkers of efficacy of BTT/GTC in human breast cancer cells, MDA-MB-468 and in the TAg animal model which may be potentially useful in clinical trials.

* To test the hypothesis that tumour oxidation status in breast cancer cells, as reflected by the malondialdehyde- DNA adduct M1dG, is affected by exposure to BTT/GTC.

* To test the hypothesis that tumour oxidation status in TAg mouse tumour tissue, as reflected by the malondialdehyde-DNA adduct M1dG, is affected by intervention with BTT/GTC.
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* To test the hypothesis that BTT/GTC affects the proliferation of breast cancer cells in vitro.

* Explain whether intervention with BTT/GTC affects the rate of proliferation of tumour tissue in TAg mice.

* To test the hypothesis that BTT/GTC induces apoptosis in breast cancer cells in vitro.

* To test the hypothesis that intervention of TAg mice with BTT/GTC induces apoptosis in tumour tissue.
CHAPTER 2

Materials and Methods
## 2.1 Materials

### 2.1.1 Materials

All chemicals were purchased from Sigma-Aldrich Company Limited (Poole, Dorset, UK) and from Fisher Scientific (Loughborough, Leicestershire, UK) unless stated otherwise. Black tea theaflavins were sent as a gift from Unilever, Sharnbrook, UK. Control Calf thymus DNA and MDA treated Calf thymus DNA was kindly supplied by Raj Singh, Leicester. Anti-M1dG antibody was kindly supplied as a gift from Professor L Marnett, Vanderbilt University.

<table>
<thead>
<tr>
<th>Source</th>
<th>Reagents, Cell Lines, Antibodies</th>
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<tr>
<td>Amersham Biosciences, Buckinghamshire, UK</td>
<td>ECL Western blot detection reagent, Hyperfilm High Sensitive film, Nitrocellulose membrane (pore size-0.1μM), Nitrocellulose membrane (pore size-0.45mM)</td>
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<td>HBL-100 cells, MDA-MB-468 cells</td>
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<td>Biocare Medical, CA, USA</td>
<td>Cleaved caspase 3 rabbit mAb</td>
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<td>Bio-Rad Laboratories, Hertfordshire, UK</td>
<td>Bio-Rad reagent, Protein ladder</td>
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<td>Beckman Coulter UK, Buckinghamshire</td>
<td>Coulter Isoton II diluent</td>
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<td>Calbiochem, San Diego, USA</td>
<td>Anti-SV40 T antigen (Ab-1) mouse mAb</td>
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<td>Caltag Medsystems Ltd, Buckingham, UK</td>
<td>Annexin FITC kit</td>
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<tr>
<td>Cell Signaling Technology, MA, USA</td>
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<td>Geneflow, Staffordshire, UK</td>
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<td>Hunan Kinglong Bio Resource Co Ltd,</td>
<td>Black tea</td>
</tr>
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<td></td>
</tr>
<tr>
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<tr>
<td>Oncogene, MA, USA</td>
<td>Goat anti-mouse IgG, peroxidase conjugate.</td>
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<td>Company/Manufacturer</td>
<td>Products/Reagents</td>
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<td>Oxoid Ltd, Hampshire, UK</td>
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<td>Supersignal West Dura Extended Duration Substrate.</td>
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<tr>
<td>Qiagen Ltd, West Sussex, UK</td>
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<tr>
<td>Raymond A Lamb, East Sussex, UK</td>
<td>DPX</td>
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<tr>
<td>Santa Cruz Biotechnology, California, USA</td>
<td>AKT 1 goat IgG polyclonal antibody, Alpha-tubulin mouse IgG, EGFR rabbit IgG, ERK (K23) (rabbit antibody), pAkt 1/2/3 (Ser 473)-rabbit IgG, pERK (E-4) mouse IgG, Donkey anti-goat antibody</td>
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<td>Peroxidase-labelled goat, anti-mouse IgG2a</td>
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<td>Whatman International Ltd, Kent, UK.</td>
<td>Blotting paper for western blotting</td>
</tr>
<tr>
<td>Unilever UK Foods, Sharnbrook, Bedford, UK.</td>
<td>Green tea</td>
</tr>
</tbody>
</table>
2.1.2 Buffers

Ammonium acetate buffer (1M, 2M)
Ammonium acetate buffer was prepared with ammonium acetate and distilled water at the required concentrations (1M, 2M) and stored at room temperature.

Annexin buffer
Annexin buffer was supplied as a 4x stock that was stored at 4°C and diluted to 1x with distilled water. Working concentration was 10mM Hepes (pH 7.4), 140mM NaCl, 2.5mM CaCl₂.

Bovine serum albumin (BSA)
BSA (1mg/ml) solution was prepared in distilled water, to produce standard curves for use in the Bradford protein assay.

Cl buffer (cell lysis buffer)
Cl buffer was obtained from Qiagen and stored at 4°C and used directly to lyse cells. The composition of the buffer was as follows: 1.28M sucrose, 40mM Tris-Cl, pH 7.5; 20mM MgCl₂; 4% Triton X-100.

Citrate buffer
Citrate buffer was composed of sodium citrate (10mM) and distilled water.

Di-potassium hydrogen orthophosphate (10mM)
A 10mM di-Potassium hydrogen orthophosphate solution was prepared with distilled water prior to use. Fresh solution was prepared for individual experiments. This buffer was also referred to as KP buffer.

Ethanol (70%)
Ethanol (70%) was diluted in distilled water and stored at room temperature.
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**G2 buffer (digestion buffer)**
G2 buffer was obtained from Qiagen and stored at room temperature. The composition of the buffer was as follows: 800mM guanidine HCl; 30mM Tris-Cl, pH 8.0; 30mM EDTA, pH 8.0; 5% Tween-20; 0.5% Triton X-100.

**Hydrogen peroxide solution (3%)**
Hydrogen peroxide solution was prepared in distilled water.

**Lysis buffer**
Lysis buffer comprised 120mM NaCl, 50mM Tris Base, 0.5% Triton X-100, 1mM EDTA, 1mM EGTA, and 1mM Na$_3$VO$_4$ in distilled water. Protease cocktail inhibitor (Sigma) was added to the buffer prior to use.

**Neutral buffered formalin (NBF)**
NBF (10%) consisted of 10% (v:v) formalin, 29mM NaH$_2$PO$_4$ and 46mM Na$_2$HPO$_4$ and made up to its desired volume with distilled water.

**Phosphate buffered saline (PBS)**
PBS was prepared with distilled water, 10 tablets in 1000ml of distilled water.

**PBS-propidium iodide solution (PBS-PI)**
The PBS-PI solution was prepared with propidium iodide and PBS, conc 1:1000 working concentration was used.

**PBS-tween**
PBS –Tween buffer comprised of PBS (conc) and 0.1% tween was prepared with distilled water and stored at room temperature.

**QBT buffer (equilibration buffer)**
QBT buffer was obtained from Qiagen and stored at room temperature. The buffer was made up of 750mM NaCl; 50mM MOPS, pH 7.0; 15% Isopropanol, 0.15% Triton X-100.
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**QC buffer (wash buffer)**
QC buffer was stored at room temperature. The composition of the buffer was as follows: 1.0M NaCl; 50mM MOPS, pH 7.0; 15% Isopropanol.

**QF buffer (elution buffer)**
QF buffer was stored at room temperature and consisted of 1.25M NaCl; 50mM Tris-Cl, pH 8.5; 15% Isopropanol.

**Reservoir buffer (10x)**
Reservoir buffer comprised Glycine (288g), Tris Base (60g), sodium dodecyl sulphate (SDS) (20g) in 2l of distilled water. The buffer was diluted down to a 1x concentration prior to running the gel.

**Running buffer (5x)**
Running buffer was prepared with Tris HCl (38.5g) and Tris base (1.5g) in 500ml of distilled water.

**Sodium bicarbonate solution (2.5%)**
Sodium bicarbonate solution was prepared by dissolving 25g of sodium bicarbonate in 1l of distilled water.

**5x Stacking buffer**
Stacking buffer was prepared with Tris HCl (55g) and Tris Base (200g) in 1l of distilled water.

**Stripping buffer**
Stripping buffer was prepared with Tris Base (3.78g), 20% (w:v) SDS (50ml) and made up to 500ml with distilled water.
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Tris-buffered saline tween buffer (TBST)
TBST was prepared with 1M Tris Base pH 7.5 (500ml), NaCl (87.8g), Tween-20 (10ml) and made up to 1l with distilled water.

Transfer buffer
Transfer buffer comprised Tris Base (29g), Glycine (14.5g), Methanol (1l), 20% (w:v) SDS (9.25ml) in 5l of distilled water.

Trypsin/EDTA (1x)
Trypsin/EDTA solution (0.025%) was prepared from a 10x stock solution in distilled water prior to use.
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2.2 Method

2.2.1 Tea preparation

2.2.1.1 Black tea preparation

For the first animal study, black tea was obtained from Hunan Kinglong Bio-resources (see figure 2.1) and theaflavins were extracted by the following method, based upon that of Tomito et al (2002).

Black tea extract (2g) was dissolved in 500ml of distilled water and heated to 50-70°C while stirring. Black tea extract was then cooled and poured into a separating funnel. Caffeine was extracted into chloroform (4 x 150ml). The bottom, chloroform layer containing caffeine was filtered off and discarded. Ethyl acetate (4 x 200ml) and 2.5% sodium bicarbonate solution was then added to the separating funnel. This was washed with 500ml of distilled water and bottom layer was collected. This extraction is composed of theaflavins and a few catechins. Majority of the catechins and thearubigins were removed during extraction. Ethyl acetate was removed from crude theaflavins under reduced pressure. (Rotavap @ 50°C) A column bed was constructed with Sephadex LH-20 medium in 35-65% acetone: water with a bed height of 15cm. An isocratic mobile phase was prepared also consisting of 35:65 acetone: water. Two crude theaflavin extractions were combined and dissolved in a minimum amount of mobile phase (usually 4-5ml) which was loaded onto the top of the sephadex column. Mobile phase was carefully added to fill the column, ensuring that the stationary phase was not disturbed. A green/yellow band corresponding to chlorophyll was eluted and discarded. Three orange bands were then eluted, the first band was mainly theaflavin and catechins and the later bands were theaflavin gallates, and theaflavin digallate respectively. A ‘theaflavin fraction’ was collected consisting of the later half of the first band and the theaflavin bands ‘theaflavin fraction’ was extracted a second time with ethyl acetate. The second and third bands were extracted with ethyl acetate (800ml). The ethyl acetate was dried over sodium sulphate and filtered using a buchner funnel and filter paper. The filtrate was evaporated using a rotavap under negative pressure at 50°C and dried filtrate was stored in a dessicator over silica gel under reduced pressure. A 70-80% yield of theaflavins was achieved and verified by HPLC (see figure 2.2). For the remaining animal studies, black tea theaflavins (BTT) were kindly
supplied as a gift from Jacek Obuchawicz, Unilever, R&D. BTT comprised theaflavin 11%, theaflavin 3 gallate 28%, theaflavin 3’ gallate 16%, theaflavin 3, 3’ digallate 45%. Compositions of both theaflavin preparations (theaflavins extracted from black tea purchased from Hunan Kinglong Bioresources and theaflavins supplied by Unilever) were comparable (see figure 2.3).

Figure 2.1 represents an hplc trace of black tea purchased from Hunan Kinglong Bioresources Ltd.

Figure 2.2 represents an hplc trace of theaflavins extracted from black tea purchased from Hunan Kinglong Bioresources Ltd.

Figure 2.3 illustrates a hplc trace of the theaflavins supplied by Unilever.
2.2.1.2 GTC preparation
Decaffeinated green tea extract was purchased from Unilever and contained a mixture of epicatechin 3 gallate (ECG 5%), epicatechin (EC 3%), epigallocatechin 3 gallate (EGCG 60%), epigallocatechin (EGC 10%). The caffeine content of GTC and BTT was <0.5 and 0% respectively. Pure catechin standards for HPLC analysis were provided by Unilever R&D.

2.2.2 Animal studies
Experiments were carried out under animal project license PPL 40/2496, granted to Leicester University by the UK Home office. The experimental design was vetted by the Leicester University Local Ethical Committee for Animal Experimentation and met the standards required by the UKCCCR guidelines. A breeding colony was established with wild type FVB mice and female and male C3(1) SV40 T, t antigen (Tag) mice on an FVB background, the latter purchased from the Jackson Laboratory (Bar Harbor, Me). Ear tissue from newborn mice was genotyped for the presence of the transgene using PCR. Genotyping was performed by Sharon Platton. Female mice (+/-) encompassing the Tag transgene were used in the study.

At four weeks of age, heterozygous transgenic female mice (group size 10-16) were randomly assigned to control and treatment groups. Littermates were separated between groups. Mice received either GTC or BTT with their drinking water, controls received drinking water alone. Solutions of GTC and BTT were freshly prepared three times a week. From 11 weeks of age mice were examined once or twice weekly for presence of tumours by palpation, tumour size was measured using calipers (see fig 2.1). Tumour volume was calculated using the equation:

\[ V = \frac{(D \times (d^2))}{2} \]

D represents the long and d the short diameter.

Blood was collected from animals by cardiac puncture under terminal halothane or isofluorane anaesthesia when tumour size, as determined by the sum of the length of
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 palpable tumours per mouse, exceeded 17mm. Animals were shaved, mammary pelt was removed, weighed and pelt or tumours were either fixed in 10% NBF formalin for histological evaluation or flash frozen in liquid nitrogen and stored at -80°C, respectively, for analysis. Liver and salivary gland were also collected from individual animals. Three different study designs were used.

Figure 2.4 shows an image of a TAG mouse bearing tumours. Callipers were used to measure tumour size.

Experimental study design 1
In this study, animals (n=10) were administered tea preparations at 0.05% (w/v) or 0.01% (w/v) from week 4 to the end of the experiment. After approximately 11 weeks, tumour size was examined by palpation and measured using calipers on a weekly basis. Animals were killed (by cervical dislocation) when tumour size, as determined by the sum of the length of palpable tumours per mouse, exceeded 17mm. Animals were shaved, mammary pelt was removed (see fig 2.2) weighed and fixed in 10% NBS formalin for histological evaluation. Blood, liver and salivary gland were removed from animal. Blood was taken from individual animals at the end of the experiment by cardiac puncture, centrifuged and plasma was stored at -80°C until analysis.
Figure 2.5 shows an image of a mammary pelt bearing tumours, removed from a TAg mouse

Experimental study design 2
Mice (n=14) received tea preparations at 0.05% (w/v) from week 4 to the end of the experiment at week 19. After approximately 11 weeks, tumour size was measured by calipers twice weekly. Measurements were recorded and mice were killed at week 19 (day 128) unless total tumour size exceeded 17mm, in which case the mice were culled in agreement with UKCCCR guidelines. Day 128 was selected as a suitable endpoint because in the first study no animals exceeded the 17mm maximum at this age. This allows comparison of the development of disease at fixed age. At the end of the experiment, animals were shaved, mammary pelt was removed, weighed and fixed in 10% NBS formalin for histology. Blood, liver and salivary gland were removed from animals. Blood was collected at the end of the experiment by cardiac puncture, centrifuged (7000g x 10min) and plasma was stored at -80°C until analysis.
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**Experimental study design 3.**

Mice (n=14) were divided into 5 groups. The control group of mice received drinking water alone until the end of the experiment. Two groups of mice received tea preparations (0.05% of GTC/BTT) from week 4 (day 28) to week 13 (day 91) followed by drinking water without tea until the end of the experiment (week 22, day 154). The last two groups received drinking water alone until week 13 (day 91) and then received drinking water supplemented with tea. All mice were killed in week 22 (day 154) and tumours were assessed twice weekly. Blood was collected at the end of the experiment by cardiac puncture, centrifuged and plasma and leukocytes were stored at -80°C until analysis. Liver was also removed, weighed and flash frozen in liquid nitrogen and stored at -80°C.

2.2.3 Investigation into MDA-MB-468 and HBL-100 cell proliferation

2.2.3.1 Cell preservation

MDA-MB-468 cells are a human derived breast cancer cell line. These cells are malignant, adherent, epithelial cells derived from a metastastic adenocarcinoma of breast. They are oestrogen receptor negative, p53 negative and Rb negative. The cells express epidermal growth factor and transforming growth factor α. Cells were maintained in RPMI-1640 medium containing glutamine, supplemented with 10% FBS (www.atcc.org).

HBL-100 cells are a human epithelial cell line harbouring SV40 genetic information and has unlimited growth potential. This transformed immortalized human breast cell line is oestrogen receptor positive, p53 positive and Rb negative and was maintained in DMEM supplemented with 10% FBS (www.atcc.org).

Each cell line was maintained in medium at 37°C, 5% CO₂ until cells achieved 70-80% confluence. Medium (20ml) was removed and cells were washed with PBS(x2). Trypsin/EDTA (1x) was added to the cells and incubated at 37°C, in a 5% CO₂ incubator for 5-10 min or until cells detached from the surface of the flask. Medium (2.5ml) was then added to cells to inhibit the effects of trypsin. The cell suspension was transferred to universal tubes and supernatant was separated by centrifugation for 5 min at 1000 rpm at 4°C. Supernatant was then removed and discarded, cell pellet was resuspended in fresh
culture medium and aliquots of this were added to flasks containing fresh culture medium and incubated in a 37°C, 5% CO₂ incubator.

2.2.3.2 Treatment with GTC/BTT
Cells were maintained in medium until confluent and trypsinised following the method in section 2.2.3.1. Supernatant was removed and cells were reconstituted in 5ml of fresh culture medium. A small aliquot (approximately 500µl) was taken, to determine the number of cells in the whole suspension. An aliquot (20µl) was added to 10ml of Isoton II solution and placed in the Beckmans Z2 Coulter counter. The coulter counter was programmed to count cells that were between 10 and 17µM in size. The number of cells in the suspension was calculated by taking the average from four readings on the coulter counter and multiplying by the dilution factor.

HBL-100 (1x10⁵) and MDA-MB-468 (2.5x10⁴) cells were transferred to individual wells of a 24 well plate and incubated in a 37°C incubator, 5% CO₂ for 24h to allow attachment. Cells were treated with various concentrations of BTT and GTC. A solution (1mg/ml) of each extract was prepared with distilled water. The extracts were sonicated for 20 min, filtered using a 0.2µM filter and diluted to the following concentrations with culture medium: 3.125µg/ml, 6.25µg/ml, 12.5µg/ml, 25µg/ml and 50µg/ml. Medium was removed from the cells and the fresh medium supplemented with tea extracts were dispensed into the wells. Cells were incubated from 24 to 192h in a 37°C, 5% CO₂ incubator.

2.2.3.3 Assessment of cell proliferation
Medium was removed and cells washed with PBS (10ml). Trypsin/EDTA solution (2.5ml) was added to wells and incubated for 5-10 min, to allow cells to detach from the surface of the plate. Medium was then added to the wells and the number of cells per well were determined using parameters as previously discussed in section 2.2.3.2. The cell count values were obtained as replicates of 3 independent experiments.

2.2.3.4 IC₅₀ values
IC₅₀ is the concentration at which 50% of cells survive. However the IC₅₀ values were determined by measuring the number of cells after exposure to BTT/GTC at each
Chapter 2: Materials and Method

concentration and at each time point relative to control, control = 1. Therefore cannot
determine whether the effect of the tea extracts was a result of reduction in proliferation or
cell death. The IC\textsubscript{50} values were determined by reading the number of cells at 50% from a
graph plotted as relative inhibition against concentration.

2.2.4 Immunohistochemical determination of PCNA in fixed tissues from
TAg mice.

2.2.4.1 Preparation of slides
Mouse pelt, which was conventionally embedded in paraffin wax was cut to give 5
transverse sections, corresponding to the 5 pairs of mammary glands. Sections consisting of
the largest lesion per mouse were analysed. Slides were prepared by Jenny Edwards (MRC,
Toxicology unit, Leicester) and stained with hematoxylin and eosin.

2.2.4.2 Dewaxing slides
Tissue sections were de-waxed (3x), to allow antibody access, by immersing xylene. The
sections were placed in containers filled with IMS (x3) and then 70% IMS for 3min each.
The tissue sections were washed in running tap water for 5 min and then in distilled water
for 3 min. Citrate buffer was prepared and adjusted to pH 6.0 with 2M NaOH and poured
into a pressure cooker. The sections were placed into the pressure cooker and heated in the
microwave in order to retrieve antigen. The time it takes to heat the slides varied depending
on the antigen of interest or PCNA detection, the tissues were heated for 20 min.

The sections were then placed in two changes of distilled water at room temp and then in
freshly prepared hydrogen peroxide solution (3%) for 20 min to inhibit endogenous
peroxidase.
Sections were washed in running water for 5 min and then in distilled water. The tissue
slides were then placed in Shandon cover plates and inserted into the Shandon Sequenza.
The reservoir was filled with PBS and checked for leakage between slide and coverplate.
2.2.4.3 Antibody reaction

The primary antibody was diluted, (see table 2.2). 100μl of the diluted antibody was added to the sections and left at room temperature for 3h. Following incubation, sections were washed with PBS (x3) by filling the reservoir and allowing it to empty.

The secondary antibody (see table 2.2 for details on dilutions) was added (100μl) to the sections and left at room temp for 1h. Incubation time varied between 1h to 2 half hour incubations according to the secondary antibody used. The sections were washed with PBS (x3), 180μl of freshly prepared 3,3’-diaminobenzidine tetrahydrochloride (DAB) (1mg/ml) was added to each section for 5 min. The sections were washed with PBS and transferred to a staining rack. The tissue sections were washed in running tap water for 3 min and counterstained with haematoxylin. The sections were then dehydrated through 70% and IMS (x3) and finally into xylene.

2.2.4.4 Analysis

The sections were analysed under the microscope. Numbers of stained nuclei in five fields per tumour section were counted (x40 magnification). The PCNA proliferation index (%) and cleaved caspase 3 indices were calculated by dividing the number of stained nuclei by the total number of nuclei x 100.

2.2.5 Study of MDA-MB-468 cell viability

2.2.5.1 Overview on the Annexin FITC detection assay

The Annexin FITC apoptosis detection kit is designed to identify a specific biochemical change in the cell surface membrane that is considered to be a major event of early apoptosis. These kits contain Annexin V which is conjugated to fluorescence isothiocyanate (FITC). It can be detected using flow cytometry or fluorescence microscopy. The counterstain, propidium iodide (PI), can be used to distinguish apoptotic cells with intact membranes, from lysed necrotic cells. Annexin V binds to phosphatidylserine (PS), which is normally present on the inner membrane leaflet of viable cells. The translocation of PS to the membrane surface is an early event in apoptosis. The cell populations were detected as follows: Viable cells were nonfluorescent and were detected in the lower left quadrant of
the fluorescence-activated cell sorting histogram. Cells in the metabolically active stages of apoptosis stained with annexin, but not with PI and were detected in the upper left quadrant and necrotic (lysed) cells with compromised membranes which were bound to both annexin and PI were those in the upper right quadrant. Necrosis or cell death by metabolic arrest and lysis is defined by a general swelling of the whole cell and its constituent organelles. Because this process is caused by early permeabilisation of the cell membrane (lysis), necrosis is easily detected in vitro by exposure to PI. (www.upstate.com/browse/productdetail.asp)

2.2.5.2 Extracting lysates
Cells (1 x 10^5) were seeded onto petri dishes and incubated for 24h in a 37°C, 5% CO_2 incubator. Following incubation, cells were treated with BTT/GTC and incubated for 24 or 48h. For extraction, medium containing the floating cells was removed and reserved in centrifuge tubes. The petri dishes were washed in PBS (x2) (5ml) and the remaining adherent cells were trypsinised for a short time with Trypsin/EDTA (1ml). An equal volume of medium was added to the petri dishes and fractions of floating cells and adherent cells were combined. Supernatant was removed by centrifugation for 5min at approximately 350 x g. Pellet was reconstituted in 4ml of fresh culture medium and incubated at 37°C for 30 min.

2.2.5.3 Apoptosis assay
Cell suspension (4ml) was transferred to a FACS tube and supernatant was removed by centrifugation. Pellet was resuspended in 1ml of annexin buffer (so that the final cell no. was approx 1 x 10^5). Annexin V FITC conjugate (4μl) was added, vortexed and incubated at room temperature for 10 min. Propidium iodide was added at a final concentration of 1.5μg/ml and incubated at room temp for 1 min. The samples were then analysed via flow cytometry (Bekcton Dickinson Flow Cytometer, BD Biosciences, Oxford, UK) as described in section 2.2.5.1.
2.2.6 Study of protein expression in MDA-MB-468 cells and TAg tumour tissue.

2.2.6.1 Protein extraction

Protein extraction from cells
Cells (1x10^6) were dispensed into petri dishes (n=16) and incubated for 24h in a 37°C, 5% CO₂ incubator. Following incubation, cells were treated with BTT or GTC and incubated between 1 and 48h depending on the desired time point. Medium was removed, cells were washed in ice cold PBS buffer (x2), (5ml) and 200-300μl of lysis buffer was added to individual petri dishes. Lysates were vortexed thoroughly, sonicated at cycle 1, amplitude 90 for 3 x 5 s bursts and left on ice for 10 min. The supernatant was removed by centrifugation at 13000rpm and transferred into clean eppendorfs. The protein lysates were stored at -80°C (long term) or -20°C (short term) prior to analysis. Protein concentrations of lysates were determined using the Bradford assay.

Protein extraction from tissues
Approximately 50mg of tissue was weighed and homogenized in lysis buffer (200μl) on ice, using a hand held homogenizer and transferred to eppendorfs. The tissue homogenates were kept on ice for 10-15 min, vortexed for 10 s, sonicated at cycle 1, amplitude 90 for 3 x 5 second bursts and incubated on ice for 10min. The supernatant was removed by centrifugation at 13000 rpm for 3 min, and dispensed into eppendorfs. The protein lysates were stored at -20°C and protein concentrations were determined using the Bradford assay.

2.2.6.2 Determine protein concentration using the Bradford assay
Stock solution of bovine serum albumin (1mg/ml) was prepared in distilled water and diluted to generate a standard curve.
Lysates were diluted 1:1000 or 1:250 with water. Diluted lysates and BSA dilutions were dispensed into eppendorfs (800μl), and 200μl of bio-rad reagent was added. Samples were vortexed and left for 10min. The absorbance was read at 595nm using the UV-Visible Spectrophotometer (Cary 50 Bio, Varian). A standard curve was produced with the
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Absorbance values for the BSA standard. The absorbance readings obtained for the lysates were determined from the standard curve and corrected for dilution factor.

2.2.6.3 Western blot technique

Overview of western blot

The western blot technique is utilized for the detection of one or more protein antigens in a mixture. The sample (protein) is loaded onto an SDS-polyacrylamide gel (SDS-PAGE). Electrophoresis causes the proteins to separate according to size and charge. The proteins are transferred, by application of an electric current to a more accessible medium, such as nitrocellulose membrane or nylon sheet. The next step involves incubating with a protein solution containing, for example casein or milk powder. This step is employed to ‘block’ nonspecific binding sites. The membrane is then incubated with a specific primary antibody and unreacted antibody is removed by washing. The secondary antibody is specific for the primary antibody.

![Diagram of antibody reaction](image)

Figure 2.6: Schematic diagram of antibody reaction.
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Preparation of gels

Gel casting apparatus (Bio-rad, mini gel apparatus) was set up utilizing Bio-rad short and spacer plates. The running gel was then poured between the plates, to 1-3 mm below the well base.

Running gel was prepared as follows:

10% gel

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide (30%) 37.5:1 ratio</td>
<td>10 ml</td>
</tr>
<tr>
<td>5x Resolving buffer</td>
<td>6 ml</td>
</tr>
<tr>
<td>SDS 10% (w:v) solution</td>
<td>300 μl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>13.4 ml</td>
</tr>
<tr>
<td>Ammonium persulfate (Amps) 10% (w:v) solution</td>
<td>230 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>15 μl</td>
</tr>
</tbody>
</table>

12.5% gel

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>12.5 ml</td>
</tr>
<tr>
<td>5x Resolving buffer</td>
<td>6 ml</td>
</tr>
<tr>
<td>SDS 10%</td>
<td>300 μl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>11 ml</td>
</tr>
<tr>
<td>Amps 10%</td>
<td>230 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>15 μl</td>
</tr>
</tbody>
</table>

The next step involves preparing the stacking gel. The stacking gel was comprised of:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>5x Stacking buffer</td>
<td>4 ml</td>
</tr>
<tr>
<td>SDS 10%</td>
<td>200 μl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>13.1 ml</td>
</tr>
<tr>
<td>Amps 10%</td>
<td>160 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>16 μl</td>
</tr>
</tbody>
</table>

Stacking gel was poured onto the running gel and a 10 well comb was inserted. Once set, the comb was removed and the cast gels were placed into a running tank and filled with 1x reservoir buffer.
Chapter 2: Materials and Method

**Sample preparation**
Lysates (25µg-100µg) were dispensed into eppendorfs and 10-15µl of loading buffer was added to the protein. Samples were heated to 100°C, centrifuged to remove particulates and loaded onto the gel wells.

**Running the gel**
Bio-rad dual range protein ladder (5-10µl) was loaded into the first well of each gel and samples were loaded into the remaining wells. The gel ran at 80V through stacker and at 100-130V through running gel.

**Transferring proteins from gel to nitrocellulose**
Four pieces of Whatman blotting paper and one piece of nitrocellulose membrane were cut to size (9x6cm for blotting papers and 8x5cm for membrane). Mini gel holder cassettes were positioned with the black side forming the bottom layer. Fiber pads, blotting papers and membranes were immersed in transfer buffer before and during use. The fiber pad was placed onto the cassette, followed by 2x whatman blotting papers. The gel (with the stacking gel removed) was removed from the glass plates and placed on top of the blotting papers. The gel surface was immersed in transfer buffer. Next, the nitrocellulose membrane was laid out on to the gel, followed by another set of whatman blotting papers and fiber pads. The cassette was inserted into the blotter assembly and into the tank with the black side of the blotter attached to the –ve electrode. The negative charge is on the same side of the gel and the positive charge is on the side of the nitrocellulose membrane. This allows binding of the negatively charged proteins to the positively charged nitrocellulose membrane. The tank was filled with transfer buffer and the gel was transferred at 30V overnight on ice or 100V for 2h on ice.

**Antibody reaction**
The transfer assembly was disassembled and the nitrocellulose membrane was placed in a container filled with TBST buffer. The membrane was washed for 5 min in TBST, then blocked with 5% or 10% of Milk powder (w:v) (made up in TBST) and placed on a rocking platform. The membrane was blocked for 2h at room temperature. Membrane was washed.
for 5min in TBST (x2) (10-15ml per membrane) and incubated with 1° antibody overnight at 4°C, placed on a rocking platform. The antibody was diluted between 1:500-1:2000 (v:v) in 5% (w:v) marvel in TBST. The actual concentrations of the various primary antibodies used in the study with respective secondary antibody dilutions are presented in the table 2.2. Following incubation with 1° antibody overnight, antibody solution was poured off and membrane was washed for 5min in TBST (x5). Secondary antibody was then added, which was diluted in 5% milk powder (w:v) in TBST. The membrane was incubated in the secondary antibody solution for 1h at room temp placed on a rocking platform. The membrane was then washed for 5 min in TBST (x5).
Table 2.1 represents the antibody concentrations used in analysing the expression of various proteins.

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Dilution in 5% Marvel</th>
<th>Secondary Antibody</th>
<th>Dilution in 5% Marvel</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKT 1 goat IgG</td>
<td>1/1000</td>
<td>Donkey anti-goat</td>
<td>1/2000</td>
</tr>
<tr>
<td>Cleaved caspase 3</td>
<td>1/1000</td>
<td>Anti-rabbit IgG</td>
<td>1/2000</td>
</tr>
<tr>
<td>(Asp 175)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFR rabbit IgG</td>
<td>1/500, 1/1000</td>
<td>Anti-rabbit IgG</td>
<td>1/2000</td>
</tr>
<tr>
<td>ERK (K23) rabbit IgG</td>
<td>1/1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pAKT 1/2/3 (ser 473)</td>
<td>1/1000</td>
<td>Anti-rabbit IgG</td>
<td>1/2000</td>
</tr>
<tr>
<td>rabbit IgG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse monoclonal PCNA</td>
<td>1/50</td>
<td>Peroxidase labeled goat, anti-mouse IgG2A</td>
<td></td>
</tr>
<tr>
<td>pEGFR (Tyr 1068) rabbit IgG</td>
<td>1/500, 1/1000</td>
<td>Anti-rabbit IgG</td>
<td>1/2000</td>
</tr>
<tr>
<td>pERK (E-4) mouse IgG</td>
<td>1/1000</td>
<td>Goat anti-mouse IgG</td>
<td>1/2000</td>
</tr>
<tr>
<td>pHER2/Erb2</td>
<td>1/1000</td>
<td>Anti-rabbit IgG</td>
<td>1/2000</td>
</tr>
</tbody>
</table>

**Developing the membrane**

The membrane was placed onto a flat surface with the protein side up, ECL (1.5ml per membrane) was prepared (ECL is a 1:1 v:v mix). The Enhanced Chemiluminescence Luminol (ECL) mix was then dispensed onto the membrane and left for 1min. The excess
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was drained off and the membrane was wrapped in cling film with the protein side smooth and placed in a darkroom cassette.

In the darkroom- a high sensitive film was placed over the membrane and allowed to expose between 1-5min. The film was then developed and protein bands were detected.

**Stripping the membrane**

In order to re-use membranes to detect other proteins they were stripped using a pre-heated mixture of stripping buffer and 400μl of β-mercaptoethanol and heated at 60°C for 45min. The membranes were washed in TBST for 5 min x3.

**Equal loading**

Membranes were re-blocked in 10% milk powder prepared in TBST, overnight at 4°C or 2h at room temperature. Membranes were washed in TBST (x3) for 5min before analysis of proteins, as described earlier, (primary antibody was made up in 5% marvel with TBST at the required dilution). Equal loading was carried out as follows:

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Dilution in 5% Marvel</th>
<th>Secondary Antibody</th>
<th>Dilution in 5% Marvel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total AKT</td>
<td>1/1000</td>
<td>Donkey anti-goat IgG</td>
<td>1/2000</td>
</tr>
<tr>
<td>Alpha-tubulin</td>
<td>1/500</td>
<td>Goat anti-mouse IgG</td>
<td>1/1000</td>
</tr>
<tr>
<td>β-actin</td>
<td>1/1000</td>
<td>Anti-rabbit IgG</td>
<td>1/2000</td>
</tr>
</tbody>
</table>

The membrane was immersed in primary antibody for 2h at room temp, placed on a rocking platform. Membrane was washed in TBST (x5) for 5min and secondary antibody solution was poured into a container encompassing the membrane. The membrane was developed as described on the previous page.
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**Densitometry**

To quantify the results obtained by western blot, developed films showing protein bands of interest were placed on a transilluminator (Gene Genome Bioimaging System, Syngene, Cambridge, UK) and each band measured by densitometry. This was repeated for bands representing the proteins used for equal loading. The final densitometry values for proteins of interest were calculated as proportions of the equal loading protein.

2.2.7 Malondialdehyde DNA adduct levels in MDA-MB-468 cells, TAg tumours and leukocytes.

2.2.7.1 Sample preparation

**Preparation of cells**

Cells (1 x10^6) were plated out onto petri dishes and incubated for 24h in a 37°C, 5% CO₂ incubator. Following 24h incubation, medium was removed, cells were washed in PBS (x2) and treated with BTT and GTC and incubated for 24h. Cells were harvested and cell pellet was resuspended in 10ml of culture medium, Supernatant was separated by centrifugation at 1000g for 3min and discarded. Pellet was reconstituted in 10ml of ice-cold PBS and supernatant was removed by centrifugation. Pellet was resuspended in 2ml of PBS, 2ml of ice-cold C1 buffer and 6ml of ice-cold distilled water. Buffer C1 lyses the cells but stabilises and preserves the nuclei. The suspension was mixed by gentle inversion and incubated on ice for 10 min. Supernatant was removed by centrifugation at 13000g for 15 min at 4°C and discarded. The pellet was resuspended in 1ml of ice cold C1 lysis buffer and 3ml of ice cold distilled water. The suspension was gently vortexed and supernatant was removed by centrifugation at 13000g for 15 min at 4°C. This wash step removes all residual cell debris from the nuclear pellet. The pellet was stored at -20°C until further use. The pellet was resuspended in 5ml G2 buffer and vortexed gently for up to 30seconds. Proteinase K (20mg/ml, 95µl) and 16µl of RNAse A (100µg/ml solution) were added to the suspension and incubated for 2h at 37°C in a water bath, with gentle shaking. Buffer G2 lyses the nuclei and denatures proteins such as nucleases, histones, and viral particles. Proteinase K digests the denatured proteins into smaller fragments. Buffer G2 and
Proteinase K, in combination, strip the genomic DNA of all bound proteins, facilitating efficient removal during purification.

Tissue preparation
In preparing the tissues for DNA extraction, tissues were weighed, <100mg of tissue was used for extraction. 100mg of tissue yields approximately 90 μg of DNA. Tissue was homogenized using a hand held homogenizer and transferred into tubes consisting of RNAase (100mg/ml) and G2 buffer (9.5ml). Proteinase K (20mg/ml, 500μl) was added to the tissue and the suspension was incubated at 50°C for 2h in a water bath, gently shaking.

Blood preparation
Whole blood was placed into a 15ml centrifuge tube, containing 1ml ice-cold C1 buffer and 3ml of ice-cold distilled water. The tube was mixed by inverting the tube several times until suspension became translucent. The suspension was then incubated on ice for 10 min. The lysed blood was centrifuged at 4°C for 15 min at 13000 rpm. Supernatant was transferred into a 50ml centrifuge tube and discarded. The pellet appears red due to residual haemoglobin. 1ml of ice-cold C1 buffer and 3ml of ice-cold distilled water were added to the pellet. Supernatant was discarded by centrifugation at 4°C for 15min at 13000g. 5ml of G2 buffer was added to the pellet and vortexed for 10-30s. Proteinase K (20mg/ml, 95μl) was added to the suspension and incubated at 50°C for 60 min.

2.2.7.2 Isolation of genomic DNA from cells, tissue and blood.
Cell, tissues and blood suspensions were purified using the same procedure. Qiagen genomic tips (100/G) were placed into a QIArack over a waste tray. The Qiagen tips were equilibrated with 4ml of QBT buffer and allowed to empty by gravity flow. Flow begins automatically due to the presence of detergent (0.15% Triton x-100) in the equilibration buffer, this reduces the surface tension. The sample suspensions were centrifuged at 5000g for 10min at 4°C to remove any particulate matter before loading onto the QIAGEN Genomic-tips. The rate of flow depends on the sample source, the genome size and the number of cells from which the DNA sample was prepared. Reduced flow rates occur due to concentrated genomic DNA lysates. The superior selectivity of the Qiagen resin and the
salt and pH conditions of the lysate ensures that only DNA binds. Degraded RNA and cellular protein are not retained and appear in the flow-through fraction. The sample was allowed to flow through until there was no evidence of the lysate in the genomic tip. The genomic-tip was washed with 7.5ml of buffer QC (x2); this removed all contaminants, such as traces of protein and RNA (e.g., RNAase A), without affecting the binding of the DNA. The wash buffer contains a low concentration of ethanol which eliminates non-specific hydrophobic interactions, further enhancing the purity of the bound DNA, without the use of phenol. The genomic DNA was eluted with 1x 5ml of QF buffer which was pre-warmed to 50°C. The genomic DNA was eluted into 10ml centrifuge tubes containing 3.5ml of isopropanol. The eluted DNA was allowed to flow through into the tubes, the tubes were inverted 10-20 times and a small DNA pellet was retrieved after centrifugation at 4000g for 20 min at 4°C. The pellet was washed with 0.5-1.5ml of 70% ethanol. Pellet and ethanol was transferred into 2ml eppendorfs using a pasteur pipette. Supernatant was removed after centrifugation at 5000g for 10 min at 4°C. The DNA pellet was dissolved in 100-200µl of distilled water and stored at -80°C for long-term storage.
Figure 2.7: illustrates the DNA purification procedure (Qiagen Genomic DNA Handbook)

2.2.7.3 Quantification of DNA
DNA was diluted in distilled water, 1:1000 dilutions were quantified in a spectrophotometer at 280nm. The purity of the DNA was determined by measuring the
ratio between the absorbance 260nm and 280nm. Acceptable purity was represented by ratio of 1.5-1.9.

2.2.7.4 Overview of the immunoslot blot assay

The immunoslot blot assay is a sensitive and specific method used for M1dG detection. It requires a relatively small amount of DNA (<5μg) and is less time consuming and laborious than other methods, thus allowing analysis of a large number of samples in a short time. Synthetic nucleoside standard, MDA-modified calf thymus DNA and oligonucleotides were prepared by Raj Singh (CSMM, University of Leicester, Leicester) by the methods described previously (Leuratti et al., 1998). Calf thymus DNA (CT-DNA) was incubated with malondialdehyde in 0.1M potassium dihydrogen orthophosphate for 4 days. Following precipitation with ethanol, DNA was redissolved in water and set aside for the immunoslot blot (ISB) assay. Oligonucleotides were precipitated using ethanol and dissolved in 0.1M potassium dihydrogen orthophosphate. Once the standard MDA-modified CT-DNA and oligonucleotides were prepared, they were digested to deoxynucelosides. The amount of M1dG was measured by HPLC- fluorescence using a calibration curve obtained with synthetic M1dG. Malondialdehye modified CT-DNA was diluted with oligonucleotides containing known amounts of adduct and used for generating standard curves. The M1dG (D10A1) primary antibody, which was obtained from LJ Marnett at Vanderbilt University was used to identify DNA adducts in both synthetic standards and unknown samples. The binding of D10A1 antibody was proportional to the amount of M1-dG in the samples. Leuratti et al (1998) investigated whether the D10A1 antibody was specific and found that this monoclonal antibody did not bind to unmodified intact DNA or RNA. It was also shown that unmodified oligonucleotides did not bind to the antibody. Competitive inhibition studies showed that cross-reactivity of antibody binding to other related exocyclic adducts was very low.

Sample preparation

Control calf thymus DNA and MDA treated calf thymus DNA (5fmol and 10fmol) were prepared to produce a series of standards.
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KP buffer (65μl) and 150μl of PBS were added to 35μl of each standard and vortexed. The standards were then sonicated for 20min and heated to 100°C for 5 min on a heating block and then cooled on ice for approximately 10 min. The standards were centrifuged at 13,000g for 2 min and 250μl of 2M ammonium acetate was added to standards before centrifugation at 13,000g for 1 min.

Approximately 3.5μg of DNA was required for the assay. DNA lysates (3.5μg) were made up to 100μl with KP buffer. PBS (150μl) was added to the sample, vortexed and centrifuged for 1 min at 13,000g. The samples were sonicated for 20min and heated to 100°C for 5 min and then cooled on ice for at least 10 min. The standards were mixed and centrifuged at 13,000g for 2 min and then 250μl of 2M ammonium acetate was added, vortexed and centrifuged at 13,000g for 1 min. The samples were prepared at the same time as the standards.

Assay

Two gel-blotting papers were bathed in 1M ammonium acetate and then placed onto the minifold immuno-slot blot apparatus (Schleicher and Schuell, Germany). The nitrocellulose filter was bathed in distilled water prior to immersion in 1M ammonium acetate. The filter was then placed on top of the blotting paper. The aspirator was turned on and 143μl of standard and sample were added to wells in triplicate (1μg of DNA into each well). Samples were allowed to run through until there was no evidence of the sample in the well. 1M ammonium acetate was added to individual wells and allowed to run dry. The filter was removed from the apparatus and baked in an 80°C oven for 1½h. The filter was then placed in a plastic container and bathed in 10ml of PBS-Tween plus 5% powdered milk and allowed to rock gently at room temperature for 1h.

Probing with antibody

The filter was washed for 5min with PBS-Tween (x2) and then incubated with the MDA-dG primary antibody. The MDA-dG antibody was diluted to 1:8000 in PBS-Tween plus 5% milk powder. The filter was incubated in primary antibody for 2h at room temperature, followed by an overnight incubation at 4°C. Filter was then washed with PBS-T for 1 min followed by a further two 5 min washes. The secondary antibody was prepared by diluting
the antibody (mouse immunoglobulins HRP) with PBS-T plus 0.5% milk powder (1/4000). The secondary antibody was poured into the container with the filter and allowed to rock for 2h. Filter was washed with PBS-T for 15min followed by a further two 5min washes. Just before being used, Supersignal Chemiluminescent substrate was prepared by mixing equal volumes (4ml) of the ultra luminal/enhancer solution with the ultra stable peroxide solution.

**Analysing membrane**

The filter was bathed in the chemiluminescent reagents for 5min and the level of DNA adducts were detected by exposure on the genegnome bioimaging system.

**Equal loading**

Following analysis, the filter was placed into a container with PBS-PI solution. The container was covered with foil and allowed to rock gently at room temperature for 3h. The filter was then washed in PBS by gentle rocking for 1h followed by a further 30min wash. The bands were analysed on the genegnome bioimaging system. Individual bands were quantified and adduct level was corrected on the basis of PI staining of each band.

**2.2.8 Statistical analysis**

Data was analysed using Excel (Microsoft Office XP Professional). Results were statistically analysed using SPSS (version 13) software package. Values were subjected to one-way analysis of variance (ANOVA) and log rank analysis and statistical significance was determined by post hoc Tukey’s pairwise comparison.
CHAPTER 3

Effects of BTT/GTC on MDA-MB-468 and HBL-100 breast cells
Chapter 3: Effects of BTT/GTC on MDA-MB-468 and HBL-100 breast cells

3.1 Introduction

The primary aim of the work described in this chapter was to study mechanisms by which BTT and GTC inhibit breast cancer cell growth and survival \textit{in vitro} and therefore may ultimately serve as biomarkers of efficacy in preclinical and clinical studies (refer to chapter 1.6). Studies on biomarkers may be particularly useful to indicate efficacy of a chemopreventive agent (Veronesi and Bonanni, 2005). In the light of the properties of BTT and GTC (see chapter 1.5), three biochemical properties of tea potentially germane to cancer chemoprevention were studied, 1, their ability to inhibit growth and survival of breast cancer cell lines, 2, their ability to interfere with signal transduction molecules EGFR, pEGFR, ERK, pERK, and pAKT and 3, their antioxidant properties. The cell lines chosen for this investigation were the human derived breast cancer cell line, MDA-MB-468 which shares properties with the TAg model, and HBL-100 cells which are a non-malignant, human derived, transformed breast cell line. The hypothesis was tested that exposure of MDA-MB-468 cells to GTC or BTT affects proliferation and survival of cells by inducing apoptosis, modulating the EGFR signal transduction pathway and affecting the levels of oxidative DNA adducts in cells, as indicated by levels of M1dG.
3.2 Effect of BTT/GTC on growth and survival of MDA-MB-468 and HBL-100 breast cells

MDA-MB-468 cells exposed to BTT or GTC (3.1-50 μg/ml) for 24h to 192h were counted using a Beckman coulter counter. The number of MDA-MB-468 cells/ml decreased dose dependently (3.1-50 μg/ml) at all time points, in the presence of either BTT (figure 3.1) or GTC (figure 3.2).

Similarly, a dose dependent (3.125-50 μg/ml) decrease in HBL-100 cells was also observed from 24h-192h when cells were exposed to BTT (figure 3.3) and GTC (figure 3.4). The IC50 values at 48h (table 3.1) show that both extracts decreased the number of cells at similar potency with no difference between malignant and non-malignant cells.

![Figure 3.1 Effect of BTT on number of MDA-MB-468 cells. Values are the mean ± SD of replicates of 4 experiments (each with n=8). Standard deviation error bars have been omitted from the figure, they were between 5% (control at 168h) and 86% (25 μg/ml of BTT at 192h) of the mean. Star indicates that values were statistically significant from control (*P<0.05, **P<0.01).](image-url)
Figure 3.2 Effect of GTC on number of MDA-MB-468 cells. Values are the mean ± SD of replicates of 4 experiments (each with n=8). Standard deviation error bars have been omitted from the figure, they were between 11% (control at 96h) and 100% (50µg/ml of GTC at 120h) of the mean. Stars indicate that values were statistically significant from control (*P<0.05, **P<0.01).
Figure 3.3 Effect of BTT on number of HBL-100 cells. Values are the mean ± SD of replicates of 4 experiments (each with n=8). Standard deviation error bars have been omitted from the figure, they were between 16% (3.125μg/ml of BTT at 192h) and 100% (50μg/ml of BTT at 144h) of the mean. Star indicates that value was statistically significant from control (*P<0.05).
Figure 3.4 Effect of GTC on number of HBL-100 cells. Values are the mean ± SD of replicates of 4 experiments (each with n=8). Standard deviation error bars have been omitted from the figure, they were between 20% (3.125µg/ml of GTC at 168h) and 100% (50µg/ml of GTC at 192h) of the mean. Stars indicate that values were statistically significant from control (*P<0.05, **P<0.01).

Table 3.1 IC\(_{50}\) values of BTT and GTC.
Cells were exposed to BTT and GTC for 48h. Cell numbers were counted and plotted, relative inhibition against concentration (see chapter 2.2.3.4). IC\(_{50}\) is the concentration at which 50% of the cells survive. This may be a result of a decrease in cell proliferation and/or increase in cell death. Values are expressed as IC\(_{50}\) ± SD, n=3.

<table>
<thead>
<tr>
<th>Treatment/Cell line</th>
<th>MDA-MB-468 (µg/ml)</th>
<th>HBL-100 (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTT</td>
<td>7.2 ± 0.26</td>
<td>9.9 ± 0.17</td>
</tr>
<tr>
<td>GTC</td>
<td>8.9 ± 0.19</td>
<td>8.1 ± 0.12</td>
</tr>
</tbody>
</table>
Chapter 3: Effects of BTT/GTC on MDA-MB-468 and HBL-100 breast cells

3.3 Effect of GTC and BTT on MDA-MB-468 tumour cell survival

The hypothesis was tested that exposure of MDA-MB-468 cells to GTC or BTT induces apoptosis. MDA-MB-468 cells were studied by flow cytometry using Annexin V FITC. The representative fluorescence scattergrams in figures 3.5 (A, B) and their quantitative evaluation in figures 3.5 (C, D) demonstrate that GTC at 12.5μg/ml and at 25μg/ml and BTT at 25μg/ml significantly decreased the number of viable cells following incubation at 48h. BTT enhanced the numbers of apoptotic cells after incubation for 24h and 48h. This increase in apoptotic cells was significant at 25μg/ml for 24h (figure, 3.5 E). Likewise GTC seemed to increase apoptotic cell numbers; however this increase failed to reach statistical significance (figure 3.5 F). The number of apoptotic cells was reduced when treated with GTC at 25μg/ml. This was possibly related to an increase in necrotic cells.
Chapter 3: Effects of BTT/GTC on MDA-MB-468 and HBL-100 breast cells

A

Control

BTT 25

GTC 3.1

BTT 3.1

GTC 12.5

Annexin V FITC

BTT 12.5

GTC 25

Propidium Iodide
Chapter 3: Effects of BTT/GTC on MDA-MB-468 and HBL-100 breast cells

B

Control

BTT 25

GTC 3.1

BTT 3.1

GTC 12.5

BTT 12.5

GTC 25

Annexin V FITC

Propidium Iodide
Chapter 3: Effects of BTT/GTC on MDA-MB-468 and HBL-100 breast cells

### Graphs

#### C

- **X-axis:** BTT conc (µg/ml)
- **Y-axis:** Viable cells (% of control)
- **Graphs:**
  - 24h
  - 48h

#### D

- **X-axis:** GTC conc (µg/ml)
- **Graphs:**
  - 24h
  - 48h

#### E

- **X-axis:** BTT conc (µg/ml)
- **Y-axis:** Apoptotic cells

#### F

- **X-axis:** GTC conc (µg/ml)
- **Graphs:**
  - 24h
  - 48h

**Legend:**
- ■ 24h
- □ 48h

**Significance:**
- * P < 0.05
- ** P < 0.01
Figure 3.5: Effect of exposure to GTC or BTT on number of viable, apoptotic and necrotic MDA-MB-468 breast carcinoma cells. Representative fluorescence scattergrams show cells after incubation for 24 (A) or 48h (B), bar diagrams show effects of BTT (C,E,G) or GTC (D,F,H) at 3.1, 12.5 or 25µg/ml on cell viability (C,D), extent of apoptosis (E,F) and necrosis (G,H) after incubation for 24 (closed bars) or 48h (open bars). Numbers above scattergrams are concentrations in pg/ml. Results were obtained by fluorescence activated cell sorting using Annexin V FITC and propidium iodide. In A and B cells in the lower quadrant are represented as values in C and D, values in E and F reflect cells in the upper left quadrant and cells in the upper right quadrant are represented as values in G and H. Figures C and D are expressed as percentage of control cells and all values are mean ± SD of 3 separate cellular incubations. Stars indicate that values were significantly different from the respective controls (*p<0.05, **p<0.01).
Chapter 3: Effects of BTT/GTC on MDA-MB-468 and HBL-100 breast cells

3.4 Effects of BTT or GTC on the EGFR signal transduction pathway

3.4.1 Effects of tea on levels of EGFR, pEGFR, ERK, pERK and pAKT

Activation of EGFR can initiate multiple cellular responses, which are associated with cell proliferation and mitogenesis (see chapter 1.6). Human malignancies including breast cancer develop through abnormalities in the expression of growth factors and their receptors. Breast cancer often overexpresses EGFR (Navolanic et al., 2003). This process can occur in the early stages of breast cancer. Therefore EGFR has been considered as a target of many breast cancer chemoprevention agents (Sah et al., 2004; Shimizu and Weinstein, 2005). Previous studies have shown a reduction in activated cellular EGFR (pEGFR) and HER2 (pHer2) levels after treatment with EGCG (Masuda et al., 2003). The purpose of this part of study was to investigate the potential of BTT or GTC to modulate the EGFR signal transduction pathway and signal transduction molecules downstream in this pathway. The signal transduction molecules analysed were EGFR, pEGFR, ERK, pERK and pAKT. In this experiment, AKT was used as a loading control. AKT protein levels were not affected by BTT and GTC.

**EGFR and pEGFR expression**

MDA-MB-468 cells were exposed to BTT or GTC at concentrations 3.125μg/ml, 12.5μg/ml and 25μg/ml for 24h and 48h. Proteins were extracted and analysed for EGFR and pEGFR as described under materials and methods. EGFR levels were unaffected by BTT or GTC at the doses investigated (figure 3.6-3.9). Although there is a 30% increase in EGFR levels compared to control when treated with BTT (25μg/ml) and approximately 20% decrease in EGFR levels when exposed to GTC (25μg/ml) at 24h (figure 3.6) and 48h (figure 3.7) the values were not statistically significant from control. The weak effects of BTT and GTC were also evident when pEGFR levels were compared to control at 24h (figure 3.8) and 48h (figure 3.9)
Chapter 3: Effects of BTT/GTC on MDA-MB-468 and HBL-100 breast cells

**ERK and pERK expression**

MDA-MB-468 cells were exposed to BTT or GTC (3.125, 12.5 and 25μg/ml) for 24 and 48h. Proteins were extracted and analysed for ERK and pERK expression levels as described under materials and method. No significant effect was observed on ERK levels at 24h, whereas GTC at 25μg/ml increased the levels of ERK by approximately 50% compared to control (figure 3.10). However this effect was not statistically significant. At 48h, GTC (25μg/ml) and BTT (12.5μg/ml) reduced the levels of ERK by 35% (figure 3.11). No significant effect on pERK expression levels was observed. Although there was a slight decrease in pERK levels at 24h with BTT (figure 3.12) and a decrease with GTC at 48h, the values were not statistically significant from control (figure 3.13).

**pAKT expression**

MDA-MB-468 cells were exposed to either BTT or GTC and analysed for pAKT at 24h and 48h. There were no significant changes in pAKT levels at 24h or 48h (figure 3.14, 3.15).

**3.4.2 Effect of tea on EGFR and phosphorylated EGFR expression**

EGFR and pEGFR levels were measured at shorter exposure times (0.5h-12h) (figure 3.16 A, B and 3.17 A, B). There were no significant effects on EGFR and pEGFR levels following treatment with tea extracts at the time points stated. EGFR and pEGFR levels were non-significantly reduced by approximately 20% at 0.5h with BTT.
Figure 3.6: Changes in EGFR levels following treatment with BTT or GTC for 24h, relative to control. A shows a representative western blot, B quantitation by densitometry of EGFR bands relative to control (1), expressed as mean ± SD, n=3. Changes in EGFR levels following exposure of cells with BTT or GTC were not significantly different from control.
Chapter 3: Effects of BTT/GTC on MDA-MB-468 and HBL-100 breast cells

Figure 3.7: Changes in EGFR levels following treatment with BTT or GTC for 48h, relative to control. A shows a representative blots of EGFR at 48h, B quantitation of densitometry readings of EGFR bands relative to control (1), expressed as mean ± SD, n=3. Levels of EGFR following exposure with BTT and GTC for 48h were not significantly different from control.
Figure 3.8: Changes in pEGFR levels following treatment with BTT or GTC for 24h relative to control. A shows a representative blot of pEGFR at 24h, B quantitation of densitometry readings of pEGFR relative to control (1) expressed as mean ± SD, n=3.

Levels of pEGFR following exposure with BTT and GTC for 24h were not significantly different from control.
Chapter 3: Effects of BTT/GTC on MDA-MB-468 and HBL-100 breast cells

Figure 3.9: Changes in pEGFR levels following treatment with BTT or GTC for 48h relative to control. A shows a representative blot of pEGFR at 48h, B quantitation of densitometry readings of pEGFR relative to control (1) expressed as mean ± SD, n=3. Levels of pEGFR following treatment with BTT and GTC for 48h were not significantly different from control.
Figure 3.10: Changes in ERK levels following treatment with BTT or GTC for 24h relative to control. A shows a representative blot, B quantitation of densitometry readings of ERK bands relative to control (1) expressed as mean ± SD, n=3. ERK levels treated with BTT and GTC for 24h were not significantly different from control.
Chapter 3: Effects of BTT/GTC on MDA-MB-468 and HBL-100 breast cells

![Graph showing changes in ERK levels following treatment with BTT or GTC for 48h relative to control. A shows a representative blot, B quantitation of densitometry readings of ERK protein, relative to control (1), expressed as mean ± SD, n=3. Changes in ERK protein treated with BTT and GTC for 48h were not significantly different from control.](image-url)
Figure 3.12: Changes in pERK levels following treatment with BTT or GTC for 24h relative to control. A shows a representative blot of pERK protein bands, B quantitation of densitometry readings of pERK, relative to control (1) expressed as mean ± SD, n=3. Changes in pERK levels treated with BTT and GTC for 24h were not significantly different from control.
Chapter 3: Effects of BTT/GTC on MDA-MB-468 and HBL-100 breast cells

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Figure 3.13: Changes in pERK levels following treatment with BTT or GTC for 48h relative to control. A shows a representative blot of pERK. B quantitation of densitometry readings of pERK protein bands at 48h relative to control (1), expressed as mean ± SD, n=3. Changes in pERK protein levels treated with BTT and GTC for 48h were not significantly different from control.
Figure 3.14: Changes in pAKT levels following treatment with BTT or GTC for 24h relative to control. A shows a representative blot of pAkt, B quantitation of densitometry readings of pAKT protein bands relative to control, expressed as mean ± SD, n=3. Changes in pAKT levels treated with BTT and GTC for 24h were not significantly different from control.
Figure 3.15: Changes in pAKT levels following treatment with BTT or GTC for 48h relative to control. A shows a representative blot. B quantitation of densitometry readings of pAKT protein bands relative to control (1) expressed as mean ± SD, n=3. Changes in pAKT levels treated with BTT and GTC for 48h were not significantly different from control.
Chapter 3: Effects of BTT/GTC on MDA-MB-468 and HBL-100 breast cells

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Chapter 3: Effects of BTT/GTC on MDA-MB-468 and HBL-100 breast cells

B

Concentration of BTT (µg/ml)

EGFR levels relative to control

0.5hr
1hr
2hr
4hr

0
1
2
3

C

Concentration of BTT (µg/ml)

EGFR levels relative to control

0.5hr
1hr
2hr
12hr

0
1
2
3

D

Concentration of GTC (µg/ml)

EGFR levels relative to control

0.5hr
1hr
2hr
4hr

0
0.5
1
1.5
2
2.5
3
Figure 3.16: Time dependent changes in EGFR levels following treatment with BTT and GTC in MDA-MB-468 cells. A, shows a representative blot of EGFR protein bands at 0.5h, 1h, 2h, 4h, 6h, 8h, and 12h. B, C, D and E quantitation of densitometry readings of EGFR protein when exposed to BTT (B and C) and GTC (D and E) relative to control, expressed as mean ± SD, n=3. Changes in EGFR levels treated with BTT and GTC were not significantly different from control.
Chapter 3: Effects of BTT/GTC on MDA-MB-468 and HBL-100 breast cells

A

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Chapter 3: Effects of BTT/GTC on MDA-MB-468 and HBL-100 breast cells

B

Concentration of BTT (µg/ml)

C

Concentration of BTT (µg/ml)

D

Concentration of GTC (µg/ml)
Chapter 3: Effects of BTT/GTC on MDA-MB-468 and HBL-100 breast cells

Figure 3.17: Time dependent changes in pEGFR levels following treatment with BTT and GTC in MDA-MB-468 cells. A shows a representative western blot of pEGFR protein at 0.5h, 1h, 2h, 4h, 6h, 8h and 12h. B, C, D and E quantitation of densitometry readings of pEGFR exposed to BTT (B and C) and GTC (D and E) relative to control (1), expressed as mean ± SD, n=3. Changes in the levels of pEGFR at the time points stated were not significantly different from control.
3.5 Effect of GTC and BTT on M1dG adduct levels in MDA-MB-468 tumour cells

Figure 3.18 shows that exposure of cells to BTT or GTC caused a concentration-dependent decrease in M1dG adduct levels, and that both tea preparations reduced M1dG adduct levels to a similar extent.

Figure 3.18: Effect of GTC (open bars) or BTT (closed bars) on levels of M1dG in MDA-MB-468 breast carcinoma cells. Cells were incubated without (0µg/ml) or with tea extracts (3.125-25µg/ml) for 48h after which M1dG was determined. M1dG levels were determined by slot blot analysis. Values are the mean ± SD of 6 separate cellular incubations. Stars indicate that values were significantly different from the respective controls (*p<0.05, **p<0.01)
3.6 Discussion

The ability of BTT or GTC to inhibit breast cancer cell growth and survival were investigated in the MDA-MB-468 and the HBL-100 cell lines. BTT and GTC dose-dependently decreased the number of MDA-MB-468 and HBL-100 cells (figure 3.1-3.4). As only cell number was measured, the effects of BTT and GTC may be due to cell proliferation or death. There was no significant difference in IC\textsubscript{50} values between the two treatments or between malignant and non-malignant cells with values between 7.2 and 9.9\mu g/ml (table 3.1). In this in vitro study, low doses of BTT and GTC were employed, because a low dose is more representative of likely tissue concentrations achieved in clinical studies. The IC\textsubscript{50} values obtained from the cell assays represented relatively low concentrations of BTT and GTC in contrast to efficacious doses (40-80\mu g/ml) used in previous investigations (Kavanagh et al., 2001). There is a growing understanding that induction of apoptosis in neoplastic or preneoplastic cells plays an important role in the chemopreventive efficacy of some diet-derived agents (Agostini et al., 2005). Here, an increase in apoptotic cells after exposure to either BTT or GTC was observed (figure 3.20 E, F). However an increase in necrotic cells was also observed. BTT (25\mu g/ml) reduced the number of cells by 30% and 50% at 24h and 48h, respectively. At 24h, BTT increased apoptotic cells by 10% and necrotic cells to 20%. Similarly, at 48h BTT (25\mu g/ml) increased apoptotic cells by 22% and increased necrotic cells by 27%. Therefore an increase in apoptotic cells is also accompanied by an increase in necrotic cells. GTC at 25\mu g/ml was toxic to the cells, demonstrated by a decrease in cell number by 90% and an increase in necrotic cells by 85% at 24h and 48h time points. An increase in apoptosis with BTT and GTC has been shown in numerous cancer cell lines (Martin, 2006) at high doses of BTT and GTC (20-80\mu g/ml). Although in this study I have shown an increase in apoptosis with low concentrations of BTT or GTC, this has also been accompanied by an increase in necrotic cells. A study by Baliga et al (2005) on 4T1 cells (a metastatic breast cancer cell line) demonstrated a significant increase in early apoptotic cells, when treated with EGCG (20-80\mu g/ml). This study found that exposing cells to EGCG at 20\mu g/ml, increased apoptosis by 7% and 15.3% at 24h and 48h respectively. This increase in apoptotic cells was accompanied by a slight increase in late apoptotic cells by 3.1% and
6.5% at 24h and 48h, respectively. A marked increase in apoptotic cells were achieved with 80μg/ml of EGCG. Apoptotic cells were increased by 32.5% and 40.9% at 24h and 48h, respectively. However late apoptotic cells were increased by only 8.9% and 8.2% at the time points investigated. Here I have shown that an increase in apoptotic cells and necrotic cells was observed after intervention with comparatively low concentrations of BTT and GTC (3.125-25μg/ml). The efficacy of tea extracts on the apoptotic pathway, and the effects of BTT and GTC on caspase 3, a marker of apoptosis were examined. MDA-MB-468 cells were exposed to BTT or GTC (3.1-25μg/ml) for 24 and 48hrs. The expression of cleaved caspase 3 was examined by western blot. Cleaved caspase 3 protein bands were not quantified (results not shown) because there was no evidence of the cleaved caspase 3 protein on the x ray film. Cells were exposed to BTT/GTC for 24 and 48h, this time exposure may have been too long for any effect of BTT or GTC on cleaved caspase 3 to occur. Cleaved caspase 3 protein may have been degraded at an earlier time point. In addition, the monoclonal antibody used in the experiment detected only cleaved caspase 3 protein and not caspase 3 protein therefore degradation of cleaved caspase 3 could not be confirmed.

MDA-MB-468 cells were exposed to BTT and GTC. The effect on levels of EGFR, pEGFR, ERK, pERK and AKT were evaluated in cells. BTT and GTC (3.1-25μg/ml) had no significant effect on EGFR, pEGFR, ERK, pERK and AKT levels at 24h and 48h. Studies have shown that EGCG (30μg/ml) decreases the levels of pHER2 in breast cancer cells (BT-474) at 24 hours (Masuda et al., 2003). Furthermore, a reduction in pHER2 levels between 13-96% was observed in MMTV-Her-2/neu cell lines, NF639 with EGCG (20-80μg/ml) (Pianetti et al., 2002).

Earlier time points were then considered and EGFR and pEGFR levels were measured at a range of incubation time points (0.5h-12h). The reason for selecting short incubation time points was because previous studies on cancer cell lines demonstrated a significant effect on numerous signalling molecules with tea extracts at shorter incubation time points. Treatment of colon cancer cells with EGCG (20μg/ml) and polyphenon E (decaffeinated green tea catechin mixture, 20μg/ml) decreased pEGFR, pHER2, pERK and pAKT levels within 6h (Shimizu et al., 2005). A significant effect was observed with EGCG (20μg/ml) and polyphenon E (20μg/ml) in colon cancer cells induced with a ligand (TGF α, 50ng/ml)
at 24h. Theaflavin-3'-gallate and theaflavin-3,3'-digallate (20µM) decreased the levels of EGFR in mouse epidermal cells (JB6 C141) and human epidermoid carcinoma cells (A431) in an 1hr. However in the studies performed here, EGFR and EGFR phosphorylation levels were not affected by BTT or GTC at the time points investigated. An explanation for this lack of activity of BTT and GTC on EGFR, pEGFR levels and proteins downstream at 24h and 48h and EGFR and pEGFR levels at shorter incubation time points, were probably a result of the low concentrations of BTT and GTC (3.1µg/ml-25µg/ml) employed in this study. Many studies show a reduction in EGFR phosphorylation when treated with EGCG at higher concentrations (>50µg/ml), and where low concentrations were used, cells were frequently stimulated with a ligand (Bigelow and Cardelli, 2006). Studies in cell culture systems that show high concentrations of tea polyphenols, such as EGCG, do not reflect the concentrations obtained in blood and tissues in vivo. Blood and tissue concentrations of EGCG are much lower than the effective concentrations observed in vitro (Hou et al., 2004). A study by Chow et al, (2005) found that the maximum plasma total catechin concentrations following oral administration of 400mg EGCG, 800mg EGCG and 1,200mg EGCG were 174.4 ± 80.6ng/ml, 360.1 ± 136.9ng/ml and 871.9 ± 625.3ng/ml, respectively. The oral bioavailability of the major green tea constituents is low, resulting in catechin levels in human plasma concentrations 5 to 50 times less than concentrations shown to exert biological activities in in vitro systems. Theaflavins and thearubigins are difficult to detect in blood or urine, and therefore there is little information regarding the bioavailability of black tea polyphenols in human or animals (Frei and Higdon, 2003). Tea polyphenols have low bioavailability because the polyphenols tend to be conjugated and converted to methylated, glucuronidated and sulphated metabolites. Therefore they are poorly absorbed and tend to be rapidly eliminated. Most of the ingested EGCG does not get into the blood and is excreted through the bile to the colon. EGC and EC appear to be more bioavailable, but the fractions of these compounds in the plasma are still very low (Lambert and Yang, 2003).

The low concentrations described here were more suitable for the identification of relevant pharmacodynamic biomarkers for monitoring efficacy in the animal studies described in chapter 4. It is more relevant to study the effects of tea extracts at low concentrations in in vitro studies which can then be extrapolated to in vivo studies.
Chapter 3: Effects of BTT/GTC on MDA-MB-468 and HBL-100 breast cells

I next studied the effects of BTT and GTC on malondialdehyde DNA adduct, M1dG levels. It has been established that tea catechins and theaflavins possess strong antioxidant properties (Leung et al., 2001). Therefore one of the mechanisms thought to contribute to their cancer chemopreventive properties is protection against oxidative damage (see introduction, chapter 1.6) Malondialdehyde is a carcinogenic and mutagenic product of two metabolic processes, lipid peroxidation and prostaglandin biosynthesis. In prostaglandin biosynthesis process cyclooxygenases (COX) catalyse the conversion of arachidonic acid to prostaglandin endoperoxides (PG). Two isoforms of COX exist, COX-1, and COX2. COX-2 can be stimulated by infection and inflammation in vivo and induced by tumour promoting stimuli in vitro. COX-2 results in production of prostaglandin E2 (PGE2) via the endoperoxide PGH2. COX-2 protein levels have been shown to be elevated in human carcinomas. MDA is produced by the spontaneous and enzymatic breakdown of PGH2 (see introduction, chapter 1.6.6) (Sharma et al., 2001). MDA forms endogenous adducts on DNA to form M1dG (Marnett, 2002). The malondialdehyde DNA adduct, M1dG was employed as an indicator of oxidative damage and coincidentally as a measure of antioxidant activity. The results presented here show that BTT and GTC both reduced M1dG levels dose-dependently. GTC reduced M1dG levels at lower concentration (12.5µg/ml) than BTT (25µg/ml). The immunoslot blot assay utilised to measure the levels of M1dG is a sensitive method which allows the detection of adducts in small amounts of DNA (~1µg) (Zhang et al., 2002). Here I have investigated the proposition that M1dG can serve as a marker of breast cancer chemoprevention and demonstrated that this marker would be suitable for assessment in in vivo experiments and perhaps clinical studies.

The mechanisms, by which tea extracts reduce M1dG levels, may be due to the possible role of tea in scavenging oxygen radicals. The flavonoids present in green and black tea can be considered as very effective radical scavengers (Rietveld and Wiseman, 2003). Therefore reducing the number of oxygen radicals and preventing lipid peroxidation. In addition, induction of detoxifying enzymes can also occur. The antioxidant activity of tea polyphenols promotes the expression of genes that encode detoxifying defence proteins, such as glutathione peroxidase (Na and Surh, 2006). Detoxifying enzymes may reduce the levels of oxygen radicals and reduce lipid peroxidation and generation of DNA adducts, such as M1dG.
Chapter 3: Effects of BTT/GTC on MDA-MB-468 and HBL-100 breast cells

Tea extracts can also act as suppressing agents by inducing apoptosis or cell cycle arrest and therefore inhibiting promotion and progression of carcinogenesis (Ahmad et al., 1997). Many studies have demonstrated induction of apoptosis or cell cycle arrest with tea polyphenols (Khan et al., 2006).

In conclusion, both BTT and GTC decreased the number of malignant and non-malignant breast cells. One mechanism that may have contributed to the growth inhibition of breast cells involves the induction of apoptosis. Although in this study, it was found that induction of apoptosis was accompanied by increase in necrosis, other studies (Baliga et al., 2005) have shown that extracts of green tea may induce apoptosis without necrosis. Therefore this may be important in chemoprevention trials. It was demonstrated that the EGFR signaling pathway was not the target for tea components at these concentrations and a dose-dependent decrease in M1dG, a marker of oxidative DNA damage was observed. Therefore, the data presented here suggests that these tea preparations may be suitable for consideration as chemopreventive agents in vivo and that M1dG and apoptotic signalling molecules may perhaps constitute as suitable pharmacodynamic biomarkers of efficacy.
CHAPTER 4

Effect of BTT and GTC on mammary tumour development in TAg mice
Chapter 4: Effect of BTT and GTC on mammary tumour development in TAg mice

4.1 Introduction

The purpose of this study was to compare the breast cancer chemopreventive efficacy of GTC and BTT in the TAg breast cancer model. These model shares characteristics with the breast cancer cell line, MDA-MB-468 (see chapter 3.1). All female TAg mice develop mammary tumours from approximately 12 weeks of age that progress from oestrogen receptor positive to oestrogen receptor negative status (see chapter 1.2). The SV40 T, t antigens, when activated, binds to p53 and Rb proteins and functionally inactivates them. Mice were treated as described under experimental study designs in methods section 2.2.2. Three different study designs were used. Mice were culled once the combined length of tumours exceeded 17mm. The main objective of the first study was to determine the effects of BTT and GTC on tumour size in TAg mice. This study was divided into two parts. The aim of the first part was to determine the effects of BTT/GTC, at 0.05%, on tumour size. Histological sections of mammary tissues were evaluated and assessed on the basis of the largest lesion. These lesions were graded according to the maximum diameter on the histological section.

For the second part of the study, the mouse experiments were repeated but with two additional groups of 16 mice, which received tea preparations (BTT/GTC) at 0.01%, in their drinking water. The purpose of this part of the study was to establish a suitable dose of BTT/GTC that may be potentially used for clinical trials. The objective of the second study was to determine the effects of BTT and GTC on mammary carcinogenesis at a fixed end point (week 19, day 133). In the final study (see experimental study designs in chapter 2.2.2), the objective was to explore the efficacy of BTT and GTC in early or late carcinogenesis. The mechanisms by which tea preparations may affect tumour size of TAg mice were examined. Histological sections were evaluated and the effects of BTT and GTC on tumour tissue proliferation were examined by measuring the levels of proliferating cell nuclear antigen (PCNA) in mammary tissue sections from mice. The hypothesis was tested that intervention with GTC or BTT induces apoptosis in mammary tumour tissue as indicated by the levels of cleaved caspase 3. The hypothesis was also tested that intervention with GTC or BTT affects levels of oxidative DNA adducts in tumour or blood,
Chapter 4: Effect of BTT and GTC on mammary tumour development in TAg mice

as reflected by levels of M1dG. Western blot was performed to analyse the effects of BTT and GTC on the SV40 transgene.
Chapter 4: Effect of BTT and GTC on mammary tumour development in TAg mice

4.2 Effect of BTT/GTC on tumour size of TAg mice

4.2.1 Effect of a single dose (0.05%) of BTT/GTC on mammary tumour development

TAg mice (10 per group) received BTT or GTC (0.05%) with their drinking water from 4 weeks of age to the end of the experiment. Inclusion of BTT or GTC with the drinking water did not affect the intake of fluid or diet of mice as reflected by body weight (table 4.1). Mice were killed when the combined length of tumour per mouse reached 17mm, which occurred between days 154 and 175 (weeks 22-25). Mammary pelt was removed from individual animals and weighed. Mammary pelt weight from individual TAg mice in the intervention groups were similar to the pelt weights removed from mice in the control group (see table 4.2). Figure 4.1 show that administration of GTC or BTT significantly increased the percentage of mice in the intervention groups when compared with mice in the control group. Mice were culled once tumours exceeded 17mm, therefore mice in the GTC group were culled a week later than mice in the control group whereas mice in the BTT group were culled two weeks later when compared to control. The average age at death of animals was 144 ± 8 days for control mice, 151 ± 8 days for mice on GTC (p=0.27 versus control) and 154 ± 16 days for mice on BTT (p<0.05 versus control). The increase in the percentage of mice in the treated groups was accompanied by a slight, but not significant, decrease in the number of mice bearing palpable tumours (figure 4.2), reduction in the number of tumours per mouse (figure 4.3), and decrease in volume of tumours in the intervention groups (figure 4.4). Tumour volume (mm$^3$) and the number of mice bearing palpable tumours were retarded by approximately 2 weeks with BTT. However, the difference in tumour volume and number of mice with tumours between the intervention groups and control group was not statistically significant.

4.2.2 Histological Evaluation

Five blocks of mammary tissue from each mouse were embedded, sectioned and stained conventionally with haematoxylin and eosin and then investigated for the presence of neoplasm by Dr Peter Greaves. In this study, an additional sixth block from the
Chapter 4: Effect of BTT and GTC on mammary tumour development in TAg mice

neck/salivary gland region was also examined. Neoplasms were assessed on the basis of the largest lesion per block. Lesions were grouped based on the following criteria, simple hyperplasia, this is when epithelial cells lining the mammary ducts were two or three layers thick. Intraduct hyperplasia is when cells lining the mammary ducts were more than three layers thick. Intraduct carcinoma is when carcinoma cells filled or expanded the ducts but were not infiltrating through the duct wall, these cells were usually less than 0.1 mm diameter. Carcinomas are when tumour cells infiltrate tissues outside duct. Carcinomas were usually 0.1 mm diameter or more. Figures 4.5 (A, B) demonstrate that there was no significant difference in diameter of most severe or largest adenocarcinoma between treatment groups and control group. The mean tumour diameter was also similar in treatment groups and control group (C).

The carcinomas were invasive mammary carcinomas of duct type and similar in all animals. They tended to be large multicentric carcinomas and often associated with extensive intraduct spread. There was no evidence of metastases into the lymphoid tissue, despite the close proximity of a few regional lymph nodes in some sections. Histological inspection did not reveal significant differences in either tumour size or cytology between the tumours in the different groups.

4.2.3 Effect of BTT or GTC at two different concentrations (0.01 and 0.05%) on mammary carcinogenesis in TAg mice

This study was conducted as described in experimental study design 1 under materials and method (chapter 2.2.2). Previously in section 4.2.1, results showed that BTT and GTC (0.05%) significantly increased the number of TAg mice as reflected by a reduction in tumour volume and number of tumours, compared to control. It is important to establish a suitable dose which would be practical for use in clinical trials therefore the effects of a lower dose (0.01%) of GTC and BTT was investigated in this experimental study. Mice were randomly assigned to a group (10-16 in each). Mice in each group received either drinking water, or drinking water supplemented with 0.01% BTT, 0.05% BTT, 0.01% GTC or 0.05% GTC. Palpable tumours were measured using calipers (twice weekly). Individual tumours were extracted and flash frozen in liquid nitrogen and stored at -80 C for M1dG analysis (see chapter 2.2.8). Fifteen mice developed tumours in the control group, 13/16
mice in 0.01% BTT group, 10/10 mice receiving 0.05% BTT, 15/16 mice in 0.01% GTC group and 10/10 receiving 0.05% GTC. The remaining mice were re-genotyped for the presence of the transgene. Results showed that the animals were not TAg mice and therefore did not develop tumours. Figures 4.6 (A and B) show that percentage of mice in the control group at day 156 was 20%, 40% in 0.01% BTT group, 30% in 0.05% BTT group, 50% on 0.01% GTC and 30% in the 0.05% GTC group. At the end of the experiment there was no difference in the percentage of mice in each group (figure 4.6 A, B). There was a slight, but not significant reduction in the number of tumours per mouse in the intervention groups compared to control (figure 4.7 A, B). Figure 4.8 (A, B) shows no difference in the percentage of mice bearing palpable tumours between the treated groups and control. The mean tumour volume (mm$^3$) per tumour bearing mouse appeared to be lower in mice receiving 0.05% BTT and 0.05% GTC (figure 4.9 A, B), compared with controls, though this difference was not statistically significant. The number of tumours per mouse (figure 4.2) and tumour volume (figure 4.4) in the previous study was compared with this repeat study (figures 4.7 and 4.9). Both experiments showed that the number of tumours per mouse and tumour volume were similar. Mice in the control group had the greatest number of tumours and a large tumour volume and mice on BTT had the lowest number of tumours per mouse and a small tumour volume in both studies. However there was a difference in the number of mice with tumours in the first study compared to repeat study. In the first study (figure 4.3) the number of mice that developed tumours at 120 days in the control, GTC and BTT (0.05%) group were 4 out of 10 mice, 3/10 and 1/10, respectively. In the repeat study (figures 4.8) the number of mice that developed tumours at 120 days in the control, GTC and BTT (0.05%) group were 10 out of 15 mice, 8/10 and 7/10 mice, respectively.

4.2.4 Effect of BTT and GTC on expression of the SV40 transgene

TAg tumour tissue was removed at the end of the experiment and proteins were analysed via western blot to ensure the SV40 transgene was not affected by tea preparations. Western blot analysis showed that neither GTC nor BTT interfered with the expression of the SV40 transgene (figure 4.10).
Table 4.1 Effect of BTT and GTC on animal weight (g). Control animals received drinking water without tea (10 per group) from 4 weeks of age (day 28) to end of the experiment. In the treatment groups, animals received BTT or GTC from 4 weeks of age (day 28) to end of the experiment. All animals were weighed once a week.

<table>
<thead>
<tr>
<th>Days</th>
<th>Average weight of control mice (g)</th>
<th>Average weight of mice in GTC group (g)</th>
<th>Average weight of mice in BTT group (g)</th>
</tr>
</thead>
<tbody>
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</tr>
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<td>175</td>
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</tr>
</tbody>
</table>
Table 4.2: Effect of GTC or BTT (each 0.05% in the drinking water) on mammary pelt weight (g) from TAg mice. Control animals received drinking water without tea (10 per group) from 4 weeks of age to end of experiment. In the treatment groups, animals received BTT or GTC from 4 weeks of age to end of experiment. At end of the experiment, all animals were shaved and mammary pelt was removed and weighed. Data represented as mean ± SD. Statistical analysis showed that the mean pelt weight of mice in treated group was not significantly different from control.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean Pelt Weight (g) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.31 ± 0.82</td>
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<tr>
<td>GTC</td>
<td>3.1 ± 0.70</td>
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<tr>
<td>BTT</td>
<td>3.07 ± 1.06</td>
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</table>

Figure 4.1 - Effect of consumption of GTC (□——□) or BTT (Δ——Δ) (each 0.05% in the drinking water) on percentage of TAg mice. Control animals received drinking water without tea preparation (■——■). Groups consisted of 10 animals that received tea
preparations from 4 weeks of age until the end of experiment. Mice were killed when the length of tumours per mouse reached 17 mm. Stars indicate that log rank analysis of the differences between mice on GTC or BTT versus control afforded p<0.05 (*) or p<0.01 (**), respectively.

Figure 4.2: Effect of consumption of GTC or BTT (each 0.05% in the drinking water) on the percentage of mice with palpable tumours. Control animals received drinking water without tea. Animals (10 per group) received tea preparations from 4 weeks of age. Tumour size was measured and the number of mice bearing tumours was recorded weekly. Animals were killed when tumour length exceeded 17mm. The numbers of mice bearing palpable tumours in the intervention groups were not significantly different from the control.
Chapter 4: Effect of BTT and GTC on mammary tumour development in TAg mice

Figure 4.3: Effect of GTC and BTT (each 0.05%) in the drinking water) on number of tumours per mouse in TAg mice. Control animals received drinking water without tea. Animals (10 per group) received tea preparations from 4 weeks of age. Tumour size was measured and the number of tumours per mouse was recorded weekly. Mice were killed when tumours per mouse reached 17mm in length Data represented as mean ± SD n =10. Statistical evaluation showed that the number of tumours per mouse in the treated groups were not significantly different from the control.
Figure 4.4: Effect of GTC and BTT (each 0.05%) in the drinking water on tumour volume (mm$^3$) of TAg mice. Control animals received drinking water without tea. Animals (10 per group) received tea preparations from 4 weeks of age. Tumour volume was measured and recorded weekly. Mice were killed when tumours per mouse reached 17mm in length. Data represented as mean $\pm$ SD n =10. The average tumour volume of mice in the intervention groups was not significantly different from the control due to the large standard deviation.
Chapter 4: Effect of BTT and GTC on mammary tumour development in TAg mice

A

![Graph showing number of mice with diameters of largest adenocarcinoma](image)

B

![Graph showing number of mice with diameters of largest adenocarcinoma](image)
Chapter 4: Effect of BTT and GTC on mammary tumour development in TAg mice

Figure 4.5: Histological investigation of the effect of GTC (solid bars) and BTT (hatched bars) (each 0.05% in the drinking water) on tumour size in TAg mice, as reflected by size of largest or most severe adenocarcinoma per mouse (A), number of mice with size of largest tumour either below or above 0.5mm diameter (B), and mean tumour diameter (C). Control animals (open bars) received drinking water without tea preparation. The largest lesion in each mouse was selected and measured. Groups (with 10 animals) received tea extracts from 4 weeks of age. Data represented as mean ± SD n =10. Tumours were measured weekly and animals were killed once tumour length exceeded 17mm. Statistical evaluation showed that the size of tumours in mice receiving tea preparations was not statistically different from the control.
Figure 4.6 Effect of BTT and GTC on percentage of TAg mice in each group. Animals received either (0.01% or 0.05%) BTT (A) or GTC (B) in the drinking water (10-16 in group). Control animals received drinking water without tea (16 in group). Tumours were measured twice weekly and animals were killed when combined tumour length exceeded 17 mm. Statistical analyses showed that there was no significant difference between the intervention groups and the control.
Chapter 4: Effect of BTT and GTC on mammary tumour development in TAg mice

Figure 4.7 Effect of BTT or GTC on the number of tumours per mouse. Animals received either BTT (A) or GTC (B) (0.01% or 0.05%) in their drinking water (10-16 per group). Control animals received drinking water without tea (16 per group). Tumours were
measured twice weekly and the numbers of tumours per mouse were recorded. Animals were killed when tumours reached 1mm in length. Values are mean ± SD, n=10-16. The number of tumours per mouse in the treated groups was not significantly different from control due to large standard deviation.

A

![Graph showing percentage of mice bearing tumours vs. mouse age (days)]
Figure 4.8 Effect of BTT or GTC on percentage of mice bearing palpable tumours. Animals received either BTT (A) or GTC (B) (0.01% or 0.05%) in the drinking water (10-16 per group). Control animals received drinking water without tea (16 in group). Tumours were measured twice weekly and the numbers of mice bearing tumours were recorded. Animals were killed when Mouse age (days) m in length. The number of mice bearing tumours in the intervention groups was not significantly different from the mice in the control group due to large standard deviation.
Figure 4.9 Effect of BTT or GTC on tumour volume per mouse. Animals received either BTT (A) or GTC (B) (0.01% or 0.05%) in drinking water (10-16 animals per group). Control animals received drinking water (16 per group). Tumours were measured twice weekly and tumour volume was measured and recorded. Animals were killed when tumours exceeded 17mm in length. Data represented as mean tumour volume/mouse ± SD,
n= 10-16. Tumour volume of mice in the treated groups was not statistically significant from the control.

**Figure 4.10** Effect of GTC (open bars) or BTT (closed bars) in the drinking water at 0.01 or 0.05% on expression of SV40 T antigen in mammary tumour tissue in TAg mice. A shows representative blots. Animals received tea extracts from week 4 until tumour size exceeded 17mm (week 22-25), after which they were killed, and tissue was obtained and examined by Western blot using an antibody against SV40 T antigen.
4.3 Effect of tea extracts on mammary carcinogenesis at fixed end point

4.3.1 Evaluation of BTT/GTC on mammary tumour development

In a second animal experimental design, mice (n=14) were assigned to one of three groups, receiving 0.05% BTT, 0.05% GTC or drinking water. Palpable tumours were measured (twice weekly) using calipers. Measurements were recorded and all mice were killed in week 19 (day 133). Number and volume of tumours were recorded and tumours obtained post-mortem were graded on histological sections according to their diameter. Day 133 was selected as endpoint because in the first study no animals exceeded the 17mm maximum at this age. Twelve out of 14 mice in the control group and 13/14 mice in the GTC and BTT group developed palpable tumours. The remaining mice were re-genotyped for the presence of the transgene. Results showed that the animals were not TAg mice and therefore did not develop tumours. At the end of experiment the mammary pelt was removed and weighed. The weights of tumour bearing pelts of the control and mice on GTC or BTT in this study were illustrated in table 4.3. The difference between the control and treatment groups suggests that BTT and GTC consumption decreased tumour load by 39% (P<0.05) and 23% respectively. Intervention with either tea preparation impeded mammary carcinogenesis, demonstrated by time of appearance of palpable tumours (figure 4.11), tumour number (figure 4.12) and tumour volume (figure 4.13), although the difference between intervention and control groups in the late stages of tumour development was statistically significant only when tumour volume was compared. Mice receiving GTC had fewer palpable tumours, and palpable tumours were not detected up until day 110, suggesting a delay in tumour onset (figure 4.12).

4.3.2 Histological Evaluation

At the end of the experiment at day 133 (week 19), the mammary pelt was removed and fixed in 10% NBF formalin for 2 weeks. The pelt was cut into 5 transverse sections representing 5 blocks per animal and evaluated by Dr Peter Greaves. Blocks were embedded, sectioned and stained conventionally with haematoxylin and eosin. The
carcinomas were similar in all animals being invasive mammary carcinoma of a ductular type though precursor lesions were also present and recorded. Carcinomas tended to be multicentric and were therefore assessed and recorded on the basis of most severe or largest lesion per block. In the control group there was an increased number of invasive tumours that were >0.5mm in size compared to GTC and BTT groups (figures 4.14). Mice in the GTC and BTT group tended to have more tumours that were <0.5mm in size and fewer invasive tumours >0.5mm (figure 4.14 A, B). Figure 4.14 C shows the mean tumour size of the largest lesion per mammary tissue block. The mean tumour size of the largest/most severe lesion per mouse was larger in control compared to treated mice. The difference in mean tumour size between control and GTC and control and BTT is statistically significant (P <0.01). In both GTC and BTT intervention groups, the size of the largest tumour lesion per mouse was significantly reduced by 42 and 40% respectively, compared to control.

Table 4.3 Effect of BTT and GTC on mammary tumour pelt weight. Control animals received drinking water. In the treated groups, animals received either BTT or GTC (0.05% in each drinking water) from 4 weeks of age to 19 weeks of age, after which they were killed. Mammary tumour pels were removed from individual animals (n=14) from all three groups. The pels were weighed and the values are the mean pelt weight ± SD of 12-13 animals. Star indicates that the mean pelt weight of mice consuming BTT was statistically significant from control (*P<0.05)

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean Pelt Weight (g) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.93 ± 1.85</td>
</tr>
<tr>
<td>GTC</td>
<td>1.44 ± 0.53</td>
</tr>
<tr>
<td>BTT</td>
<td>1.18 ± 0.27 *</td>
</tr>
</tbody>
</table>
Figure 4.11 Effect of GTC and BTT (each 0.05% in the drinking water) on percentage of mice bearing palpable tumours. Control animals received drinking water without tea (12 per group). Animals (12-13 per group) received tea preparations from weeks 4 to 19, after which they were killed. Tumour size was measured and the numbers of mice bearing tumours were recorded. Statistical analysis was conducted on the number of tumours per mouse in each group at each time point recorded. There was no significant effect of BTT and GTC on the number of mice bearing tumours in the treated groups compared to control.
Figure 4.12 Effect of consumption of GTC and BTT (each 0.05% in the drinking water) on the number of tumours per tumour bearing mouse. Control animals received drinking water without tea (12 per group). Animals (12-13 per group) received tea preparations from weeks 4 to 19, after which they were killed. Data represented as mean number of tumours per mouse ± SD of 12-13 animals in each group. There was no statistical significance between the number of tumours per tumour bearing mouse in the treated group and control.
Figure 4.13 Effect of GTC and BTT on mammary tumour volume per mouse. Animals received tea preparations, BTT and GTC (0.05%) in their drinking water (13 animals per group). Controls received drinking water without tea (12 animals per group). Tumour size was measured weekly and animals were killed at day 128 (week 19). Data represented as mean tumour volume per mouse ± SD, n=13-14. Tumour volumes in the intervention groups on day 100 and at day 103 (indicated by stars) were significantly lower than those in the controls (*P<0.05).
Chapter 4: Effect of BTT and GTC on mammary tumour development in TAg mice

Figure 4.14 Histological investigation of the effect of GTC (solid bars) and BTT (hatched bars) (each 0.05% in the drinking water) on tumour size in TAg mice, as reflected by size of largest tumour per mouse (A), number of mice with size of largest tumour either below or above 0.5mm diameter (B), and mean tumour diameter (C). Control animals (open bars) received drinking water without tea preparation. The largest lesion in each mouse was selected and measured. Groups (with 12-13 animals) received tea extracts from week 4 to 19, after which they were killed. Star indicates that difference between intervention and controls was significant (p≤0.01).
4.4 Effect of early and late intervention with BTT and GTC on mammary carcinogenesis in TAg mice

Tea preparations may exert their effect on mammary carcinogenesis during either early or late stages of tumour development. In order to explore efficacy in early or late carcinogenesis, GTC or BTT were added to the drinking water either from week 4 to 13 (day 28-91) to be subsequently replaced by water without tea until week 22 (day 154), or from week 13 to 22 (day 91-154) preceded by drinking water without tea from week 4 to 13 (day 28-91). Each group comprised 14 mice. Tea consumption of either BTT or GTC weakly decreased tumour number per mouse compared to controls, irrespective of whether administration was during early or late carcinogenesis (figure 4.15 B). In contrast, there was no significant difference between any of the intervention groups and controls, when groups were compared in terms of volume or number of tumours or numbers of tumour-bearing mice (figure 4.15 A, B, C).
Chapter 4: Effect of BTT and GTC on mammary tumour development in TAg mice

(ii)

![Graph showing percentage of mice bearing tumours vs mouse age (days).]

B (i)

![Graph showing number of tumours/mouse vs mouse age (days).]
Chapter 4: Effect of BTT and GTC on mammary tumour development in TAg mice

(ii)

![Graph showing the effect of BTT and GTC on tumour development.]

C (i)

![Graph showing the effect of BTT and GTC on tumour volume.]

Mouse age (days)

Number of tumours/mouse

Tumour volume/mouse (mm$^3$)

Mouse age (days)
Figure 4.15 Effect of GTC and BTT (each 0.05% in the drinking water) on early or late phases of mammary carcinogenesis in TAg mice, as reflected by (A i, ii), percentage of mice bearing tumours. (B i, ii), Number of tumours per mouse. (C i, ii), Tumour volume per mouse. Control animals received drinking water without tea preparations. Animals (14 per group) received either GTC or BTT from day 28 (week 4) for 63 days (9 weeks) to day 91 (week 13), then pure drinking water without tea for 63 days (9 weeks) from day 91 (week 13) to day 154 (week 22), or pure drinking water from day 28 to day 91 (week 4 to 13), then GTC or BTT for 63 days to day 154 (week 22). Mice were killed after 154 days (week 22) except for three, which were killed earlier, because the size of their tumours exceeded 17mm. Statistical analysis using one way ANOVA showed that the percentage of mice receiving BTT from day 90 to 114 days was significantly higher than control,(*P<0.05) (A,i) and number of tumours per mouse (B) from day 120 to 154 (week 19-22) were significantly lower (p<0.01) in the intervention groups than in the control group, except for animals which received GTC from beyond 91 days (week 13), for which the difference between intervention and control was significant (p<0.05) in weeks 21 (day 146) and 22 (day 153).
Chapter 4: Effect of BTT and GTC on mammary tumour development in TAg mice

4.5 Effect of GTC and BTT on tumour tissue proliferation

Mammary tissue sections of the largest severe adenocarcinoma were obtained from control and intervention groups at week 19 (day 133). In a preliminary experiment Richard Edwards (MRC Toxicology Unit, Leicester) stained tumour sections from the control group for PCNA and Ki67, to determine the most suitable proliferation marker. PCNA was considered the most suitable proliferation marker as there were more sections stained with PCNA than Ki67. All tumour sections were then stained for PCNA, a nuclear protein required for the catalytic activity of DNA polymerase-δ, and inspected microscopically. The PCNA labeling index in tumours of control mice and treated mice are illustrated in figure 4.16 A. In the light of the typical cut-off point of between 30 and 45% used to differentiate between “low” and “high” PCNA labeling indices, (Chu et al., 1998) this value suggests that TAg mouse mammary tumours are characterised by a high proliferation rate (figure 4.16B). Consumption of BTT reduced proliferation significantly by 11% (P< 0.005). PCNA levels were represented by brown staining.
Chapter 4: Effect of BTT and GTC on mammary tumour development in TAg mice

B

<table>
<thead>
<tr>
<th>Group</th>
<th>PCNA labeling index % (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>53 ± 13</td>
</tr>
<tr>
<td>GTC</td>
<td>50 ± 12</td>
</tr>
<tr>
<td>BTT</td>
<td>47 ± 10 *</td>
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</table>

Figure 4.16 Effect of BTT and GTC on PCNA labelling index (%). Animals (12-13 per group) received either BTT or GTC (0.05%) in their drinking water from 28 days (4 weeks) of age to 133 days (19 weeks) of age, after which animals were killed. Control animals (12 per group) received drinking water without tea. Five transverse sections of control (i), BTT (ii) and GTC (iii) were cut and the largest lesion per mouse was stained with PCNA antibody (A). All optical fields per experimental group (n=70 per group) were compared. The PCNA labeling index was calculated (see experimental methods and materials) for each group. Values (B) are the mean proliferation index per group ± SD, n= 12-13 per group. Star indicates that the PCNA labeling index for animals in the BTT group were significantly reduced compared to animals in the control group (p<0.005).
4.6 Effect of BTT/GTC on apoptosis in TAg mouse tumours

The hypothesis was tested that intervention with GTC or BTT induces apoptosis in mammary tumour tissue. In chapter 3.3, it was established that BTT and GTC increased the number of apoptotic cells in MDA-MB-468 cells. In mammary tissue of TAg mice, which had received tea preparations at either 0.01 or 0.05% for their lifetime, both tea preparations induced apoptosis as reflected by an increase in levels of cleaved caspase 3. GTC at the lower dose and BTT at the higher dose elevated protein levels by 67% and 38%, respectively (Figure 4.17 A, B).

A

<table>
<thead>
<tr>
<th></th>
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<tr>
<td>0</td>
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<td>0.01%</td>
</tr>
<tr>
<td>0</td>
<td>0.05%</td>
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</tr>
</tbody>
</table>

Cleaved Caspase 3

\[\beta\text{-actin} \]

19 kDa

17 kDa
Figure 4.17 Effect of GTC (open bars) or BTT (closed bars) in the drinking water at 0.01 or 0.05% on expression of cleaved caspase 3 protein in mammary tumour tissue in TAg mice. A shows representative blots B quantitation by densitometry of cleaved caspase 3 bands in 10-16 individual mice. Values in B are expressed as arbitrary densitometry readings, control expression is shown by the hatched bars. Animals received tea extracts from week 4 until tumour size exceeded 17mm (week 22-25), after which they were killed, and tissue was obtained and examined by Western blot using an antibody against cleaved caspase 3. Star indicates that values were significantly different from control (p<0.05).
Chapter 4: Effect of BTT and GTC on mammary tumour development in TAg mice

4.7 Effect of GTC and BTT on oxidative DNA adduct levels in TAg mouse tumour and blood

4.7.1 Effect of BTT/GTC on MldG adduct levels in TAg tumour tissue and blood leukocytes

In chapter 3.5, it was shown that BTT and GTC significantly reduced MldG adduct levels in MDA-MB-468 cells. In this experiment mammary tumour tissue and blood leukocytes from TAg mice, which had received tea preparations at either 0.01 or 0.05% for their lifetime, were analysed for MldG adducts. Tumours were divided into two groups according to the size of tumour; <0.5mm in diameter and >0.5mm diameter. Tumours were divided into two groups because MDA can also be generated via the cyclooxygenase pathway of arachidonic acid metabolism (see chapter 1.6.6) and a previous study showed that COX 2 expression in adenomas in Apcmin mice was size-dependent (Sale S, 2005). Therefore MldG adduct levels in tumours of different sizes were analysed to establish whether size was a determining factor in adduct levels. Figure 4.18A shows that there was a significant decrease in MldG adduct levels in tumours <5 mm in size. BTT (0.05%) and GTC (0.01%) reduced MldG adduct levels. The difference in MldG adduct levels in the treated group compared to control was statistically significant (P = < 0.01). In tumours >0.5mm in size, 0.05% BTT significantly decreased MldG adduct levels compared to control (P= <0.01), (figure 4.18B). However, MldG adduct levels in the control and treated group were not affected by the size of tumours (figure 4.18 A, B).

Fig 4.18C shows that when results for MldG adduct levels in tumours <5mm and >5mm were combined, intervention with either tea preparation significantly decreased MldG adduct levels in tumour tissue. Adduct levels in tumours of mice receiving GTC at the 0.01 or 0.05% dose levels were reduced by 50% (p<0.05) and 78% (p<0.001), respectively, compared to controls, whilst MldG in mice on BTT was decreased by 71% (p<0.001) and 63% (p<0.05), respectively. Thus, there was a dose-dependent decrease in MldG adduct levels with GTC. In contrast, there was no difference in MldG levels in blood leukocytes collected at the end of the experiment between control mice and mice of tea on either preparation (figure 4.18 D)
4.7.2 Effect of GTC and BTT given early or late on M1dG adduct levels in mammary tumours of TAg mice.

Tumour tissue was obtained from mice at the end of the experiment (day 154, week 22) and analysed for levels of M1dG adducts. Intervention with either tea preparation administered before day 91 (week 13) or after 91 days (week 13) had no effect on M1dG adduct levels (figure 4.19). Tissue was also removed from the mammary pelt of a TAg mouse that possessed no tumours. This normal breast tissue was also analysed for M1dG adduct levels and produced similar values to TAg tumour tissue (3.6 fmoles x 10^8 nucleotides).

A

B
Figure 4.18 Effect of GTC (open bars) or BTT (closed bars) on levels of M1dG mammary tumour tissue and leukocytes in TAg mice. Tumours were separated according to their size, <0.5mm in diameter (A) or >0.5mm in diameter (B). C represents the levels of M1dG adducts in TAg tumour tissue independent of size. D shows the effects of BTT/GTC on M1dG adduct levels in leukocytes. The concentration of GTC or BTT in the drinking water was 0.01 or 0.05%, control animals received drinking water without tea extracts (hatched bars). Animals received tea extracts from day 28 (week 4) until tumour size exceeded 17mm (day 133- 154, week 22-25), after which they were killed and tumour tissue was obtained. M1dG levels were determined by slot blot analysis. Values are the
mean ± SD of groups of between 10 mice (0.05% BTT/GTC) and 16 mice (control and 0.01% BTT/GTC). Stars indicate that values were significantly different from the respective controls (*p<0.05, **p<0.01, ***p<0.001).

Figure 4.19 Effect of GTC (open bars) or BTT (closed bars) administered before day 91 (week 13) or after 91 days (week 13) on levels of M1dG mammary tumour tissue in TAg mice. Mice received either GTC or BTT in the drinking water from 28 days (4 weeks) of age to day 91 (week 13) or from day 91 (week 13) to day 154 (week 22) (14 per group). Control animals (14 per group) received drinking water without tea extracts (hatched bars). Mice were killed after 154 days (week 22) except for three, which were killed earlier, because the size of their tumours exceeded 17mm. M1dG levels were determined by slot blot analysis. Values are the mean ± SD of groups of 14 mice. M1dG adduct levels in mice in the intervention groups were not statistically different from the control.
Chapter 4: Effect of BTT and GTC on mammary tumour development in TAg mice

4.8 Discussion

The results described show for the first time that extracts of black and green tea delay mammary carcinogenesis in the TAg mouse model. The effects of BTT and GTC on tumour size were investigated (see results chapter 4.2). Both tea preparations significantly increased the percentage of TAg mice in the intervention groups, compared to control. Mice in the GTC or BTT group possessed smaller tumours and thus were culled a week or two weeks later than mice in the control group. This was accompanied by a slight, but not significant decrease in volume and number of tumours, and a reduction in the number of mice bearing tumours as compared to control. The experiments were repeated following study design 1, (see chapter 2.2.2) but with an additional dose (0.01%) of BTT and GTC. There was no significant difference in the percentage of mice present in the treated groups compared to control and the number of tumours per mouse between the intervention groups and control. There was a slight decrease in tumour volume in mice exposed to BTT (0.01%, 0.05%) and GTC (0.05%). However this decrease failed to reach significance. The results suggest that retardation of mammary carcinogenesis was only achieved with BTT and GTC at the 0.05% dose level. Animals were then culled at week 19 (day 133), to determine the effects of BTT/GTC on mammary carcinogenesis at a fixed end point. The mammary pelt weight was reduced in mice exposed to either tea preparation. Both tea preparations significantly decreased tumour volume and reduced the number of tumours per mouse, compared to control.

The effect of tea on tumour size was detected by careful pathological analysis. This analysis failed to reveal cytological differences between malignancies in mice in the intervention or control groups. Although cytological differences were not observed, histological analysis showed that the mean tumour diameter of mice in the treated groups was lower than the control group and this may be a result of reduced PCNA levels. The difference in tumour diameter between the intervention groups and the control group suggests that tea extracts delayed mammary carcinogenesis in a subtle rather than a dramatic fashion. The delay in mammary carcinogenesis was broadly similar for both GTC and BTT intervention groups, and it resembles that found previously in this model with 2-
difluoromethylornithine and dehydroepiandrosterone (Green et al., 2001). In this study, mice (21-25 per group) were supplemented with 2-difluoromethylornithine (DFMO) and dehydroepiandrosterone (DHEA) in their feed at beginning of week 7. Both agents reduced mammary tumour incidence, mammary tumour number and average tumour weight. Pathological analysis revealed that the number of mammary intraepithelial lesions (MIN) lesions at 14 weeks of age was similar in the control and in the treated groups.

Mice received tea preparations for 9 weeks (63 days) during early or late stage of tumour development. A slight delay of mammary carcinogenesis was observed. However, this retarding effect was seen only when intervention groups were compared in terms of tumour number per mouse, thus the effect of tea was not as robust as that observed in mice on tea for the whole duration of the experiment. A significant increase in the percentage of mice bearing tumours, was observed in mice receiving BTT from 90 days to 114 days, compared to control. However when mice received BTT from 90 days to 154 days, the results were not significantly different from control. This increase may be explained by the large interindividual variation between mice. The findings that tea extracts can delay mammary carcinogenesis in rodents is consistent with previous observations using the 7,12 dimethylbenz[a]anthracene (DMBA)-induced rat mammary carcinogenesis model (Kavanagh et al., 2001; Rogers et al., 1998; Tanaka et al., 1997). Rats received green tea either in the diet, (Tanaka et al., 1997) in drinking water (Baliga et al., 2005; Kavanagh et al., 2001; Rogers et al., 1998; Weisburger et al., 1997; Zhou et al., 2004) or intraperitonealy injected into nude mice (Liao et al., 1995). Green tea significantly increased mean latency to first tumour and reduced tumour burden and number of invasive tumours per tumour bearing animal (Kavanagh et al., 2001; Tanaka et al., 1997). Mammary gland carcinogenesis was not affected by black tea in rats fed a normal diet (AIN-76A). However, rats fed a high fat diet showed a reduction in tumourigenesis, as reflected by a reduction in tumour burden compared with rats drinking water (Rogers et al., 1998; Weisburger et al., 1997).

In nude mice, green tea inhibited the growth of tumours in a MCF-7 xenograft breast cancer model, whereas BTT had no effect on tumour growth, compared to control mice. In three of these previous studies (Rogers et al., 1998; Weisburger et al., 1997; Zhou et al., 2004),
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The significant effect of tea on mammary carcinogenesis was only achieved with concentrations above 1%, expressed as concentration of tea polyphenols in drinking water. The dose of BTT and GTC shown to delay mammary carcinogenesis in the study described here was 0.05%.

The results described in these studies show a slight delay in mammary carcinogenesis in the TAg model. However in this study, there is variation between experiments. The volume of tumours or number of tumours in mice in one experiment is different from the repeated experiment. This variation is probably due to the large standard deviation between individual animals. The variation may be resolved by using more animals in the experiments. Using more than 20 animals in each group may reduce the standard deviation and increase the statistical significance between treated groups and control. Epidemiological data has suggested that green tea may prevent breast cancer (chapter 1.4). It would be interesting to evaluate whether tea combined with other chemopreventive agents, such as tamoxifen can prevent breast cancer in this TAg model. Green tea increased the inhibitory effect of tamoxifen on proliferation of MCF-7 cells and in animal experiments (Sartippour et al., 2006). Mice treated with both green tea and tamoxifen developed small MCF-7 xenograft tumours, as compared with each agent alone and demonstrated the highest level of apoptosis in tumours Green tea may also reduce the toxic effects of tamoxifen.

A variety of mechanisms have been proposed to contribute to the putative cancer chemopreventive properties of tea polyphenols, including modification of the cell cycle machinery (Ahmad et al., 1997), induction of apoptosis (Hsu et al., 2003), inhibition of epidermal growth factor receptor tyrosine kinase (Sah et al., 2004) and modulation of matrix metalloproteinases (Demeule et al., 2002) causing blockade of metastasis and angiogenesis.

The delay in tumour development by tea preparations described (chapter 4.6) may be related to their ability to interfere with mammary tumour cell survival signalling. The effect of BTT/GTC on induction of apoptosis was examined in tumour tissue to confirm that the mechanism which was observed in MDA-MB-468 cells \textit{in vitro} also occurred \textit{in vivo}. BTT and GTC increased the number of apoptotic MDA-MB-468 cells \textit{in vitro} and induced
apoptosis by increasing tumour levels of cleaved caspase 3 protein. A significant increase in cleaved caspase 3 levels was only achieved with BTT. In the case of BTT, efficacy may also be related to the slight but significant decrease in rate of proliferation. Apoptosis can play a crucial role in eliminating preneoplastic and hyperproliferating cells from the organism, thus its induction may contribute to prevention of tumour progression. The results presented here are consistent with previous reports in which green tea polyphenols induced apoptosis in mammary cells *in vitro* (Zhao et al., 2006) and increased levels of cleaved caspase 3 in mammary tumour tissue. Green tea increased the ratio of Bax/Bcl-2 in tumours and increased the activation of caspase 3 in 4T1 tumours in BALB/c mice (Baliga et al., 2005). Green tea treatment also inhibited cell proliferation in 4T1 cell-induced breast tumours as evident by the inhibition of PCNA expression in tumours. However in this paper, efficacy was seen using dietary doses which were higher than those employed in this study. The induction of apoptosis in the TAg model was consistent with a study, in which mullerian inhibiting substance suppressed tumour growth (Gupta et al., 2005), as indicated by a decrease in the number of animals bearing tumours compared to control. This observation is consistent with the increase in apoptosis and decrease in proliferation of TAg tumour tissue in the mullerian inhibiting substance-treated group compared to controls. Previous studies have demonstrated an induction of apoptosis accompanied with an increase in Bax/Bcl2 ratio, APAF-1 and PARP protein. It would have been interesting to investigate the effect of BTT/GTC on Bax/Bcl2 ratio, Apaf-1 and PARP protein in the TAg model.

One mechanism thought to contribute to cancer chemoprevention by tea polyphenols, is their anti-oxidative activity. The results described (chapter 4.7) demonstrate that intervention with either GTC or BTT significantly reduced M1dG levels, which reflect oxidative DNA changes, in mammary neoplastic tissue. These findings are consistent with reports (Leung et al., 2001) in which green and black tea constituents possessed similar antioxidant activity and increased human plasma antioxidant capacity *in vivo*. In addition, high levels of putative MDA adducts have been measured in normal breast tissues from cancer patients than those found in non cancer controls (Wang et al., 1996). Freese et al (1999) investigated the levels of MDA in healthy women fed a diet rich in linoleic acid.
Women ingested encapsulated green tea extract (3g/day) and MDA levels were measured in plasma. Green tea decreased MDA levels compared to the placebo group. This amount corresponds to approximately 10 cups a day. The results presented here and the evidence provided from previous studies (Freese et al., 1999; Wang et al., 1996) hint at the possibility that M1dG levels may serve as a marker of efficacy of tea polyphenols in breast cancer, even though the lack of effect on leukocyte M1dG levels is incompatible with their potential usefulness as a surrogate marker.

Some studies show no significant effect with green tea extracts (Whitsett et al., 2006) and black tea extracts in mammary carcinogenesis (Zhou et al., 2004). Other studies show a significant effect with green tea extracts (Hirose et al., 2003; Kavanagh et al., 2001; Tanaka et al., 1997) and black tea (Rogers et al., 1998; Weisburger et al., 1997). The differences in these studies may be a result of the administration methods employed. For example, green tea may have been prepared with fresh leaves infused with hot water, filtered, and given to the animals as a drink or dissolved in the drinking water. Green tea has also been mixed with the diet or intraperitoneally injected into mice. These administration methods influence the catechins both quantitatively and qualitatively. The concentration of catechins also varies in the original tea leaves according to the variety, origin and growing conditions. In addition, during preparation, the total amount of catechins may not be extracted from the leaves, thus the concentration may differ from the absolute values determined through the complete extraction of leaves. Moreover, catechins are relatively unstable and could be quantitatively and qualitatively modified during the time frame of the experiment (Crespy and Willamson, 2004).
CHAPTER 5

Final Discussion
5.1 The value of the TAg mouse in experimental breast cancer chemoprevention

Most preclinical breast cancer chemoprevention studies to date have been performed using chemical carcinogenesis models that have limitations (see chapter 1.2). The molecular targets and their relevance to human cancer in chemically-induced transformation are not well characterised. This transgenic model may be employed in favour of over other breast cancer models such as xenograft models or carcinogen induced models because transgenic mice possess a functional immune system, a carcinogen is not used and tumour formation tends to have a relatively short latency. Thus, they can be considered as a popular model for preclinical evaluation of potential chemotherapeutic and chemopreventive agents. The phenotypes of mammary tumours from such other models generally do not have histologic characteristics which resemble human breast cancer (Cardiff, 2001) suggesting that the oncogenic pathway may differ from those in human breast cancer. In contrast, one might argue that the TAg mouse may turn out to be a reasonable and physiologically robust model of an insidious type of human oestrogen receptor-negative breast cancer. ER-ve breast cancer is associated with poor prognosis (chapter 1.5.1). The TAg model shares similar features to human DCIS, and there is currently no generally accepted clinical management option for women presenting with DCIS. Therefore the TAg model may ultimately turn out to be a valuable model, which offers potential insights into strategies for the prevention and/or treatment of a grave and fairly untreatable human condition. This hypothesis is perhaps worth further investigation.

Results such as those presented here obtained in the TAg mouse indicate clinical prevention strategies which may delay progression from DCIS to full-blown mammary carcinoma. However it is important to note that some results described here hint at disadvantages associated with this model. Results of intervention studies were extremely variable, for example, when the experiment was repeated within 6 months of the first intervention study, tea preparations showed no significant effect on the number of TAg mice, as reflected by tumour size, in contrast to a significant effect observed in the first study. Statistical significance was often not achieved in the results, due to large interindividual variation between mice. Individual mice from each group developed
tumours at different time, therefore increasing variability. But then, one may argue that such variability is perhaps reminiscent of the human disease counterpart.

Breast carcinogenesis in rodents can differ from human breast cancer. For example, the biology of rodents and their tumours differs from human breast cancer, e.g. number of tumours, size, the degree of differentiation and maturation. Tumours in animals must have a rapid programme of progression as mice can develop very malignant tumours within a short time period and thus have a shorter lifespan which is different to human breast cancer. In addition, human breast cancer starts with the local lymph nodes and usually spreads via the lymphatic system, followed by distant metastases predominantly to the bone, the brain, the adrenal glands, liver and lungs. In contrast, in this TAg model mammary cancers metastasise predominantly to the lungs. However in this experimental study, metastasis did not occur, this was due to the fact that mice were culled once tumours exceeded 17mm. A limitation of this study was that exposure to tea was not measured therefore the ingested quantity cannot be precisely evaluated. However by injecting tea intraperitoneally into mice would allow an accurate measurement of the volume of tea ingested.

It is unlikely that any single mouse model will be able to mimic all aspects of human breast cancer, as breast cancer consists of a heterogeneous group of diseases which can be characterised by different sets of genetic mutations, histopathological types and metastatic potentials which can be within the same primary tumour mass. However this does not invalidate the use of rodent models in studying specific aspects of the disease (Gusterson et al., 1999). The use of transgenic models such as the TAg model for preclinical testing, may offer a means to overcome many of the difficulties encountered in human trials. Trials are quite expensive, require large cohorts and it can be difficult to control actual dietary consumption leading to imprecise records of dietary intake.

The use of animal models provides a tool to perform studies in a controlled experimental environment in which chemopreventive agents can be measured.
The use of M1dG and apoptotic signaling molecules as suitable pharmacodynamic biomarkers of efficacy

A pharmacodynamic biomarker can be described as a marker that may be altered by chemopreventive agents and the biological alterations are represented as the early and intermediate stages of carcinogenesis. A suitable biomarker of efficacy would be one that is associated with development of cancer, the change in the biomarker is related to carcinogenic progression, is mechanism based, it can be detected in target tissue and can be accessible for multiple biopsies (Sharma and Farmer, 2004). It must be possible to measure the biomarkers on samples that are readily obtained, which generally means blood or urine (Collins, 2005).

The use of M1dG as biomarker of efficacy has been investigated in many studies (Sharma and Farmer, 2004, Zhang et al., 2002, Everett et al., 2001). The results presented here show that green tea and black tea theaflavins reduced M1dG levels and increased apoptosis in TAg tissue and in breast cancer cell lines. However the mechanism by which this reduction occurs has not been studied in detail. It would be interesting to investigate whether tea extracts induce detoxifying enzymes and affect COX2 expression. The effect of tea extracts on the apoptotic signaling pathway can also be studied in detail. In addition, due heterogeneity of tumours the proposed biomarkers may have some limitations. The complexity and heterogeneity of tumours may cause variability in measuring biomarkers of efficacy as there are a number of mechanisms involved in carcinogenesis.

A study by Guiseppe et al (2005) investigated the effect of curcumin on patients with colorectal cancer. In this study M1dG levels were affected by the specific manner in which tissue samples were obtained. Pretreatment biopsy samples which consisted predominantly of malignant tissue were obtained from the surface of the tumour. Samples obtained at surgery consisted of a “wedge” of tissue which included the tumour core. It was hypothesised that levels of M1dG could differ in tumour samples taken from the edge or the middle of the tumour, due to differences in exposure of tissue to oxidants or variation in blood supply. To test this hypothesis, M1dG adduct levels were measured in three patients who had not received curcumin. In preoperative biopsies specimens, levels of M1dG were 4 fold higher than surgically obtained tumour core and M1dG levels in postoperative
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tumour edge were approximately twice as high as in tumour core obtained post surgery. Thus, it would seem that differences in M1dG levels may be affected by origin of the sample. The daily fluctuations of MDA are also significant when collecting samples. MDA levels were increased in patients with early stage breast cancer when compared to controls. However, the significant difference in MDA levels was due to the high levels measured at 20.00h in the breast cancer patient group. Highest plasma MDA levels in the control group were found during 12.00-16.00h and lowest levels at 4.00h, while the time period for lowest levels of MDA were similar in both groups, the highest levels of MDA were found during night time in patients with early breast cancer (Akbulut et al., 2003). The results presented here show that both tea extracts reduced M1dG levels in TAg tumour tissue, however no effect was observed in the leukocytes. In addition, tea extracts at the two doses investigated (0.01% and 0.05%) reduced M1dG levels, however a delay in mammary carcinogenesis was only observed at the 0.05% level. There may also be limitations in the assay. This method is sensitive and requires small amounts of DNA (<5μg) and all samples are analysed in triplicate on the same ISB. Thus, the assay is much less laborious and time-consuming than other assays and allows routine analysis of a large number of samples in short times. Quantitation of the adduct in human samples is based on a calibration curve obtained using a standard present on the same filter as the real samples, thus standardising results obtained on different ISB. However, when using an assay based on adduct detection by direct antibody binding, the possibility of non-specific binding and the quality of the antibody has to be taken into consideration.

5.3 Comparison of effects of BTT and GTC on mammary carcinogenesis

The work described in this study demonstrates the effect of BTT and GTC on mammary carcinogenesis. Both tea extracts reduced cell number, induced apoptosis and necrosis and reduced the levels of M1dG adducts in breast cancer cells to a similar extent. Both tea preparations were also shown to delay mammary carcinogenesis in the TAg mouse model, accompanied by an increase in the number of mice, decrease in tumour volume and tumour burden. BTT significantly reduced proliferation of TAg tumour tissue, as reflected by the
decrease in PCNA levels and significantly increased cleaved caspase 3 levels. However, the effect of GTC on proliferation and cleaved caspase 3 levels failed to reach significance, suggesting that BTT was slightly more effective than GTC. The dose of GTC and BTT used in this study to delay mammary carcinogenesis was 0.05%. This value corresponds to approximately 3-6 and 15-30 cups of green and black tea, respectively, per day. This dose is based on the polyphenol content in dried solids from aqueous tea extracts and is extrapolated from mice to humans (70 kg) using the body surface area calculation described by Freireich et al (1966). The content of theaflavins in black tea extracts tends to be between approximately 3 and 6% and the black tea preparation used in the study described here consisted exclusively of theaflavins, which explains why a high cuppage of black tea is required. The results presented here suggest tentatively that perhaps the role which black tea constituent's play in breast cancer chemoprevention has hitherto been underestimated. If further studies support the beneficial health effects of theaflavins, it may be worthwhile to consider increasing the theaflavin content in commercial black tea products, which can be achieved through selective processing techniques, or formulating a theaflavin tablet which could be taken two or three times a day and would perhaps have the same effect of drinking 15-30 cups of black tea per day. A green tea tablet has already been on the market. Each tablet is 500mg and contains 96% green tea extract, corresponding to two cups. A study in healthy volunteers was conducted where volunteers administered 5 tablets a day (equivalent to 10 cups/day) and the results indicated that 10 cups of green tea is tolerable (Fujiki et al., 2002).

Many in vitro studies on tea use high concentrations (see discussion, chapter 3) and thus do not reflect typical concentrations found in human or animal plasma. It is difficult to extrapolate these results to in vivo situations. The oral bioavailability of the major green tea constituents, green tea catechins is low in humans many fold less than the effective concentrations determined in in vitro systems. Further studies need to be conducted. Greater oral bioavailability of free catechins was demonstrated by Polyphenon E capsules on an empty stomach after an overnight fast.

Currently there is little information on the potential cancer chemopreventive properties of black tea. The work described here shows for the first time that an extract of
black tea significantly increases TAg mouse survival, decreasing tumour pelt weight and tumour volume. Both tea preparations were shown to delay mammary carcinogenesis in this model at a dietary dose of 0.05%. This dose can potentially be used in clinical studies and the data presented here suggests that these tea preparations may be suitable for consideration as chemopreventive agents in clinical studies and that oxidative DNA adducts such as M1dG and apoptotic signaling molecules may perhaps constitute suitable pharmacodynamic biomarkers of efficacy. The low toxicity, low cost and ease of consumption of tea render such a strategy attractive.

5.4 Future Studies

It would be worthwhile to investigate mechanisms in detail. The effects of tea on detoxifying enzymes and on COX2 can be studied. The effects of tea on apoptotic signaling molecules, Bcl-2 and Bax, Parp, cytochrome c may be interesting to investigate. Animal studies with larger number of animals may reduce the variability in individual studies. Green tea combined with theaflavins may have more of a beneficial effect than either agent alone and can be explored in this TAg model. In addition, it would be interesting to establish the effects of dietary agents such as tea combined with chemopreventive agents tamoxifen, or aromatase inhibitors (anastrozole). The complexity and heterogeneity of known mechanisms of carcinogenesis provides a strong basis for combining drug interventions which could target different signaling pathways, thereby mediating synergistic effects to inhibit tumour progression (Brenner and Gescher, 2005).
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