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Ability of Triclocarban (3,4,4'- trichlorocarbanilide) to induce premalignant breast cell carcinogenesis and enhance 2-Amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP)-induced breast cell carcinogenesis

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To the Graduate Council:

I am submitting herewith a dissertation written by Shilpa Sood entitled "Ability of Triclocarban (3,4,4'- trichlorocarbanilide) to induce premalignant breast cell carcinogenesis and enhance 2-Amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP)-induced breast cell carcinogenesis." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

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Shilpa Sood

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DEDICATION

Dedicated to my mother Mrs Satya Prabha Sood with love

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ABSTRACT

Chronic exposure to environmental carcinogens may trigger development of sporadic breast cancer, a multistep tumorigenesis process involving aggregation of genetic and epigenetic alterations, resulting in a serial progression from a non-cancerous to precancerous stage and eventually malignant breast cancer. In this study, I used a cellular model that simulates induction and progression of chronic breast cell carcinogenesis using concentrations of environmental carcinogens which are achievable in blood or plasma. Process of carcinogenesis was measured by certain detectable constitutive and transient end points including acquisition of cancer-associated properties, increased ROS prodiction, cell proliferation, DNA damage and activation of Erk-Nox pathway which could be targeted by non-cytotoxic dosages of dietary compounds.

Triclocarban (TCC) is an antimicrobial agent present in personal care products. This study was designed to investigate the ability of TCC to act as a co-carcinogen and therefore induce premalignant breast cell carcinogenesis and enhance 2-Amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP)-induced breast cell carcinogenesis in MCF10A cells. My results showed that repeated treatments with TCC induced cancer-associated properties of reduced dependence on growth factors and anchorage-independent growth in MCF10A cells. Concurrently, there was increased ROS production, cell proliferation and upregulation of Erk-Nox pathway in response to single as well as long term exposure to TCC. Curcumin, a polyphenolic component extracted from the rhizome, *Curcuma longa*, was able to suppress these transient and constitutive endpoints induced in breast cells exposed to TCC. MCF10A cells were also subjected to either individual or combined TCC and PhIP exposure. Cells co-treated with TCC and PhIP had significantly

higher levels of cancer-associated properties, ROS production, cell proliferation and Erk-Nox pathway activation as compared to cells treated with PhIP alone. The transient and constitutive endpoints induced by co-treatement with TCC and PhIP were significantly suppressed by mimosine, a non protein amino acid isolated from *Mimosa pudica* and Ergosterol, a sterol found in cell membranes of fungi. These results proved that TCC has the ability to induce breast cell premalignancy and enhance breast cell carcinogenesis induced by PhIP in MCF10A cells. Furthermore, curcumin, ergosterol and mimosine could play promising roles in prevention of sporadic breast cancer.

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ABBREVIATIONS

ATCC American Type Culture Collection

B[a]P Benzo[a]pyrene

BrdU 5-bromo-2□-deoxyuridine

CDK Cyclin-dependent kinase

CM-H2DCF-DA Chloromethyl-dichlorodihydrofluorescin-diacetate

CM Complete MCF10A medium

EC Epicatechin

ECG Epicatechin-3-gallate

EGC Epigallocatechin

EGCG Epigallocatechin-3-gallate

ELISA Enzyme-linked immunosorbent assay

EMT Epithelial to mesenchymal transition

EpCAM Epithelial cell adhesion molecule

ERK Extracellular signal regulatory kinase

H2O2 Hydrogen peroxide

IC50 50% inhibitory concentration

LM medium Low-mitogen medium

MTT Methyl Thiazolyl Tetrazolium xi

NAC N-acetyl-L-cysteine

NNK 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone

Nox-1 NADPH oxidase

PBS phosphate buffer saline

PhIP 2-amino-1-methyl-6-phenylimidazo [4, 5-*b*] pyridine

PE Phycoerythrin

ROS Reactive oxygen species

RTK Receptor tyrosine kinase

TCC 3,4,4'-trichlorocarbanilide

Chapter 1

Background and Overview

1.1 Breast cancer

According to National Cancer Institute and American cancer society, cancer is a term used for a group of diseases in which abnormal cells divide without control and are able to invade other parts of body through the blood vascular and lymphatic systems [1]. There are many different types of cancers which can arise in any organ of the body but share the common characteristic of uncontrolled growth. Human breast is a glandular structure divided into lobules which secrete milk to be delivered via ducts to the nipple. Fat, connective tissue, and lymphatics constitute the rest of breast. Cancer of breast is broadly divided into two subtypes: those originating from ducts are known as ductal carcinomas; those originating from lobules are known as lobular carcinomas. Breast cancer is the most common type of cancer among women worldwide. Cases of breast cancer in developed countries far outnumber those in the developing countries (Figure1.1). Breast cancer is also the most common type of cancer and second leading cause of deaths among American women after lung cancer [1]. It is projected by American cancer society, that approximately 232,340 cases of invasive breast cancer and 64,640 new cases of in situ breast cancer would be diagnosed in 2013 [1]. In 2013, it is estimated that there would be about 39,620 breast cancer related deaths among women [1]. Also, about 2,240 cases of invasive breast cancer are expected to occur among men in 2013 which is about 1% of all breast cancer cases and 410 deaths are projected to occur in men because of breast cancer this year [1].

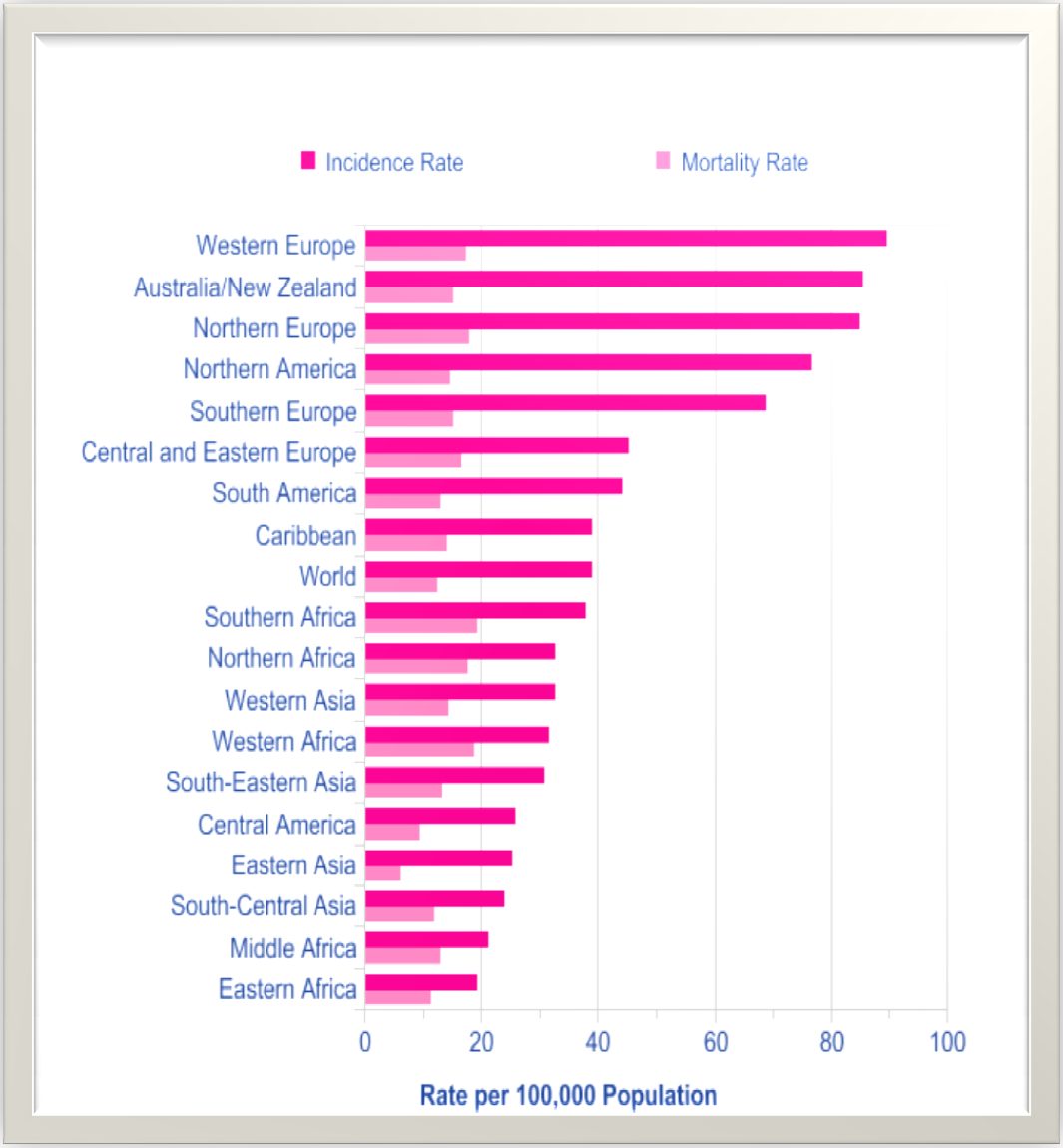


Figure 1.1: Worldwide incidence and mortality rates of breast cancer

Breast Cancer 2008 Estimates, World Age-Standardized Incidence and Mortality Rates per 100,000 Population, Females, World Regions

Modified from cancer research UK, http://www.cancerresearchuk.org/cancer-info/cancerstats/world/breast-cancer-world/ assessed April, 2013

1.1.1 Stages of breast cancer

The various stages of breast cancer are classified as follows (Figure 1.2):

Stage 0: This stage is also called as carcinoma *in situ* and is of the following three types:

a) Paget's disease of the nipple: In this condition abnormal cancerous cells are confined to the nipple.
b) Ductal carcinoma *in situ* (DCIS): cancer cells are found in the ductular lining of the breast tissue and the cells have not invaded the surrounding tissues.
c) Lobular carcinoma *in situ* (LCIS): It is the cancer affecting lobules of breast and cancerous cells do not invade surrounding tissue.

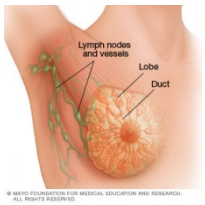
Stage I

Stage I is further subdivided into stages IA and IB.

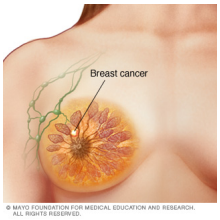
In the former sub stage, the tumor size is 2 centimeters or smaller. The tumor is present only in the breast tissue and has not spread outside the breast and has not invaded the lymphnodes. In the second substage tumor clusters ranging from 0.2 millimeters - 2 millimeters may be found in the adjoining lymph nodes.

Stage II: Here the tumor size is larger than the previous stage and varies between from 2 to 5 mm but has not yet spread to other far away locations but maybe found in lymph-nodes of the adjoining area like the axillary lymph nodes.

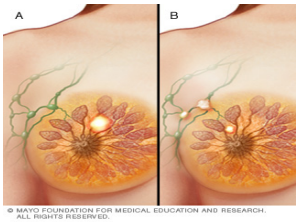
Stage III: Tumor size is larger than stage II and is larger than 5 mm. Tumor clusters measuring 0.2-2mm may be found in the adjoining lymph-nodes or lymph-nodes of collar bone. Tumors regardless of their size that have managed to infiltrate and reach the chest wall or that reach breast skin are also categorized as stage III breast cancer.



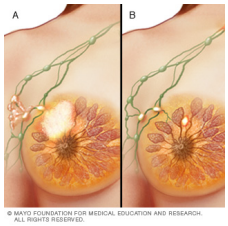
NORMAL BREAST



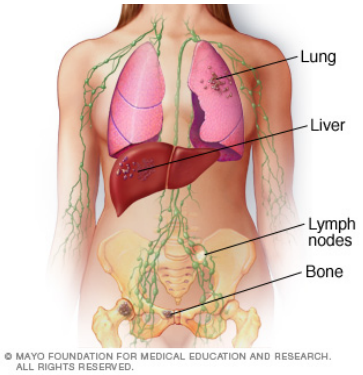
STAGE I BREAST CANCER



STAGE II BREAST CANCER



STAGE III BREAST CANCER



STAGE IV BREAST CANCER

Modified from Mayo Clinic website http://www.mayoclinic.com/

Figure 1.2. Stages of breast cancer

Stage IV**:**

It is known as the metastasizing stage of breast carcinoma. Cancer cells are present in surrounding lymph nodes and have also invaded the distant parts of the body, such as the lungs, distant lymph nodes, skin, bones, liver, or brain.

1.1.2 Classification of breast cancer:

Breast cancer is disease entity which encompasses varied subtypes on the basis of distinctness of patho-morphological characteristics and clinical outcome. Newer diagnostic tools such as immunohistochemistry, cytogenetics and microarray are employed for accurate diagnosis and prognosis [2, 3, 4, 5 , 6]. Complementary DNA microarray analysis has been used to classify breast cancer into five major subtypes which are as follows: Luminal A, Luminal B, Normal breast like, Her2 and basal like breast cancer. However, newer subtypes have also been recently added [2, 6,] (Table 1). This categorization takes into account the two major types of epithelial cells found in human mammary gland- luminal epithelial cells and basal epithelial cells, the latter are also known as myo-epithelial cells. These two types of epithelial cells can be differentiated immuno-histochemically on the basis of expression of different kinds of hormone receptors by these cells. For example, luminal cells express estrogen receptors (ER receptors) or progesterone receptors (PR receptors) and are found to be positive for cytokeratins 8/18. On the contrary the basal cells are devoid of estrogen receptor. The latter are however, found to be positive for the expression of cytokeratins 5/6, and 17. The basal type of breast cancers are also sometimes called as triple negative breast cancers because they are found to lack Human epidermal growth factor receptor 2 (HER2

receptors) too. Nonetheless there are some recognized differences between the basal type breast cancers and triple negative breast cancers. Approximately seventy five percent of basal like cancers have been found to be triple negative but the remaining twenty five percent of the basal cancers have been found to exhibit either the cell surface receptor Her2 or the two hormone receptors. Around three fourths of the triple negative breast cancers are considered as basal like breast cancers [2, 7]. Triple negative character is exhibited by the following breast cancer subtypes: basal like cancers, normal breast like tumors and breast cancers occurring due to the deficiency or abnormality of BRCA1 (Breast cancer 1, early onset) gene. Triple negative cancers are defined as tumors that lack ER, PR and HER2 expression. Moreover, triple negative cancers account for approximately ten to twenty-five percent of all the cancers of the breast tissue [8, 9] and can show different kinds of histologic patho-morphology. Triple negative cancers are found to occur predominantly in younger women less than fifty years of age and women belonging to African American ethnicity.

1.1.3 Risk factors for Breast cancer:

A risk factor may be defined as any stimulus that increases a person's chance of getting a disease. Different risk factors are associated with different types of cancers. There are some known risk factors for breast cancer. According to National Cancer Institute, 2011, being born as a female is an overwhelming risk factor for the development of breast cancer in women [1]. Many of the other risk factors for breast cancer include the following: age at menarche, age at first live birth, and age at menopause. Age is a significant risk factor with the chances of developing breast cancer increase with

increasing age in women. Only about 6% of breast cancers in the United States are known to occur in women under the age of forty years. However, in contrast to the women who are more than forty years of age, younger women are often found to be less affected with breast cancer which is generally a much more severe disease in younger women with a poorer prognosis and which generally progresses to advanced stages. Also, these younger women affected with breast cancer have higher rates of breast cancer recurrence and breast cancer related mortality [10]. Sixty seven percent of the cases categorized as invasive breast cancers occur in women who are fifty five years or are older. Breast cancer seldom occurs in females younger than twenty five years of age, but there can be an exception to this fact when breast cancer runs in the family. Hormonal levels influence the development of breast cancer and are important risk factors for the development of this disease. Many factors are known to influence proliferation and growth of cells of mammary gland. The most important among them are estrogen and progestins. Others might include transforming growth factor alpha and epidermal growth factor family proteins [11]. Levels of endogenous steroid sex hormones are strong determinants of breast tumor development risk. Infact, female menopausal breast cancer patients were found to have higher levels of steroid hormones including androgens and estrogens in their circulation [12]. Chances of acquiring mammary gland neoplasia may be affected by other hormone- related events. There is an elevation in the risk of breast cancer development for a small time period following delivery of a newborn. After that there is lessening of predisposition to developing mammary neoplasia for an extended period of time post-delivery, especially in case of younger females [13, 14, 15]. In one study, women who experienced a first full-term pregnancy before the age of twenty

years, had their chances of developing breast cancer reduced by half when compared to women who never became pregnant. The former also had significantly lower chances of breast cancer development than the females who successfully completed their first pregnancy at the age of 35 years or later [16, 17]. Age at which women start their menstruation cycles, also affects breast cancer development risk later in their lives. Women reaching puberty at an earlier age, for example, if they attained puberty when they turned 11 years or an earlier age, were found to be more at risk of developing breast cancer by about 20% as compared to women who reached puberty when they turned 14 years or later [18]. Women reaching menopause at a later age than that is normal for most women also have significant chances of developing breast cancer. Obese postmenopausal women have been shown to be more predisposed to develop breast cancer [19]. In addition, there are other factors associated with reproduction that may also influence breast cancer development. Ovarian hormones may influence development of breast cancer. Ovariectomy is known to reduce breast cancer risk by around three quarters. This kind of reduction can be greatly influenced by a women's age, her weight, and number of children she bears. It was seen that ovariectomy was most useful in reducing breast cancer development risks in cases of younger women who were not overweight and didn't have any children [20, 21]. The removal of uterus and ovaries was also shown to reduce the risk of breast cancer [22]. Individual genetic makeup also predisposes women for developing breast cancer. Moreover, the inheritance of defective genes has the capability to enhance the potential of known mutagenic agents and factors that are known to promote growth and proliferation of breast tissue ultimately leading to tumorigenesis in breast tissue. About 5%-10 of breast cancers are known to be

hereditary in nature and a prior familial history of breast cancer i.e. women with other close relatives afflicted with breast cancer like mother, siblings or aunts, predisposes them towards mammary gland neoplasia. Mutations in the following two genes: BRCA1 & BRCA2 accounts for the majority cases of hereditary breast cancers [23, 24, 25]. Even though women with a familial history of breast cancer are more predisposed to breast cancer development, yet only one in five women with relatives affected with breast cancer has been shown to carry germ line mutations in BRCA1 and BRCA2 genes [26, 27]. Mutations affecting BRCA1 are much more common than those affecting the related BRCA2 gene [28]. Mutations in the latter gene have been associated with breast cancer development in men [29].

Most of the women afflicted with Li- fraumeni syndrome are known to suffer from breast cancer. In women suffering from Li- fraumeni syndrome, germ line mutations are found in P53, which is an important tumor suppressor gene [30]. Women younger than twenty years of age who underwent radiation for treatment of other types of cancers were shown to have a high risk of up to 20% to 30% more, for the development of breast cancer as late as twenty to thirty- years after radiation therapy [31]. Exposure to ionizing radiation enhances the breast cancer development risk [32]. Other surgical and medical procedures utilizing radiation as diagnosis or treatment have been linked to higher risks of breast cancer development [33]. Moreover, women who are known to be heterozygotes for ataxia-telangiectasia gene possess enhanced predisposition to develop malignancies of the breast tissue [34]. Race is an important risk factor for the development of breast cancer. White women are more at risk of developing breast cancer as compared to the women of African-American, Hispanic, and Asian origin, even though the breast cancers

affecting black women have been found to be highly aggressive. Obesity and being overweight are other important factors associated with increased risks of breast tissue cancers [35]. Higher levels of leptin hormone secreted by adipose tissue cells, especially, those forming breast tissue microenvironment are postulated to influence development of breast cancer by aiding in angiogenesis and can also enhance metastasis and mortality due to breast cancer in obese women [35]. Obese women are not only predisposed to develop breast cancer but are also likely to have a poorer prognosis of the disease [36]. Obesity also happens to be a risk factor for women for breast cancer recurrence. Being overweight can not only increase the risk of the breast cancer recurrence in women who have had the disease, but it also is known to increase the risk of breast cancer development among postmenopausal women. Researchers have tried to shed light on the probable link between obese post-menopause state and breast cancer in recent years [19], where they have found that systemic and local mammary metabolism which gets upregulated in obese subjects, leads to activation of progesterone receptors which in turns aids in the development of post-menopausal PR positive breast cancer. Other factors like the use of hormone replacement therapy, alcohol consumption, cigarette smoking and sedentary lifestyle with no physical activity, also enhance the risk for development of breast cancer which will be discussed in the subsequent sections. Since less than ten percent of breast cancer is of hereditary origin and more that 90% is attributable to non-genetic factors, therefore, it becomes imperative to study in detail, the role played by environment agents in predisposing women to develop breast cancer [37].

1.1.3.1 Environmental Carcinogens:

Carcinogens are defined as any of the natural or synthetic substances that have the potential to cause cancer. Humans are subjected to an exposure from a wide range of chemicals from the environment each day through air, water, soil and food. Also, humans are exposed to hazardous chemicals in their work environment on a day today basis. Even, individual lifestyle factors are important contributors affecting our general health and predisposition to diseases like breast cancer [38]. Further as stated above, greater than 90% cases of breast cancer are non-hereditary and are classified as sporadic breast cancers [37]. In order to formulate an effective strategy for prevention and control of sporadic cancers, knowledge of everyday chronic exposures to low doses of environmental pollutants is of utmost importance especially for understanding pathogenesis of carcinogenesis process which ca then be used to devise preventive measures that can intervene with the process of carcinogenesis attributable to environmental agents. The American Cancer Society [1] has put forth a classification of some of the environmental factors which possess the potential to cause the non-hereditary form of breast (Table 2) cancer which are classified into the following categories according to their mode of exposure: carcinogens related to lifestyle (smoking, alcohol, diet, sedentary life style), naturally occurring substances (ultraviolet rays, infectious agents, radon), chemicals found in the home and workplace, infectious agents, carcinogens coming through medical treatments (hormone replacement, immune-suppressing treatments, exposure to radiation) and others.

1.1.3.2 Risk factors related to lifestyle

Risk factors associated with our lifestyle in general are not the causative or etiological factors for breast cancer but act as predisposing factors. High dosage exposure to carcinogens can be associated with a particular occupation or accidental exposure to a potentially hazardous material. Individual behavioral peculiarities such as being under any kind of addiction can also expose human body to direct cancer causing agents i.e. carcinogens [39, 40]. In the same study it was also noted that among other factors, habitual smoking, heavy drinking, unhealthy and imbalanced diet consumption, obesity and complete absence of any form of physical activity are prominent contributors for cancer in high-income countries [39].

1.1.3.2.1 Tobacco smoking

Cigarette smoking is a well-known and significant risk factor for initiation and progression of carcinogenesis [41]. Thousands of components found in cigarette smoke have been found to be mutagenic since it contains both promoters as well as initiators of carcinogenesis process and hence tobacco smoke can be considered as a complete carcinogen [40]. Habitual smoking is a major cause of cancer mortality worldwide and either directly causes or predisposes humans towards many different types of cancers such as those affecting lungs, upper digestive tract, esophagus, stomach, pancreas, liver, bladder, kidney, mammary and cervical cancers, bone marrow [English et al 1995. Despite the fact that there is no direct evidence of smoke originating from tobacco acting as a direct cause of breast cancer, recent reports suggest that components of smoke from tobacco, such as NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone) and

Benzoapyerene, can together play the role of co-carcinogens in sporadic breast cell carcinogenesis [42]. Moreover, more recently, evidence has accumulated to link chronic habitual smoking and being a smoker from a very young age with risk of breast neoplasia [43]. In addition, passive smoking may increase the risk of breast cancer in post-menopausal women [43].

1.1.3.2.2 Alcohol consumption

Role of alcohol as a carcinogen is under constant scrutiny. Habitual drinking can play the role a risk factor for carcinogenesis of various organs as was revealed by meta-analysis of over two hundred studies [44]**.** Alcohol has been classified as a human carcinogen. Alcohol can promote carcinogenesis by various ways which may include the following: induction of enzymes like CYP2E1, an enzyme involved in the metabolism of xenobiotics in the body, to generate carcinogens from pro-carcinogens, activation of MAPK signaling, cellular oxidative stress, and in breast cells alcohol may cause increase response to endogenous estrogens [45]. Moreover, data from several epidemiological studies shows that alcohol consumption is indeed associated with increased risk of breast cancer occurrence [46]. It has also been shown to act as a potentiator of carcinogenic effects of others like tobacco [46]. Some researchers however, consider it only as a tumor promotor or a co-carcinogen [47].

1.1.3.2.3 Diet

With busier and high tech lifestyles of the present day, quality of diet, especially in thedeveloped world, has deteriorated and has become ever more unhealthy with fast food

and fried food forming major chunk of food. Unbalanced diet which is high in fat, protein and carbohydrates and low in fiber is associated with high prevalence of cancers especially that affecting colon, prostate, endometrium and breast. But this can be countered by a regular balanced consumption of different kinds of natural antioxidants present in a wide array of fruits and vegetables [48, 49]. Diet can also be an unwanted source of carcinogens. Among the various dietary carcinogens , 2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine (PhIP), is one of the most abundant heterocyclic amines, found in high-temperature cooked meats or well done meats such as grilled/barbecued meats [50, 51, 52]. Consumption of well-done meats containing PhIP has been suggested to act as a risk factor for development of breast cancer in some epidemiological studies [53].

1.1.3.2.4 Overweight, obesity and sedentary lifestyle

Obesity is touted as an important risk factor for cancer. Some authors have suggested that obesity can exacerbate cancer mortality [54]. Rates of breast cancer in western world are considerably higher than that found in the developing world. But, the risk of breast cancer in immigrant populations after their settlement in US has steadily risen after migration which is mostly attributed to changed life style after migration. Even breast cancer risk in relatively developed Asian countries like Japan and Taiwan, has also increased in recent times. A prospective cohort study suggested that energy imbalance with high dietary intake and less consumption by means of physical inactivity may be associated with high risk of breast cancer particularly in premenopausal women [55].

1.1.3.3 Naturally occurring substances:

1.1.3.3.1 Ultraviolet rays

Ionizing radiation and ultraviolet rays have the potential to damage DNA and cause cancer. UV irradiation is one of the foremost risk factors for the development of skin cancer [56, 57, 58]. Tumors induced in response to UV radiation exposure, have shown a sharp rise in the United States, Europe and Australia [59]. Recently, however, exposure to UV radiation has been associated with a decreased risk of cancer in children [60]

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1.1.3.3.2 Infectious agents

The role of viruses first came to the fore when Bitters in 1936, found that a filterable agent, after being transmitted via milk caused breast cancer in suckling mice [61], which was later identified as a retrovirus. Since then there have been efforts to find whether there is a virus that specifically causes human viral mammary tumors but there is not concrete evidence of a direct link in this regard [62]. A significant 15% to 20% of neoplastic conditions are known to be caused by infectious agents including viruses, bacteria and parasites [63]. Some infections can enhance tumorigenicity induced by another primary agent wherein they themselves only cause immunosuppression related to infection induced inflammation. Certain infectious agents are known to be causative agents of cancer for example, *H. pylori* ,a bacterium found in stomach, is an important risk factor for the subsequent development of stomach cancer [64] and human papilloma virus (HPV) is known to be associated with cervical cancer [65]. Recent research has uncovered newer emerging pathogens capable of causing neoplasia in humans [66].

Moreover recent studies show that polycyclic aromatic hydrocarbons (PAH) and tumorigenic viruses can cause carcinogenic synergy [67]. In the later study it was shown that in response to PAH there was ROS-induced DNA damage and, human polyoma virus T antigen expressing cells impaired DNA repair, to potentiate overall the carcinogenicity.

1.1.3.3.3 Radon

Radon, a colorless, odorless, radioactive gas produced due to radioactive decay of uranium or thorium, is the second leading cause of lung cancer, after smoking. Radon is responsible for about 21,000 lung cancer deaths in US every year [68]. Radon is found in soil rock; even it is present indoors, with the maximal levels found to be present in basements areas from where it seeps via gaps in floors or walls. Even recent studies have found links of levels of radon exposure and lung cancer [69]. Recently, radon related lung cancers were found to occur more frequently among smokers [70].

1.1.3.4 Chemicals in the home and workplace

As per National Institute of Environmental Health Sciences description, chemical carcinogens are substances and circumstances that either are "known" or are "reasonably anticipated" to produce cancer. There is a long list of chemicals that are considered as carcinogens. Certain pollutants of the environment are thought to behave like hormones for example organo-chlorine pesticides can exhibit estrogen like activity and therefore might induce or influence development of breast cancer [71]. Chemicals such as benzene, auto exhaust, cigarettes, industrial processes and some consumer goods, have been associated with development of cancers while many others are being researched for

possessing potential carcinogenic activity. Some important carcinogenic chemicals are listed as under:

1.1.3.4.1 Diesel exhaust: This is produced by diesel motor driven vehicles used at construction sites or those used on roads and on farms. It not only causes environment pollution but also has been linked to the risk of lung cancer development [72, 73]. Inhalation of passive smoke from the surrounding environment, which was found to contain several thousand chemicals, out of which greater than sixty have been associated with the risk of developing cancer including lung cancer [74, 75, 76]. Asbestos is another chemical, exposure to which predisposes people to developing several diseases of lungs including cancer, mesothelioma, laryngeal cancer and gastrointestinal cancers and over 200, 000 deaths were expected to occur in 2010 due to asbestos induced cancers of the lung and mesothelium. The total deaths in US due to asbestos-related lung cancer, mesothelioma and asbestosis from workplace exposure resulted is estimated to be over 200,000 for year 2010 [77, 78]. Recent studies have shown people working in ship breaking business are predisposed to several types of cancers including oral and nasopharyngeal tumors, lung cancer and liver cancers because of possible exposure to asbestos and other metals [79].

1.1.3.5 Medical treatments (hormone replacement, immune-suppressing treatments)

To treat postmenopausal discomfitures, hormone replacement therapy (HRT) is widely used in women. It has also been used for treatment of osteoporosis and ailments of cardiovascular impaired cognitive functions and dementia in menopausal women.

However, several studies have raised concerns over its purported effectiveness in such treatments [80, 81]. Some studies report that use of hormones can increase the risk of breast cancer [82]. This type of therapy can increase the risk of other types of cancers like ovarian, colorectal and brain cancers [83, 84, 85]. In case of organ or graft transplant patients, immunosuppressive drugs are administered to reduce the chances of graft rejection. But since these agents are known to lower the immunity in the patients, such patients are highly at risk to contact infections and even cancers, [86, 87]. Since extreme immunosuppression has been suspected to be related to certain drugs used for treating cancers of skin and lymphoma [81, 88], these drugs should be used with extreme care and caution.

1.1.3.6 Others

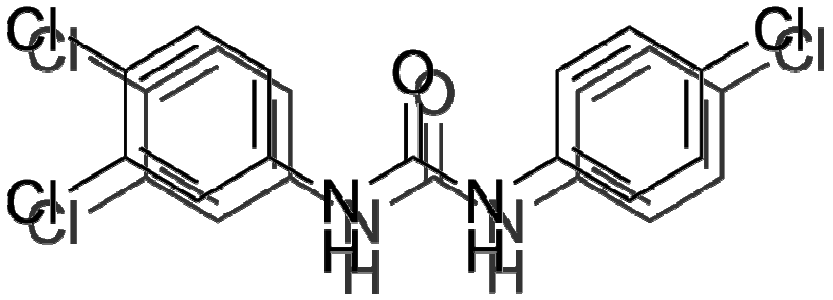
Silica, nickel and cobalt have been related to cancer causation [89]. Moreover, some other chemicals used in day to day life have been suspected to play important role in carcinogenesis process which includes among others the following: antiperspirants, talcum powder, hair dyes, and cosmetics [90]. However, some researchers suggest that a pronounced carcinogenic effect only is reflected if the exposure occurs at an occupational level [91]. In addition, there are other well known carcinogens like 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and benzo[a]pyrene (B[a] P) that can affect breast cancer development [42].

In this discussion of carcinogens so far, I have reviewed only some of the chemicals known to be carcinogenic. Moreover, apart from the chemicals that are known carcinogens, there are many other chemicals whose carcinogenic potential is unknown.

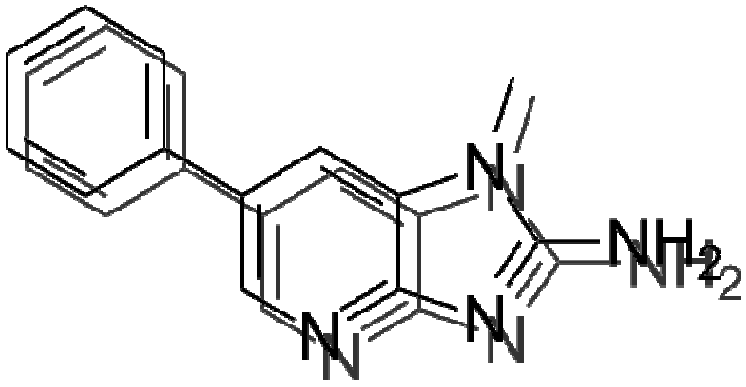
In this study, I use two chemicals to which human bodies are exposed continuously on an everyday basis: Triclocarban (TCC), or 3, 4, 4'-trichlorocarbanilide, an antimicrobial agent and the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo [4, 5-*b*] pyridine or PhIP.

1.1.3.7 Triclocarban

Triclocarban (TCC), or 3,4,4'-trichlorocarbanilide (Figure 1.3), is an antibacterial agent commonly used in personal care products such as disinfectants, soaps, body washes deodorants, detergents, cleansing lotions, wipes etc [92]. Antibacterial activity is attributed to its ability to disrupt cellular membranes [93] by inhibition of the enzyme enoyl-acyl carrier protein reductase (ENR) which is only present in some bacteria and fungi. The enzyme prevents the synthesis of fatty acids needed for building cell membranes and other vital functions. Many researchers have detected the presence of TCC in surface waters and sewage sludge [94, 95, 96, 97, 98]. These studies therefore suggest that the widespread use of personal care products containing TCC may pose risks for human health. But, adverse effects of trichlocarban on humans are largely unknown. A study involving rats [99], revealed that continuous incorporation of TCC in a diet at 3,000 or 10,000 ppm for six months lead to degenerative changes in seminiferous tubules and oligospermia. However, testicles didn't develop any pathological changes in rats fed TCC at 1000 ppm. In a study conducted in 1979 [100], a group of rats was fed various levels of a diet containing TCC and 3-trifluoromethyl-4, 4'- dichlorocarbanilide in the ratio of 2:1.



Triclocarban



PhIP

Figure 1.3 Chemical structures of carcinogens

The cohort that was fed the diet comprising of mixture at 0.25% was found to exhibit a reduction in the conception rate, number of live births and survival rate among those that were born. Recent research has shown that TCC can act as an endocrine disrupter. It has been shown to augment the activities of certain steroid hormones [101]. In recent in vitro studies, which used reporter gene assays, it has been reported that although TCC did not possess any agonist activity for estrogen receptor or androgen receptor in recombinant estrogen or androgen responsive cells, yet in the presence of estrogen and testosterone, TCC was found to amplify their hormonal actions. Another study by Chen *et al* [102], reported that in the presence of testosterone hormone, TCC was found to enhance testosterone's hormonal effects. However, TCC failed to affect protein expression of androgen receptor or AR mediated signaling. Also, TCC didn't interfere with testosterone for its binding to androgen receptor. Simultaneous administration of testosterone and TCC in diet to castrated male rats at 0.25% for 10 days lead to a significant increase in weight of accessory sex organs in comparison to rats that were exposed to either testosterone or TCC alone. TCC was shown to increase embryo production in freshwater mud snails, in a way, mimicked an exposure to certain environmental estrogens [103]. Researchers [104] have shown that TCC at nanomolar concentrations predisposes rat thymocytes to oxidative stress. The Material Safety Data Sheet (MSDS) for TCC states that TCC is a hazardous chemical that may cause cancer and damage to the genetic material [105] due to the possible presence of p-chloroaniline, a Proposition 65 carcinogen. TCC was shown to strongly augment the overexpression of aromatase AroB, which is a known target gene for estrogen, in response to treatment with exogenous estrogen in zebrafish brains. TCC exposure alone could however, stimulate

AroB expression only slightly [106]. Even though TCC has been postulated to act as an endocrine receptor, recently, [107] it was shown that TCC mediated activation of xenobiotic metabolism. Authors in this study [107] used a luciferase based reporter assay, to demonstrate that TCC activated nuclear xenobiotic receptors: ER-alpha and constitutive active/androstane receptor (CAR). In the same study, it was also shown that TCC activated ER-alpha in female rat ovaries leading to induction of *Cyp1b1* and also induced promoter activity of estrogen receptors. Moreover, TCC treatment in mice also resulted in induction of CAR target gene: UGT1A and Cyp2b10.

Direct dermal exposure to TCC results in transdermal absorption and accumulation in underlying tissues, including mammary tissues [108.]. A single whole-body shower using TCC-containing soap results in blood levels as high as ~500 nmol/L within 3 h [109, 110]. In addition, TCC is resistant to both wastewater chemical and biological treatments, and agricultural use of TCC-containing bio solids renders TCC into the food chain, thus increasing human exposure to TCC [101, 111, 112, 113, 114]. Although the role of TCC in increasing breast cancer risk has been questioned, it has not yet been addressed. It is imperative to clarify the role of low-dose TCC in sporadic breast cancer development and to identify effective agents for intervention and this precisely is the objective of this study.

1.1.3.8 PhIP

2-amino-1-methyl-6-phenylimidazo [4, 5-*b*] pyridine (PhIP) (Figure 1.3) is heterocyclic amine (HCAs) abundantly found in high temperature cooked meats particularly produced during grilling and barbequeing procedures [115, 116, 117]. Consumption PhIP via such

meats has been associated with an enhanced risk of breast neoplasia by epidemiological studies [118, 119]. People who took PhIP at microgram level concentrations via ingestion, had PhIP available systemically at picomolar and nanomolar concentrations [117]. Gastric administration of milligram levels of PhIP induced tumors of mammary gland [117]. Several studies have reported that PhIP is genotoxic to human cells and a concentration of PhIP as low as 450 nmol/L induced DNA adduct formation [117, 118, 119, 129]. Recently, it was reported that long-term exposure of breast cells to PhIP at physiologically-achievable, picomolar to low nanomolar doses induced carcinogenesis and tumorigenicity [121].

In one study, rats were fed PhIP at the rate of 75mg/Kg for 10 continuous days and this treatment induced proliferation of ductular epithelium, formation of carcinoma in situ and also mammary gland neoplasia in which high occurrence of H Ras gene mutations were found [122]. Induction of expression of oncogenic H-Ras in breast epithelial MCF10A cells confers to MCF 10A cells the capability of invasion which was accompanied by enhanced expression of metalloproteinases (MMP-2 and MMP- 9) [123] and ERK (extracellular signal-regulated kinase) pathway activation [124]. Activation of the ERK pathway causes increased expression of NADPH oxidase-1 (Nox1) and the enzyme Nox-1 in turn mediates generation of reactive oxygen species (ROS), which causes increase in cell proliferation, cellular motility, enhanced power of invasion, and neovascularization [124, 125]**.** Transient exposure of MCF 10A cells at nanomolar doses of PhIP caused increased cellular proliferation and activated the ERK pathway [126]. During this activation of ERK pathway, a cascade ensues wherein activation of downstream members of the ERK pathway members like Raf, Mek, and Erk which

ultimately generated signals for cell proliferation, cell survival and cellular differentiation and constitutively activated ERK pathway predisposes cells to malignant transformation [127]. Repeated exposures to physiologically achievable levels of PhIP in the range of picomolar to nanomolar concentrations effectively induced progressive carcinogenesis of human breast epithelial MCF10A cells from a non-cancerous stage to premalignant and malignant stages in a concentration and exposure dependent way [121]. Step wise induction of carcinogenesis was accompanied by acquisition of cancer-associated properties of reduced dependence on growth factors, anchorage-independent growth, acinar-conformational disruption, increased cellular proliferation, migration, invasion and carcinogenesis in mouse models. In addition an upregulated H-Ras gene expression, ERK pathway activation with downstream elevation of Nox-1 expression, and elevated reactive oxygen species production was noted [121]. Epicatechin gallate and epigallocatechin gallate, the green tea catechins, were found to suppress PhIP stimulated cellular carcinogenesis and mammary gland neoplasia [121]. Dipyridamole (DPM), at a non-cytotoxic physiologically achievable dose of 10nmol/L, effectively blocked breast cell carcinogenesis induced by cumulative exposures to three different carcinogens including PhIP [128]. DPM could block H-Ras upregulation, and downstream ERK pathway activation, reactive oxygen species (ROS) generation, and DNA damage induced by transient and chronic exposures to PhIP [128]. PhIP at sub nano molar concentrations has been shown to possess estrogenic activity and was shown to promote metastatic potential in mammary cancer cell lines and T47D and MCF 7 cells, as seen by the enhanced ability of the cells to digest and migrate through reconstituted basement membrane model [129].

1.2 Precancerous Model of Cancer Progression

Since breast cancer is an important cause of deaths among women especially in the developed world and more than 85% of breast cancers are sporadic in nature attributable to long-term exposure to small quantities of environmental carcinogenic agents that progressively induce non-cancerous cells of breast to acquire pre-malignancy and gradually to malignancy in a multi-year, multi-step, and multi-path disease process [130, 131, 132, 133, 134]. Hundreds of chemical carcinogens have been identified which have the ability to intensively induce malignancy in mammary cells [135]. While carcinogens are capable of inducing cellular malignancy, co-carcinogens are capable of inducing cell pre-malignancy or enhancing cellular malignancy. Investigations into co-carcinogens, at low-doses (pico- to nano-molar), capable of chronically inducing cellular pre-malignancy have been overlooked in past studies of sporadic breast cancer development and early prevention. In this work I wanted to study whether TCC at physiologically achievable doses can act as a co-carcinogen to induce premalignant state of breast cancer. Also, whether, acting as a co-carcinogen, TCC can potentiate or enhance the carcinogenicity induced by physiologically relevant dosage of PhIP. In previous studies it was demonstrated that NNK, B[a] P, and PhIP at physiologically achievable doses were capable of inducing cellular transformation of human breast epithelial cells to premalignant and malignant stages in breast epithelial cells [42, 121]. So we have developed a cellular model that tests the potency of various chemicals to induce premalignant or malignant carcinogenesis which actually mimics everyday exposure to environmental carcinogens such as daily exposure to tobacco smoke in habitual smokers [42, 136, 137, 138]. In this approach we use immortalized, non-cancerous human breast

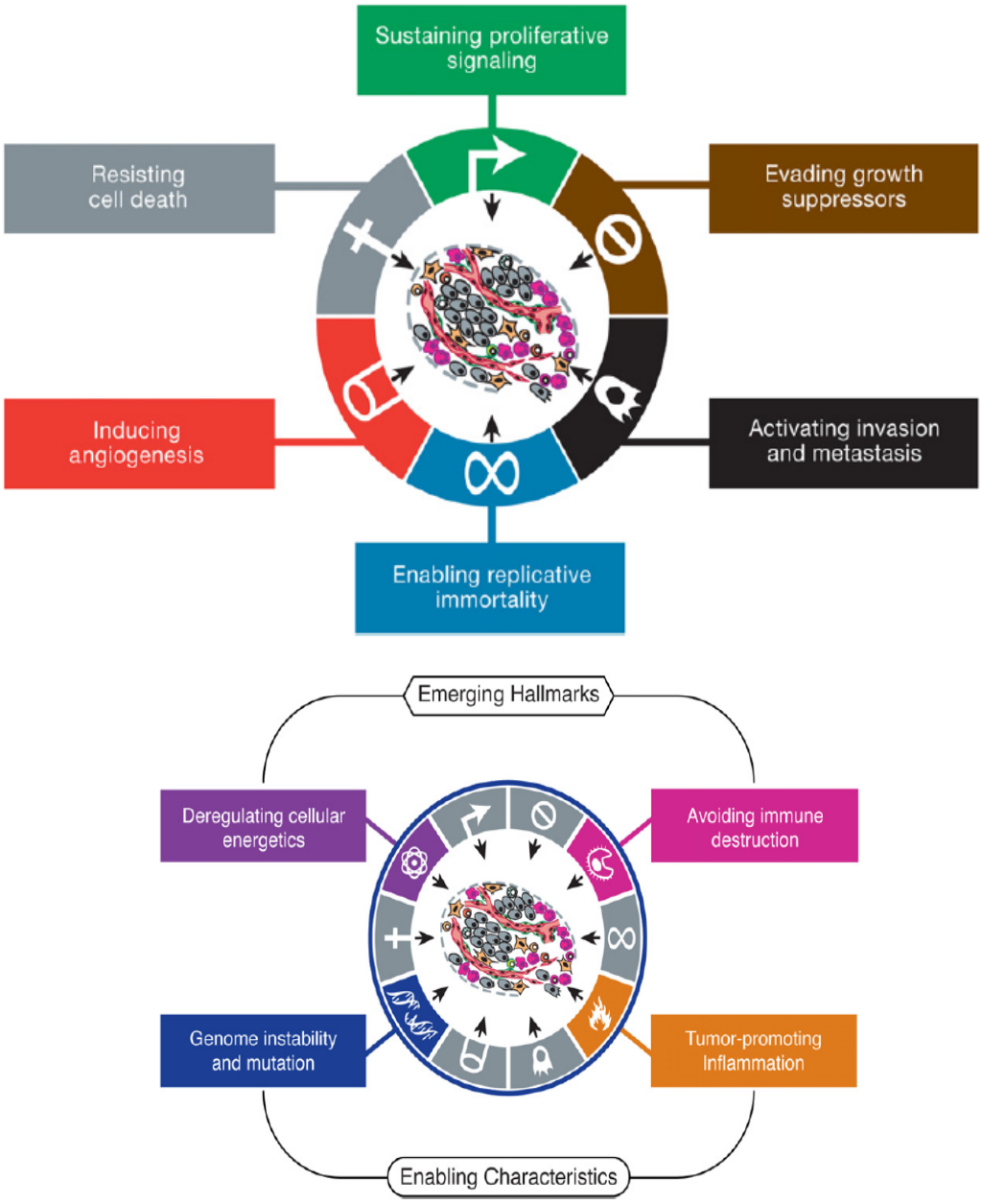
epithelial MCF10A cells [139]. MCF10A cells were repeatedly subjected to treatment with carcinogens at picomolar to nanomolar concentrations. This cumulative exposure to environmental carcinogens induces breast cells to progressively acquire several identifiable cancer-associated properties simulating chronic exposure of breast cells in human body to low doses of an environmental carcinogen in daily life. Using this model we repeatedly exposed immortalized, noncancerous, human breast epithelial MCF10A cells to TCC and PhIP to nanomolar concentrations and measured the acquisition of cancer-associated properties and the underlying molecular mechanisms for induction of cellular carcinogenesis as previously done [42, 121, 140, 141, 142, 143, 144]. Our model system identifies chemicals capable of inducing premalignant or precancerous and malignant or cancerous stages in normal cells. The model is advantageous in analyzing step by step development and progression of carcinogenesis in normal breast epithelial cells MCF 10A. This model subsequently focusses on identifying preventive compounds present in our diet that can inhibit multistep carcinogenesis process induced by repeated exposures to identified co-carcinogens or carcinogens. More importantly, this model emphasizes the relevance of subduing or preventing the process of carcinogenesis at the initial “pre-malignant” stage instead of therapeutically targeting transformed cells. The latter approach can have nonspecific targets and can thus harm the normal body cells too.

1.3 Hallmarks of cancer

The process of neoplasia involves progressive acquisition of certain token characteristics by normal cells to become tumorigenic and these properties are called hallmarks of cancer cell [145, 146]. Cancer cells have the ability to grow autonomously and they defy

normal control mechanisms that are generally put in place to check cell proliferation and growth. Hanahen and Weignberg, in 2000, initially charted out six cancer cell hallmarks (Figure 4). They are as follows: ability to activate signaling pathways responsible for conferring on cells the potential to proliferate abnormally, ability to escape restrictions imposed on the cell division of normal cells by elements called tumor suppressors, ability to invade surrounding tissues and metastasize to distant organs, ability to acquire the potential to replicate with unlimited potential and become immortalized, arrange for blood supply for the growing mass of tumor i.e. angiogenesis and avoiding apoptosis. Subsequently in the year 2011, some more hallmarks were added to the original list which are termed emerging hallmarks and are as follows: ability to alter normal cell metabolism to accommodate continuous cancerous growth and secondly, the ability to evade normal host immune response including destruction by cells of the immune system.

In the present study, assays were used to determine the acquisition of cancer related characteristics by normal cells after their treatment with co-carcinogen and carcinogen.



Modified from: Hallmarks of Cancer: The Next Generation 144(5), 2011, 646–67

Hanahan D and Weinberg RA

Figure 1.4. Hallmarks of cancer

The acquired biological properties that were measured and used as indicators of acquisition of cancerous properties included reduced dependence on growth factors, anchorage-independent growth, ability to proliferate abnormally, capability to induce ROS and activate Erk-Nox cell signaling pathway. The latter two characteristics drive cells towards abnormal proliferation. Lastly, the potential to migrate and invade surrounding tissues was also measured. Cancer cells do not respond to signals that usually regulate cell growth and division. These properties were used as targeted endpoints to determine the capability of preventive agents to suppress cellular carcinogenesis.

1.4 Detection of Cancer Progression

1.4.1 Reduced dependence on growth factors:

The cancer cells by definition divide uncontrollably and their proliferation is not restricted by the amount of growth stimulatory factors present in their surrounding environment. In addition, growth of cancer cells is not dependent on external stimulatory cues i.e. those present in the culture media. The cancer cells proliferate without any checks leading to other cancer cells. So, cancerous cells grow proficiently even in the absence of growth stimulatory signals that normal cells require from their environment. Cancer cells do not need stimulation from external signals (in the form of growth factors) to multiply [145, 147]. Whereas, in the case of normal cells, there is an absolute requirement for the presence of growth stimulatory factors in the culture media or the cell surroundings, for the cells to divide and proliferate. In the absence of growth factors, the

cells die out by the process of apoptosis. So, when the normal cells gain the capability to survive and proliferate under the conditions which lack adequate growth factors, this indicates acquisition of a property associated with cancer cells. Hence reduced dependence on growth factors is a cancer-associated property [42, 121, 140, 141, 142, 143, 144].

1.4.2 Anchorage independent growth

Normal cells require adherence to extracellular matrix for their survival in a multicellular setting. In the absence of contacts or adhesion to extracellular matrix cells fail to survive and undergo apoptosis [145]. Unusually enhanced cell survival which causes anchorage-independent cell proliferation is another cancer associated property that can promote tumorigenic transformation [145, 146]. For instance, primary fibroblastic cell cultures have an absolute requirement for attachment to external surroundings. So cell lines for example, NIH-3T3 cannot grow on agar rose or cell suspension. But when these cells can grow in suspension or on soft agar, they are considered to have acquired the property of anchorage-independence highly suggestive of acquisition of cancerous traits [148, 149, 150]. Anchorage independence gives the ability to cancer cells to form colonies in soft agar which can be used to determine progression of cellular carcinogenesis [42, 121, 140, 141, 142, 143, 144].

1.4.3 Migration and Invasion

Normal breast cells grow over basement and do not invade or escape through the basement membrane. When normal cells become motile and migrate and invade the

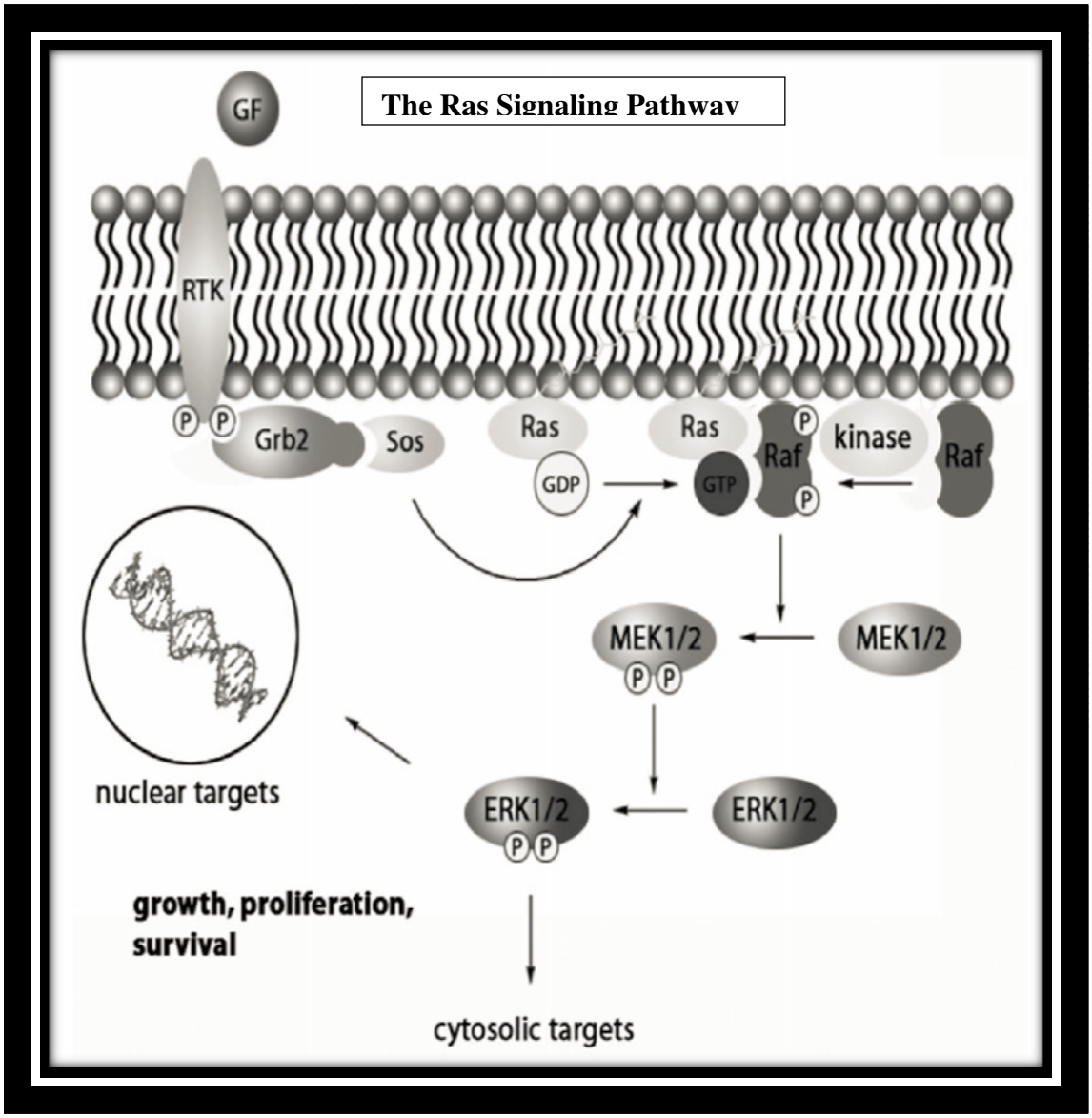
basement followed by invasion into blood stream or lymphatics, they are thought to have acquired cancer related properties. By being motile and invasive cancer cells can escape the site of primary tumor formation and via blood or lymphatics reach distant organs and tissues and colonize there [145, 152].

1.5 Role of ERK Pathway in Cancer

Cellular signal transduction involves conveying messages from extracellular environment to its intracellular environment through an intricate network of proteins which regulate numerous cell processes governing cell fates like cell survival, proliferation, differentiation, migration or apoptosis etc. The cellular signaling mechanism involves ligand like factors such as, growth factors, steroid hormone, cytokines and G protein receptor ligands in the extracellular environment impinging on cell surface proteins called as receptors, and in turn activating them after which ensues a cascade of downstream cytoplasmic protein kinase activation ultimately leading to conveying of messages to the nucleus. These protein kinases involved in this signal transduction are serine threonine kinases and referred to as mitogen activated protein kinases or MAPKs [153]. In mammals, at present 14 MAPKs have been recognized which have been allocated to seven groups [154]. Out of these the most studied and conventional MAPKS include the following: ERK1/2, JNKs, and p38 isoforms [155].

However, the most important and widely studied MAPK in breast cancer is ERK1/2 MAPK pathway [156]. This pathway involves signal transduction via activation of cell surface receptors mostly tyrosine kinase receptors followed by activation of Ras/Raf/MEK/ERK cascade ultimately conveying signals via transcription factors which

regulate expression of a variety of genes [157] (Figure 1.5). Cell surface receptors most commonly involved in breast cancer include tyrosine kinase receptors of the family: epidermal growth factor receptors (EGFR). These include EGFR1, EGFRII, EGFR III and EGFR IV. When appropriate ligand binds to the receptor, dimerization of the receptor occurs and the receptor subsequently undergoes auto phosphorylation on tyrosine residues in its cytoplasmic domains. These sites become homing location for several key adaptor proteins with SH2 or PTB domains e.g. GrB2 which can contact SOS (son of sevenless) with its SH3 domain and activate it. SOS acts as a guanine nucleotide exchange factor and helps Ras to exchange its bound GDP for GTP. Ras which now is able to interact with Raf. Raf is a serine threonine kinase then undergoes conformational change in response to its interaction with Ras and gets activated. Also, it is postulated that other kinases can activate it [158]. It then phosphorylates MEK which is a dual specificity kinase which in turn phosphorylates ERK. ERK upon phosphorylation goes to nucleus where it can phosphorylate various transcription factors like AP1, ETS, ELK1 etc. which drive transcription of genes important for cell proliferation [158]. The Ras GTP is reverted back to its inactive Ras GDP form by the hydrolysis of GTP to GDP by GTPase activating proteins (GAP) [157].



Adapted from *Yap et al., ChemMedChem. 2011 January 3; 6(1): 38–48.Small Molecule Inhibitors of the ERK Signalling Pathway: Towards Novel Anti-cancer Therapeutics*

Figure 1.5. EGFR-Ras-Erk Pathway

The importance of MAPK activation in breast cancer tissues was first demonstrated by Sivaraman *et al*. [159]. In their study they studied MAPK pathway activation in tissues obtained from primary breast cancer patients and compared these with benign breast tissues using western immunoblotting techniques and substrate based MAPK enzyme assays. They found that all tissues from breast cancer patients showed marked activation of MAPK signaling.

Some researchers [160] prepared extracts from 23 human breast cancer tissues and normal surrounding areas from the same tissue. Then using immune-precipitation assay and substrate enzyme assay they found that there was up to 2.5 times greater activity of ERK1 and ERK 2 in 50 percent of the examined breast cancer samples. Also, they showed increased MEK protein expression and activation for the first time in breast cancer tissues. In another study by Muller *et al*., all of the 131 breast cancers examined had significantly higher activation of MAPK protein expression and kinase activity as compared to the 18 normal tissues surrounding the breast cancer tissues analyzed [161]. Even though MAPK protein expression correlated with MAPK activity in only a fraction of the tissues analyzed. Tissues obtained from node positive cancer patients showed higher MAPK activity as compared to tumor tissues from the node negative individuals. Moreover, patients with a replase of the disease had higher MAPK activity as compared with patients with no replase. So, MAPK activation was suggested to be a potential marker for a relapse free survival. Vonlinting *et al.,* demonstrated that in the presence of overexpression of EGFR and HER2 receptors, Ras was found to be overly activated in 11 out of 20 primary breast cancer tissues examined as compared to non-tumor samples indicating that in the presence of ligands and overexpression of their cognate receptors

Ras upregulation may be the driving force for an activated MAPK pathway, hence suggesting a possible mechanism for upregulation of MAPK in breast cancer specimen [162]. In an attempt to clarify the mechanisms behind upregulated MAPK activity and protein expression in tumor tissues, some people [163], showed that in the primary mouse mammary cultures, presence of epidermal growth factor caused activation of MAPK. Hence, current research clearly shows the importance of activated ERK Pathway in breast cancer [156]. Studies have also shown that ERK pathway activation can result in response to an exposure to environmental carcinogens at a high concentration [164]. Recent research has also shown that long term exposure to low dose of environmental carcinogens such as those present in diet, tobacco smoke etc. can induce carcinogenesis in normal breast epithelial cells [42, 121, 141], which is accompanied by upregulation of MAPK pathway. Also, there exists a need to assess the potential hazards of a long term exposure to various chemicals that humans are exposed to in their day today life, on the basis of their ability to cause upregulation of MAPK pathway in normal cells especially those of breast tissues.

1.6 Role of Reactive Oxygen Species in Cancer

Reactive oxygen species are a class of reactive chemicals derived from oxygen and generated during mitochondrial respiration and oxidation-reduction enzymatic reactions in the cytoplasm. Main types of ROS include: superoxide anion; hydrogen peroxide; hydroxyl radical; hydroxyl ion; and nitric oxide [165, 166]. ROS producing compounds have the capability to cause or modulate progressive carcinogenesis induction [167]. As is evident by recent research, reactive oxygen species plays a prominent role in

tumorigenesis in many organs like in case of prostate cancer [168, 169]. Various sources of cellular ROS include mitochondria, peroxisomes and cytochromes. Exogenous sources of ROS generation are exposure to UV radiation, inflammatory processes and infectious agents. ROS thus generated introduces tremendous oxidative stress on the cellular machinery leading to damage to DNA, RNA and lipid components of the cell, protein denaturation, genomic instability and alteration in gene expression profile. Cellular enzyme Nox (NADPH oxidase) is also responsible for generation of ROS. Environmental agents capable of generating ROS include benzopyene, PhIP, phorbol ester, heterocyclic amines like PhIP, chlorinated compounds, inorganic metals like nickel and chromium etc., which can induce oxidative stress and consequent damage to cellular machinery, as has been shown in many studies [167]. Studies have documented that non-genotoxic chemicals may be able to induce carcinogenesis by virtue of their ability to induce oxidative stress. Environmental agents capable of inflicting both acute and chronic oxidative insult on the cell have the potential to cause cancer [170]. Most frequently encountered ROS induced DNA and RNA lesions include 8-hydoxyguanine and 8-hydroxydeoxyguanine [171]. Moreover, 8-hydoxyguanine and 8-hydroxydeoxyguanine have been touted as the most detrimental in forming DNA adducts and mutations [172]. Studies have described formation of more than 100 different types of adducts affecting purine or pyrimidine rings and DNA backbone structures [173, 174, 175, 176]. It is believed that oxidative damage to DNA machinery in man occurs at an alarming rate of 10^4 lesions/ cell/ day [173, 177]. ROS can not only be produced by exposure to high dosage of carcinogens [178], but also, recent studies have shown that low dose chronic exposure to environmental carcinogens, especially at their

physiologically relevant concentrations, can cause significant ROS production leading to DNA damage and breast cellular carcinogenesis [42, 121]. Role of Erk –Nox pathway in generating ROS and beast cell carcinogenesis has been shown in some studies but needs to be further validated. Similarly, there is a need to see if low level of ROS generated in response to exposure to physiologically achievable doses of chemicals, with which we come in contact on a day to day basis, would be able to induce breast cellular carcinogenesis. Furthermore, it needs to be determined whether TCC can induce ROS in breast cells.

1.7 NADPH Oxidase-1

Reactive oxygen species is generated by action of family of enzymes called as NADPH oxidases [nicotinamide adenine dinucleotide phosphate (NADPH) oxidase or Nox family oxidases]. ROS produced by NOX family of enzymes can in turn affect several cellular functions like migration, invasion, proliferation etc. Currently there are seven well known isoforms of NOX family Nox1 to Nox 5 and DUOX 1-2 [179]. A divergent variety of cells in the body are known to express Nox-1 like: epithelial cells, retina, smooth muscle, bone cells, endothelium and others[180, 181, 182, 183, 184, 185]. Over expression of Nox1 was found to cause cell growth, proliferation, cell transformation and tumorigenicity in nude mouse models [186]. Over expression of Nox1 has been reported in cases of colon cancer [187, 188]. Nox -1 levels were found to be elevated in breast cells chronically exposed to dietary carcinogen PhIP [121]. Nox-1 were found to be over expressed in more than 85 percent of breast and 70 percent ovarian tumors and its expression levels were tightly regulated by mitochondria. Moreover, deregulation of

mitochondrial control over nox-1functioning contributed to ovarian and breast tumors. [189].

1.8 Cancer preventive substances in diet

Due to difference in lifestyle, climatic and environmental conditions including diet in different regions of the world, wide variations in incidence of cancer and cancer related deaths are noted across different geographical regions of the world [190]. Dynamic studies and migrant studies done by various authors have compared cancer rates among native and migrant populations in host country and have linked different rates of cancer among these two populations to differences in dietary, environmental and other lifestyle factors [191, 192]. Diet may contribute to cancer related deaths [193]. A large number of studies have studied the link between intake of fruits and vegetable to occurrence of cancers of the various organs, in the body including breast cancer [194, 195]. Naturally occurring agents such as flavonoids, polyphenols, etc. present readily in fruits, vegetables, and spices' owing to their ability to suppress cancers, have been in sharp focus of both general public as well as the scientists world over and, the scientific evidence that supports their suspected anti-cancer activities is being explored in the current ongoing research. Recent research has made it clear that the diet plays a pivotal role in the arena of cancer prevention [196].

Various compounds with anti-tumor activity have been identified in different kinds of fruits and vegetables [197]. Dithiolthiones and isothiocyanates in high quantities are present in cruciferous vegetables and their anti-tumor activity is due to their ability to enhance the activity of enzymes involved in cleansing the metabolically harmful forms of

carcinogens and xenobiotic substances [198]. The diallyl sulfide and allyl methyl trisulfide present in onions and leeks are also involved in inducing enzymatic detoxification machinery [199]. Genistin, an isoflavonoid extracted from soyabeans, could inhibit enzyme tyrosine kinase activity thus impeding signals downstream of cell surface receptors known to be highly upregulated in tumor cells [200]. Compounds with anti-oxidant activity, such as carotenoids and xanthophylls, are reported to be present in leaves of green vegetables [201]. Beta-carotene present in colored fruits and vegetables like mango, carrots pumpkin and papaya, can protect a cell against oxidative damage induced by reactive oxygen species [202]. Polyphenolic anti-oxidant flavonoids like quercetin, which are present in fruits, vegetables and tea, can facilitate efflux of intracellular carcinogens and also activate the cellular enzymatic machinery responsible for detoxifying toxins [203, 204, 205]. Fiber rich foods such as vegetables and fruits have preventive activity against colon cancer [206]. Intake of a healthful diet consisting of large amounts of fruits and vegetables can help thwart cancer development. Polyphenolic compounds present in green tea have shown promising anti-cancer activity [207, 208, 209, 2010]. Green tea has preventive effect against various types of cancer and was shown to be effect in preventing skin cancer in animal models, angiogenesis, tumor growth and its invasion and metastasis to distant organs [211, 212, 213, 214, 215]. Epicatechin gallate (ECG) a flavonoid present in green tea extract can interfere with the process of carcinogenesis in a variety of ways including activation of cellular detoxification machinery, inhibition of enzyme responsible for producing active form of carcinogen like cyp1A1, blocking inflammation and inhibiting metastasis [216, 217, 218, 219, 220]. Epigallocatechin (EGC) also has numerous preventive properties [221, 222].

Epigallocatechin-3-gallate (EGCG), another polyphenolic compound present in green tea, has been shown to possess cancer preventive activity which is attributed to its antioxidant activity [122]. Hence, by counteracting reactive oxygen species produced by a variety of different carcinogens, EGCG was shown to prevent chronic breast cell carcinogenesis [42, 121]. It has been used as a therapeutic agent in various types of cancers like cervical and bladder cancers. Grape seed extract (GPSE) is enriched in proanthocyanidins, which possesses anti-oxidant activities that were shown in both cell culture studies and animal model studies to be responsible for its anti-tumor activities [223, 224, 225, 226]. In addition, GPSE prevented mammary carcinogenesis induced by carcinogen dimethylbenz[a]anthracene (DMBA). Grape seed proanthocyanidins were found to prevent chronic breast cell carcinogenesis induced by NNK ans B[a]P [141].

This study focusses on the significance of naturally occurring compounds including those that can be taken easily through daily diet like curcumin, and others like Mimosine and Ergosterol, in prevention of sporadic breast cancer and more specifically chronic breast cell carcinogenesis.

1.8.1 Curcumin

Indian yellow herb turmeric derived from Curcuma longa, has been commonly used since ancient times in Asian food diets as a yellow coloring powder and spice [227, 228, 229]. Turmeric mainly contains the following three curcuminoids: diferuloylmethane; demethoxycurcumin, and bisdemethoxycurcumin, along with volatile oils (tumerone, atlantone, and zingiberone), sugars, proteins, and resins [230]. Curcumin, is the major phenolic curcuminoid in the extracts made from turmeric and the one which is

responsible for the yellow color of turmeric. Moreover, since ages curcumin has been used in the traditional Indian medicine to treat cancer, rheumatic disorders, inflammation, gastrointestinal and ocular diseases [229, 231, 230]. Curcumin has been shown to inhibit proliferation of triple negative MDA-MB-231 breast cancer cells by inhibiting EGFR signaling pathway with down regulation of pERK1/2 and pEGFR protein expression [232]. MDA-MB-231 cells showed slower proliferation rates after treatment with curcumin. In addition, curcumin treatment also caused these cells to undergo cell cycle arrest at the G2/M stage and subsequently apoptosis. Also, cell cycle inhibitor, p21, was found to be over expressed along with an elevation of bax to bcl2 ratio in these cells [233]. It has been shown that curcumin is able to inhibit growth and metastasis of breast cancer cells *in vitro* [234, 235] and *in vivo* [235]. Curcumin was able to inhibit the in vitro migration in MDA-MB-231 cells through down-regulating the protein expression of NF-kappaB [234]. Curcumin has been shown to limit the metastatic potential of triple negative breast cancer cell lines [233]. In clinical trials, curcumin treatment is being applied to a number of patients with breast cancer, rheumatoid arthritis, Alzheimer's disease, colorectal cancer, psoriatic, etc. Several of these clinical trials have reported the curcumin treatment to be safe and effective [230, 236, 237]. Curcumin can target and affect expression of a variety of proteins including those involved in cell survival, proliferation, migration, invasion, and angiogenesis. Thus, there exists an extensive evidence substantiating curcumin’s capability as an anticancer agent in a wide variety of tumors and, it has been shown to inhibit the proliferation of tumor cells in vitro, can suppress carcinogenesis in rodents, and has been shown to decrease growth of human tumors in both orthotopic and xenograft tumor models [237]. Curcumin has been shown

to suppress mutagenic effects induced by a wide variety of carcinogens [238, 239, 240]. Curcumin was shown to inhibit benzo[a]pyerene-induced tumorigenesis in fore stomach in mouse models [238, 241], genotoxicity induced by cooked food heterocyclic amines in *Salmonella typhimurium* strains [240], and bio activation of benzo[a]pyerene diol in oral squamous carcinoma cells [242]. However, the activity of curcumin in prevention of sporadic breast cancer has not been investigated. Moreover, despite its efficacy and potential as a therapeutic anticancer agent, it is yet to be approved as a therapeutic agent because of its poor bioavailability [243, 244, 245]. But the use of various adjuvants like piperine which inhibits hepatic and intestinal glucuridation machinery, have been shown to dramatically improve bioavailablity of curcumin [245, 246]. Similarly, nanoparticle based delivery systems [247] and liposomal drug delivery subsystem has been shown to be promising agents in improving bioavailability of curcumin. Liposomal curcumin was shown to inhibit pancreatic carcinoma growth and angiogenesis in *in vivo* tumor models [248]. Curcumin structural analogues have also been developed to enhance curcumin bioavailability. Curcumin analogues which have no β-diketone moiety such as mono-carbonyl analogs could enhance its bioavailability and stability [249].

1.8.2 Ergosterol

Ergosterol forms a major constituent of fungal cell membrane. Its structure is shown in Figure 1.6. It plays a hormone like role in fungal cells and stimulates growth of fungus. The antitumor activity of ergosterol might be due to direct inhibition of angiogenesis induced by solid tumors [250]. Ergosterol extracted from Japanese edible mushroom *Hypsizigus marmoreus* showed inhibitory activity against 12-O-tetradecanoylphorbol-13-

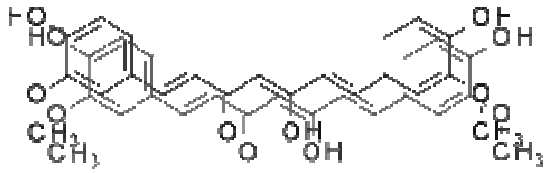
acetate (TPA) induced ear inflammation and tumor promotion in mice in a two stage experimental carcinogenesis study and hence could be used as a potential anticancer agent [251]. Ergosterol Peroxide (EP) is found in lichens and mushrooms and it possesses immunosuppressive anti-viral, anti-inflammatory and anti-tumor activities [252, 253, 254, 255, 256]. Ergosterol peroxide present in oil rich fraction isolated from *Ganoderma lucidum* showed promising results in overcoming the drug-resistance of tumor cells attributed to the expression of miR-378 [257]. Ergosterol peroxide exhibits anticancer activity in multiple myeloma U266 cells. It was shown to be an anti-cancer agent which targets JAK2/STAT3 signaling pathway and inhibits angiogenesis against multiple myeloma cells. However, there is paucity of knowledge regarding the ability of ergosterol to act as a preventive agent in cases of breast cancer.

1.8.3 Mimosine

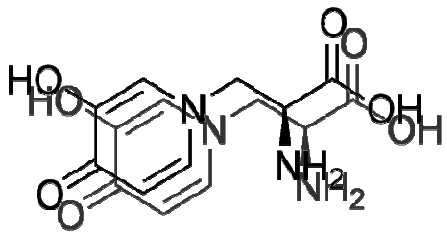
Mimosine or leucenol is an alkaloid, β-3-hydroxy-4 pyridone amino acid which is chemically very similar to tyrosine. Its structure is shown in Figure 1.6. It was initially isolated from *Mimosa pudica* and is found to occur in some members of genus *Mimosa* and all members of the related genus *Leucaena* [258].

It is a non-protein, plant origin free amino acid that is toxic to non-ruminants and un-adapted ruminants. Mimosine is an iron chelator and that inhibits DNA synthesis and mammalian cell proliferation by blocking cell cycle at the late G_1 phase [259, 260, 261, 262]. It has been shown to inhibit cell proliferation in prostate carcinoma cells [263]. Mimosine was specifically shown to inhibit cyclin D1 expression [264], and upregulate

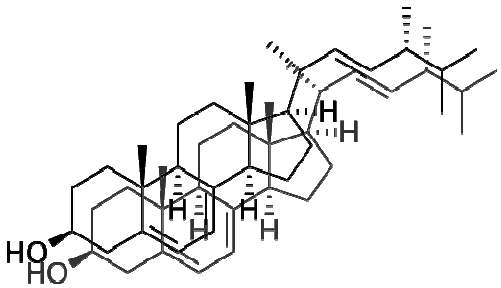
the protein expression levels of p27 [265]. In vivo studies have also demonstrated that it has an anticancer effect on human lung cancer xenografts [266]. Studies have been conducted that gave an evidence of the ability of mimosine to suppress the growth of human pancreatic cancer xenografts that were subcutaneously transplanted in nude mice and a flow cytometric analysis of the tumor cells yielded a significantly increased sub-G1 fraction indicating occurrence of apoptosis [267]. Since its role in inhibiting cancer of breast cells has not been studied, this study was done to determine if Mimosine could inhibit breast cellular carcinogenesis.



Curcumin



Mimosine



Ergosterol

Figure 1.6 Chemical structures of preventive agents

1.9 Hypothesis and Objective

I have already discussed that more than 85% of breast cancers are sporadic and attributable to long-term exposure to small quantities of environmental carcinogenic agents that progressively induce non-cancerous cells to pre-malignancy to malignancy in a multi-year, multi-step, and multi-path disease process. Carcinogens are capable of inducing cellular malignancy, and co-carcinogens are capable of inducing cell pre-malignancy or enhancing cellular malignancy. Very few studies have been done to investigate the potential of co-carcinogens to induce the state of cellular pre-malignancy or enhance carcinogenicity of other known carcinogens. There are a plethora of chemicals that humans are exposed to daily, however, the effects of their exposure on human health are largely unknown. Many such chemicals possess the hitherto unknown capacity to act as mitogens, induce cellular damage and capability to act as co-carcinogens in initiation and progression of various kinds of cancers including breast cancer. Hence, there exists a need to characterize such chemicals on the basis of their potency to induce premalignant or malignant breast cell carcinogenesis. Moreover, since exposure to some of these potential carcinogens cannot be avoided, there is an imminent need to search for compounds occurring in nature, which have the preventive activity to thwart the process of carcinogenesis initiated by exposure to carcinogens or co-carcinogens. Furthermore, early prevention of breast cancer obviates the need for therapeutics and its subsequent complications. Triclocarban is a chemical to which body is exposed via a number of ways everyday *viz.* through shower, hand washes, antiseptics, deodorants etc. Though, recently there has been a lot of focused research to determine the effects of TCC on human health, yet the potential of TCC to act as a co-carcinogen

has not been addressed so far. Cooked meat carcinogen PhIP has been shown to cause breast carcinogenesis. But whether certain co-carcinogens can enhance the carcinogenicity of PhIP when body is exposed to them simultaneously, this has not been verified till date. So, the cumulative impact of a bodily co-exposure of PhIP and TCC through different unavoidable sources on breast cells has not been addressed before. Also, there is a relative lack of work seeking to uncover the potential of naturally occurring preventive agents that can counteract the role of co-carcinogens and carcinogens and block progression of premalignant breast cell carcinogenesis. Hence to address the above needs this study was undertaken with the following central hypothesis: Long-term exposure to TCC can cause breast cell carcinogenesis and co-exposure to TCC and PhIP can enhance PhIP-induced breast cell carcinogenesis

Then working towards the central hypothesis the study was divided into four broad objectives:

- To determine if chronic exposure to physiologically achievable doses of TCC can cause breast epithelial cells to acquire the cancer-associated properties.
- To study potential of curcumin in intervention of TCC-induced breast cell carcinogenesis.
- To determine if TCC can enhance PhIP-induced breast cell carcinogenesis.
- To determine whether mimosine and ergosterol can block breast cell carcinogenesis induced by co-treatment of TCC and PhIP.

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TABLE 1: MICOARRAY CLASSIFICATION OF BREAST CANCER (MODIFIED FROM Krishnamurthy *et al*., 2012)

Subtypes	ER/PR /Her2 Status	Other Immuno-Histochemical Features	Cell Of Origin	Other Characters
Luminal A	ER or PR +ve or both Her2 -ve	keratin 8/18 +ve	luminal epithelial cell	Best prognosis Higher survival rate
Luminal B	ER or PR +ve or both, Her2 +ve	keratin 8/18 +ve	luminal epithelial cell	Higher tumor grade, Poorer prognosis
Basal Like	ER/PR –ve, Her2 +ve/-ve	keratin 5/6/17 +ve EGFR +ve	Basal/myoepithelial/ bipotentl progenitor	15%
Her 2+	ER/PR –ve, Her2 +ve	-	Late luminal progenitor	20-25%, Poorer grade and prognosis
Normal Breast Like	Tumors that don't fill any of the other categories	-	Luminal epithelial cell	6-10%, associated with fibroadenomas, Good prognosis
Claudin Low	All –ve	mesenchymal markers	Stem cells	Typically triple –ve 5-10% of all tumors

ER: Estrogen resceptor, PR: Progestron receptor, Her2; Human epidermal growth factor receptor2

Table 2:List of risk factors	
Hereditary and non-modifiable risk factors	**Environmental and modifiable risk factors**
Age	**Physical inactivity**
Gender	**Alcohol**
Genetic factors (BRCA1 and BRCA2 gene mutation)	**Smoking**
Race	**Unhealthy diet**
Menstrual and reproductive factors	**Exposure to ionizing radiation**
Proliferative breast conditions	**Hormonal replacement therapy**

Chapter 2

Induction of Human Breast Cell Carcinogenesis by Triclocarban

Research described in this chapter is slightly modified version of an article that has been submitted to Molecular Carcinogenesis in April, 2013

Induction of Human Breast Cell Carcinogenesis by Triclocarban by

Shilpa Sood, Shambhunath Choudhary, and Hwa-Chain Robert Wang

In this paper “our” and “we” refers to me and co-authors. My contribution in the paper includes the following:

(1) Selection of the topic (2) Compiling and interpretation of the literature (3) Designing experiments (4) understanding the literature and interpretation of the results (5) providing comprehensive structure to the paper (6) Preparation of the graphs and figures (7) Writing and editing

2.1 Abstract

More than 85% of breast cancers are sporadic and attributable to long-term exposure to environmental carcinogens and co-carcinogens. To identify co-carcinogens with abilities to induce cellular pre-malignancy, we studied the activity of triclocarban (TCC), an antimicrobial agent commonly used in household and personal care products. Here, we demonstrated, for the first time, that chronic exposure to TCC at physiologically-achievable nanomolar concentrations resulted in progressive carcinogenesis of human breast cells from non-cancerous to pre-malignant. Pre-malignant carcinogenesis was measured by increasingly-acquired cancer-associated properties of reduced dependence on growth factors, anchorage-independent growth and increased cell proliferation, without acquisition of cellular tumorigenicity. Long-term TCC exposure also induced constitutive activation of the Erk-Nox pathway and increases of reactive oxygen species (ROS) in cells. A single TCC exposure induced transient induction of the Erk-Nox pathway, ROS elevation, increased cell proliferation, and DNA damage in not only non-cancerous breast cells but also breast and bladder cancer cells. Using these constitutively- and transiently-induced changes as endpoints, we revealed that non-cytotoxic curcumin, a component of turmeric extracted from the Indian herb *Curcuma longa*, was effective in intervention of TCC-induced cellular pre-malignancy. Our results lead us to suggest that the co-carcinogenic potential of TCC should be seriously considered in epidemiological studies to reveal the significance of TCC in the development of sporadic breast cancer. Using TCC-induced transient and constitutive endpoints as targets will likely help identify non-cytotoxic preventive agents, such as curcumin, effective in suppressing TCC-induced cellular pre-malignancy.

2.2 Introduction

Breast cancer is the most common cancer and the second leading cause of cancer deaths among women in northern America and Europe [1]. More than 85% of breast cancers are sporadic and attributable to long-term exposure to small quantities of environmental carcinogenic agents that progressively induce non-cancerous cells to pre-malignancy to malignancy in a multi-year, multi-step, and multi-path disease process [2–5]. Over 200 chemical mammary carcinogens have been identified by measuring their ability to intensively induce malignant cells or tissues [6]. While carcinogens are capable of inducing cellular malignancy, co-carcinogens are capable of inducing cell pre-malignancy or enhancing cellular malignancy. Investigations into co-carcinogens, at low-doses (pico- to nano-molar), capable of chronically inducing cellular pre-malignancy have been overlooked in past studies of sporadic breast cancer development and early prevention.

One potential co-carcinogen, triclocarban (TCC, or 3,4,4'-trichlorocarbanilide) is a lipophilic, antimicrobial compound widely used in household and personal care products, such as disinfectants, soaps, deodorants, detergents, and wipes, etc [7]. TCC exhibits endocrine-disrupting activity to induce estradiol-dependent activation of estrogen receptor (ER)-responsive gene expression but alone lacks agonistic activity [8,9]. Direct dermal exposure to TCC results in transdermal absorption and accumulation in underlying tissues, including mammary tissues [10]. More specifically, a single whole-body shower using TCC-containing soap results in blood levels as high as ~500 nmol/L within 3 h [11,12]. In addition, TCC is resistant to both wastewater chemical and biological treatments, and agricultural use of TCC-containing biosolids renders TCC into

the food chain, thus increasing human exposure to TCC [7,13–15]. Although the role of TCC in increasing breast cancer risk has been questioned, it has not yet been addressed. It is imperative to clarify the role of low-dose TCC in sporadic breast cancer development and to identify effective agents for intervention.

One possible hindrance for such sporadic breast cancer development is curcumin, the major phenolic compound in turmeric extracts. Curcumin has been used in Asian countries for hundred years to treat various diseases, including gastrointestinal, retinal, and rheumatic disorders, as well as inflammation and cancer [16–20]. It has been shown that curcumin is able to inhibit growth and metastasis of breast cancer cells both *in vitro* and *in vivo* [21–23]. Curcumin has also been shown to inhibit stomach tumors induced by benzo[a]pyerene (B[a]P) in mice [24,25], genotoxicity induced by heterocyclic amines in *Salmonella typhimurium* [26], and bioactivation of B[a]P-diol in oral squamous carcinoma cells [27]. However, the activity of curcumin in prevention of sporadic breast cancer has not been addressed.

We have developed a cellular model to identify carcinogenic agents, at physiologically-achievable levels, capable of chronically inducing human breast epithelial cell carcinogenesis [28–33]. We revealed that and cellular, biochemical, and molecular changes transiently induced by single exposure to carcinogenic agents, and changes constitutively induced by cumulative exposures play essential roles in induction of cellular carcinogenesis and maintenance of acquired cancer-associated properties, respectively [28–33]. We then used these transient and constitutive changes/endpoints as targets to identify non-cytotoxic agents, such as green tea catechins and grape seed extract, effective in intervention of cellular carcinogenesis [29–36]. In this

communication, we used our model system, for the first time, to reveal the ability of TCC, at nanomolar levels, to induce breast cell pre-malignancy. We also studied the activity of curcumin, at non-cytotoxic levels, effective in intervention of TCC-induced cellular carcinogenesis.

2.3 Materials and methods

2.3.1 Cell Cultures and Reagents

MCF10A (American Type Culture Collection [ATCC], Rockville, MD) and derived cell lines were maintained in complete (CM) medium (1:1 mixture of DMEM and HAM's F12, supplemented with 100 ng/mL cholera enterotoxin, 10 µg/mL insulin, 0.5 µg/mL hydrocortisol, 20 ng/mL epidermal growth factor, and 5% horse serum) [28–36]. Human breast cancer MCF7 and urinary bladder carcinoma J82 cells (ATCC) were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum [33]. All cultures were maintained in medium supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin in 5% CO_2 at 37°C. Stock aqueous solutions of TCC (Sigma-Aldrich, St. Louis, MO), chloromethyl-dichlorodihydrofluorescein-diacetate (CM-H_2DCF-DA) (Invitrogen, Carlsbad, CA), U0126 (Cell Signaling, Beverly, MA), and curcumin (MP Biomedicals, Solon, Ohio) were prepared in DMSO and diluted in culture medium. Stock aqueous solutions of *N*-acetyl-*L*-cysteine (NAC) (Alexis, San Diego, CA) were prepared in H_2O and diluted in culture medium for assays.

2.3.2 Chronic Induction of Cellular Carcinogenesis

Twenty-four h after each subculturing, MCF10A cells were exposed to TCC for 48 h, as one cycle of exposure for 10 to 20 cycles; cultures were subcultured every 3 d [28–33]. After exposures to TCC, cells were assayed to detect acquired cancer-associated properties.

2.3.3 Reduced dependence on growth factors

Five × 10^3 cells were seeded in 60-mm culture dishes and maintained in low-mitogen (LM) medium containing reduced total serum and mitogenic additives to 2% of the concentration formulated in CM medium. Cell colonies (≥ 0.5 mm diameter) grown in LM medium were counted microscopically [29–33].

2.3.4 Anchorage-independent Growth

Five x 10^3 cells were mixed with soft-agar consisting of 0.4% SeaPlaque agarose (Sigma-Aldrich) in a mixture (1:1) of CM medium with 3 d conditioned medium prepared from MCF10A cultures, plated on top of the 2% SeaPlaque agarose base layer in a 60-mm culture dish, and maintained for 20 d to develop cell clones. Cell colonies (≥ 0.1 mm diameter) grown in soft-agar were counted microscopically [29–33].

2.3.5 Cell Viability Assay

A Methyl Thiazolyl Tetrazolium (MTT) assay kit (ATCC) was used to measure cell survivability in cultures. Five x 10^3 cells were seeded into each well of 96-well culture plates for 24 h. After indicated treatments, cells were incubated with MTT Reagent for 4 h, followed by incubation with detergent reagent for 24 h. Reduced MTT reagent in cultures was quantified with an ELISA reader (Bio-Tek, Winooski, VT) [37,38].

2.3.6 Cell Proliferation

Cell proliferation was determined by of 5-bromo-2'-deoxyuridine (BrdU) incorporation into cellular DNA using the BrdU cell proliferation ELISA kit (Roche, Indianapolis, IN). Five x 10^3 cells were seeded into each well of 96-well culture plates for 24 h. After treatment, cells were labeled with BrdU for 12 h, fixed, incubated with peroxidase-conjugated BrdU-specific antibodies, and stained with peroxidase substrate. Quantification of BrdU-labeled cells was determined with an ELISA reader (Bio-Tek) [37–39].

2.3.7 Intracellular ROS

As performed previously [32–36,40], cells were incubated with 5 µmol/L CM-H_2DCF-DA for 1 h to detect ROS level by flow cytometry; the mean fluorescence intensity of DCF was quantified using Multicycle software (Phoenix).

2.3.8 DNA Damage

DNA damage was measured by a comet assay [33,41]. Cells were mixed with 1% low-melting agarose and placed on agarose-coated slides. Slides were then immersed in lysis solution (1.2 mol/L NaCl, 100 mmol/L Na_2EDTA, 1% Triton X-100, and 0.3 nmol/L NaOH, pH 13) at 25°C for 1 h and rinsed three times with alkaline buffer (2 mmol/L Na_2EDTA and 300 mmol/L NaOH) for 20 min each. After electrophoresis in the same alkaline buffer at 20V for 30 min, slides were stained with 2.5 µg/mL of propidium iodide for 20 min and examined with a Zeiss fluorescence microscope (Thornwood, NY) equipped with an excitation filter of 546 nm and barrier filter of 590 nm. Fifty nuclei per

slide were scored for tail moment (% of DNA in the tail × tail length) using CometScore software (Tritek, USA).

2.3.9 Western Immunoblotting

Equal amounts of cellular proteins were resolved by electrophoresis in 10% SDS-polyacrylamide gels and transferred to nitrocellulose filters for immunoblotting [42–44]. Specific antibodies were used to detect H-Ras, phosphorylated Mek (p-Mek), Mek, phosphorylated Erk1/2 (p-Erk1/2), Erk1/2, Nox-1, and β-Actin (Santa Cruz Biotechnology, Santa Cruz, CA). Antigen-antibody complexes on filters were detected by the Supersignal chemiluminescence kit (Pierce, Rockford, IL).

2.3.10 Statistical Analysis

The Student *t* test was used to analyze statistical significance, indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; a P value of ≤ 0.05 was considered significant.

2.4 Results & Discussion

2.4.1 Induction of Breast Cell Carcinogenesis by TCC

To investigate the ability of TCC to induce breast cell carcinogenesis, we repeatedly exposed MCF10A cells to TCC at various concentrations for 10 and 20 cycles. Normal epithelial cells require growth factors to grow and survive, and cell adhesion to extracellular matrix is essential for survival in a multi-cellular environment; in contrast, cancerous cells acquire the abilities of a reduced dependence on growth factors and an

independence from cell adhesion to matrix (anchorage-independent growth) to survive [45,46]. We used these two cancer-associated properties as constitutive endpoints to measure the progress of cellular carcinogenesis induced by cumulative exposures to various carcinogenic agents [32,33]. As shown in Figure 2.1A and 2.1B, higher numbers of survived colonies, which acquired both cancer-associated properties, remained in cultures exposed to TCC at higher concentrations than in cultures exposed to lower concentrations (1000 > 500 > 200 nmol/L), and higher numbers of colonies survived in cultures exposed to TCC for 20 cycles than in cultures exposed for 10 cycles. These results indicate that cumulative exposures of MCF10A cells to TCC resulted in progressively-increased acquisition of these two cancer-associated properties/constitutive endpoints in a dose- and exposure-dependent manner.

Our previous studies [32,33] revealed that the Erk-Nox pathway, as a constitutive biochemical endpoint, is involved in maintaining acquired cancer-associated properties of pre-malignant and malignant breast cells induced by chronic exposure to carcinogenic agents, including B[a]P, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). Investigating signaling pathways involved in TCC-induced cellular carcinogenesis, we detected that As shown in Figure 1C, chronic exposure to TCC also resulted in constitutive activation of the Erk-Nox pathway, indexed by increased phosphorylation of Erk1/2 and increased level of Nox-1 (Figure 2.1C). The level of induced Erk-Nox pathway (Figure 2.1C) was in concert with the degree of acquired cancer-associated properties (2.1A & B).

Legend for Figure 2.1. Dose- and exposure-dependent induction of carcinogenesis by TCC. MCF10A cells were repeatedly exposed to DMSO (0) or 200, 500, or 1000 nmol/L (nM) TCC for 10 and 20 cycles. (A) To determine acquisition of the cancer-associated property of reduced dependence on growth factors (RDGF), cells were maintained in LM medium. (B) To determine acquisition of the cancer-associated property of anchorage-independent growth (AIG), cells were seeded in soft-agar. Cell colonies were counted microscopically. *Columns*, mean of triplicates; *bars*, SD. Statistical significance is indicated by ** $P < 0.01$, *** $P < 0.001$. (C) Cell lysates isolated from cultures exposed to TCC for 20 cycles were analyzed by immunoblotting using specific antibodies to detect levels of phosphorylated Erk1/2 (p-Erk1/2), Erk1/2, and Nox-1, with β-Actin as a control; these levels were quantified by densitometry. The level of specific phosphorylation of Erk1/2 (p-Erk/Erk) was calculated by normalizing the level of p-Erk1/2 with the level of Erk1/2, the level set in control cells (0 nM TCC) as 1 (X, arbitrary unit). The level of Nox-1 (Nox/Actin) was calculated by normalizing with the level of β-Actin, the level set in control cells as 1 (X, arbitrary unit). All results are representative of three independent experiments.

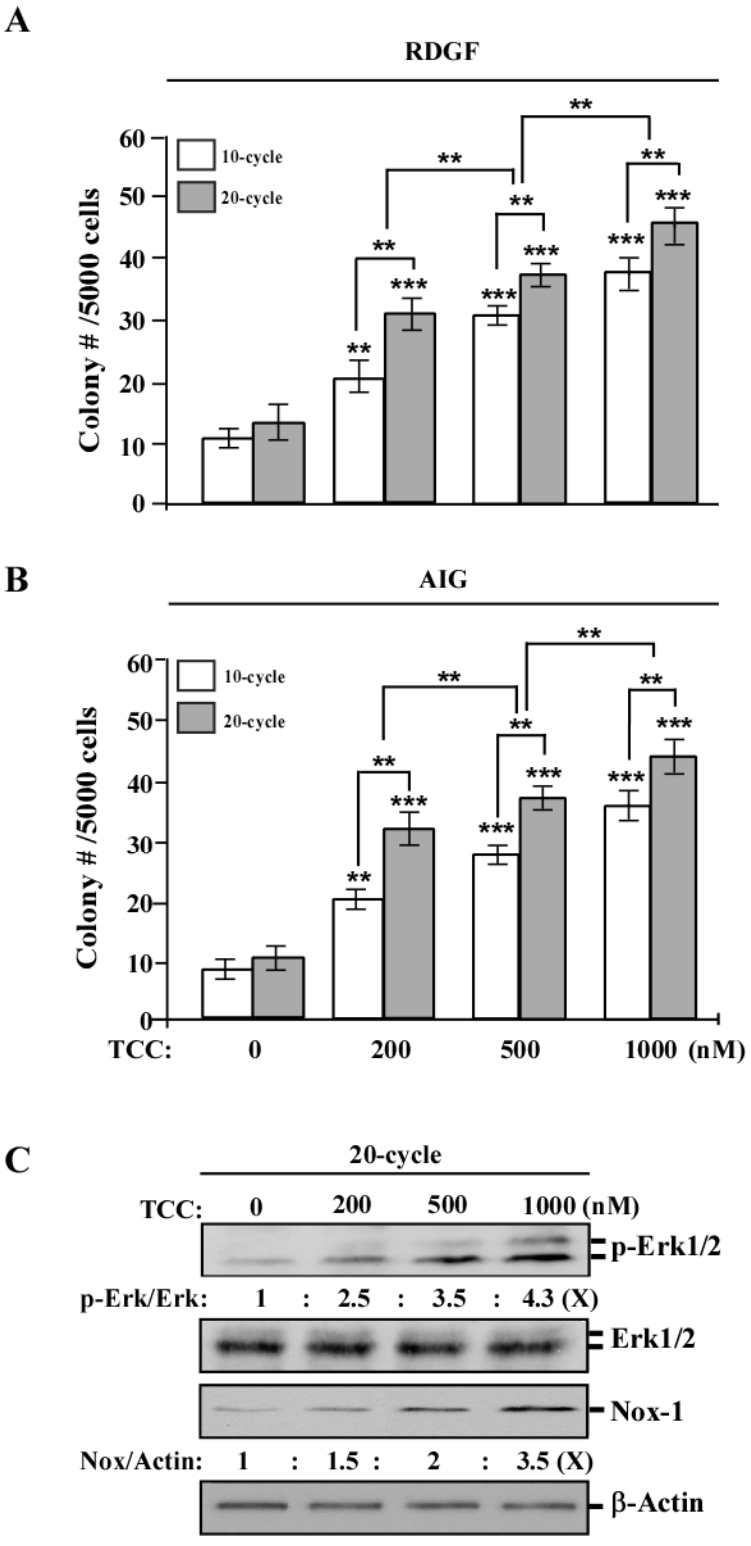

Figure 2.1. Dose- and exposure-dependent induction of carcinogenesis by TCC

These results are consistent with our published findings of constitutive endpoints induced by B[a]P, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), and/or 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in breast cell carcinogenesis [32,33]; thus, the cancer-associated properties of reduced dependence on growth factors and anchorage-independent growth can also be used as constitutive cellular endpoints in the measurement of TCC's ability to induce cellular carcinogenesis, and the Erk-Nox pathway can be used as a constitutive biochemical endpoint to detect TCC-induced cellular carcinogenesis.

2.4.2 Transient Endpoints Induced by TCC

Our previous studies revealed that a single exposure of MCF10A cells to B[a]P, NNK, or PhIP induces transient endpoints, including Erk-Nox pathway activation, ROS elevation, increased cell proliferation, and DNA damage, which play essential roles in initiation of carcinogenesis in each exposure, accounting for the mechanisms of these agents in chronic induction of carcinogenesis [32,33]. To detect transient endpoints induced by TCC, we exposed MCF10A cells to 200 nmol/L TCC for 48 h. Because studies showed that a single whole-body shower using TCC-containing soap frequently results in blood levels at ~200 nmol/L [11,12], we chose to use the physiologically-achievable level of 200 nmol/L TCC to study its activity and mechanisms in induction of cellular carcinogenesis.

As shown in Figure 2.2A, phosphorylation of Erk1/2 was transiently induced, and the maximal level of phosphorylated Erk1/2 was reached in 24 h, indicating a transient activation of the Erk pathway by TCC.

Legend for Figure 2.2. Transient endpoints induced by TCC. (A) MCF10A (10A) cells were treated with 200 nmol/L TCC (T) for the indicated periods. (B1 to B5) 10A cells were exposed to TCC in the absence or presence of 10 μmol/L U0126 (U) or 5 mmol/L NAC (N) for 24 h. (C1 to C3) MCF7 and J82 cells were treated with 200 nmol/L TCC for 24 h. (A, B1, B2, & C1) Cell lysates were prepared and analyzed by immunoblotting to detect levels of p-Erk1/2, Erk1/2, and Nox-1, with β-Actin as a control, and these levels were quantified by densitometry. The level of specific phosphorylation of Erk1/2 (p-Erk/Erk) was calculated by normalizing the level of p-Erk1/2 with the level of Erk1/2, then the level set in control cells as 1 (X, arbitrary unit). The level of Nox-1 (Nox/Actin) was calculated by normalizing with the level of β-Actin and the level set in control cells as 1 (X, arbitrary unit). (B3 & C2) Relative ROS levels were measured with CM-H_2DCF-DA labeling and normalized by the fluorescence intensity determined in control cells, set as 1 (X, arbitrary unit). (B4) Relative cell proliferation was determined and normalized by the value of BrdU detected in control cells, set as 100%. (B5 & C3) Relative DNA damage was measured by a comet assay and normalized by the value of average tail moment determined in control cells, set as 1 (X, arbitrary unit). Representative images of DNA damage in the comet assay are shown in B5. *Columns*, mean of triplicates; *bars*, SD. Statistical significance is indicated by * $P < 0.05$, ** $P < 0.01$. All results are representative of three independent experiments.

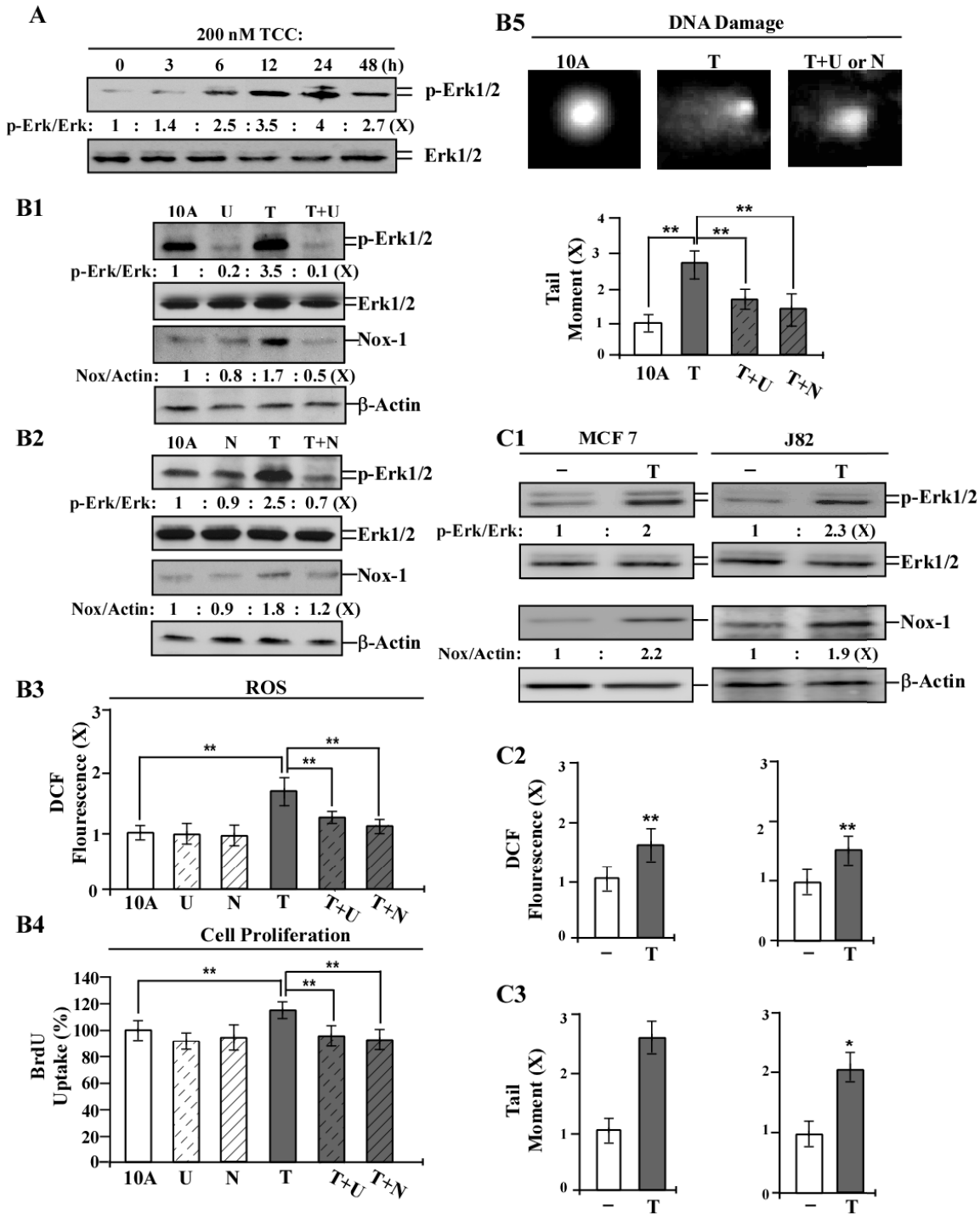

Figure 2.2 Transient endpoints induced by TCC

Using the Mek inhibitor U0126 to block the Erk pathway, Nox-1 was reduced to a lower level than in parental cells and TCC-exposed cells (Figure 2.2B1), indicating that TCC induced Nox-1 through activation of the Erk pathway, thereby verifying the ability of TCC to induce transient activation of the Erk-Nox pathway. Using NAC to block ROS, we detected that the Erk-Nox pathway was suppressed in TCC-exposed cells (Figure 2.2B2), indicating ROS was induced by TCC in activation of the Erk-Nox pathway. Additionally, blocking the Erk-Nox pathway by U0126 resulted in significant reduction of TCC-induced ROS (Figure 2B3), suppression of ROS by NAC or U0126 resulted in significant reduction of TCC-induced cell proliferation (Figure 2.2B4), and blockage of the Erk-Nox pathway or ROS resulted in suppression of TCC-induced DNA damage (Figure 2.2B5). These results indicate that TCC was able to induce the Erk-Nox pathway and Nox-dependent ROS elevation, as well as Nox-independent ROS elevation to induce increased cell proliferation and DNA damage. TCC-induced, Nox-independent ROS also played a role in activation of the Erk-Nox pathway.

To address if TCC's ability to induce these transient endpoints was limited to the ER-negative MCF10A cells, we treated human breast cancer ER-positive MCF7 cells and urinary bladder cancer J82 cells with TCC. We detected that TCC was able to induce the Erk-Nox pathway (Figure 2C1), ROS elevation (2C2), and DNA damage (2C3) in both types of human cancer cells. The results indicate that TCC-induced transient endpoints were not specific to breast cells nor to ER status.

2.4.3 Constitutive Endpoints Induced by TCC

To assess carcinogenic potency of TCC-exposed breast cells, we used the tumorigenic P-20 cell line, which resulted from cumulative exposure of MCF10A cells to PhIP for 20 cycles [33], and a highly-tumorigenic, oncogenic Ras-expressing MCF10A-Ras cell line [33,44] as controls. As performed previously, cumulative exposures of MCF10A cells to 200 nmol/L TCC for 10 and 20 cycles resulted in T-10 and T-20 cell lines, respectively. Then, we measured degrees of constitutive endpoints acquired by T-10 and T-20 cells in comparison with P-20 and MCF10A-Ras cells. As shown in Figure 3, the constitutive endpoints of reduced dependence on growth factors (2.3A), anchorage-independent growth (2.3B), increased cell proliferation (3C), ROS elevation (2.3D), and Mek-Erk-Nox pathway activation (3E) were increasingly induced in T-10 and T-20 cells in an exposure-dependent manner; however, none of these constitutive endpoints was induced to a comparable level to those acquired by tumorigenic P-20 and MCF10A-Ras cells. Inoculation of T-20 cells into mammary fat pads of immunocompromised nu/nu mice did not result in any xenograft tumors in 90 days in contrast to xenograft tumors derived from P-20 and MCF10A-Ras cells, indicating that the T-20 cell line was not tumorigenic (data not shown). These results indicate that long-term exposure to TCC was able to induce cellular pre-malignancy.

Legend for Figure 2.3. Constitutive endpoints induced by exposure to TCC. MCF10A (10A) cells were repeatedly exposed to 200 nmol/L TCC for 10 and 20 cycles, resulting in T-10 and T-20 cell lines, respectively. 10A cells were repeated exposed to 10 nM PhIP for 20 cycles to generate P-20 cells. 10A cells were stably transfected to ectopically express oncogenic H-Ras, resulting in the MCF10A-Ras cell line (Ras). (A) Cells were maintained in LM medium to determine acquired levels of reduced dependence on growth factors (RDGF). (B) Cells were seeded in soft-agar to determine acquired levels of anchorage-independent growth (AIG). (C) Relative cell proliferation was determined and normalized by the value of BrdU detected in control parental cells, set as 100%. (D) Relative ROS levels were measured with CM-H_2DCF-DA labeling and normalized by the fluorescence intensity determined in control parental cells, set as 1 (X, arbitrary unit). (E) Cell lysates were prepared and analyzed by immunoblotting to detect levels of p-Mek, Mek, p-Erk1/2, Erk1/2, and Nox-1, with β-Actin as a control, and these levels were quantified by densitometry. The levels of specific phosphorylation of Mek (p-Mek/Mek) and Erk1/2 (p-Erk/Erk) were calculated by normalizing the level of p-Mek and p-Erk1/2 with the level of Mek and Erk1/2, respectively, then the level set in control parental cells as 1 (X, arbitrary unit). The level of Nox-1 (Nox/Actin) was calculated by normalizing with the level of β-Actin and the level set in control parental cells as 1 (X, arbitrary unit). *Columns*, mean of triplicates; *bars*, SD. Statistical significance is indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. All results are representative of three independent experiments.

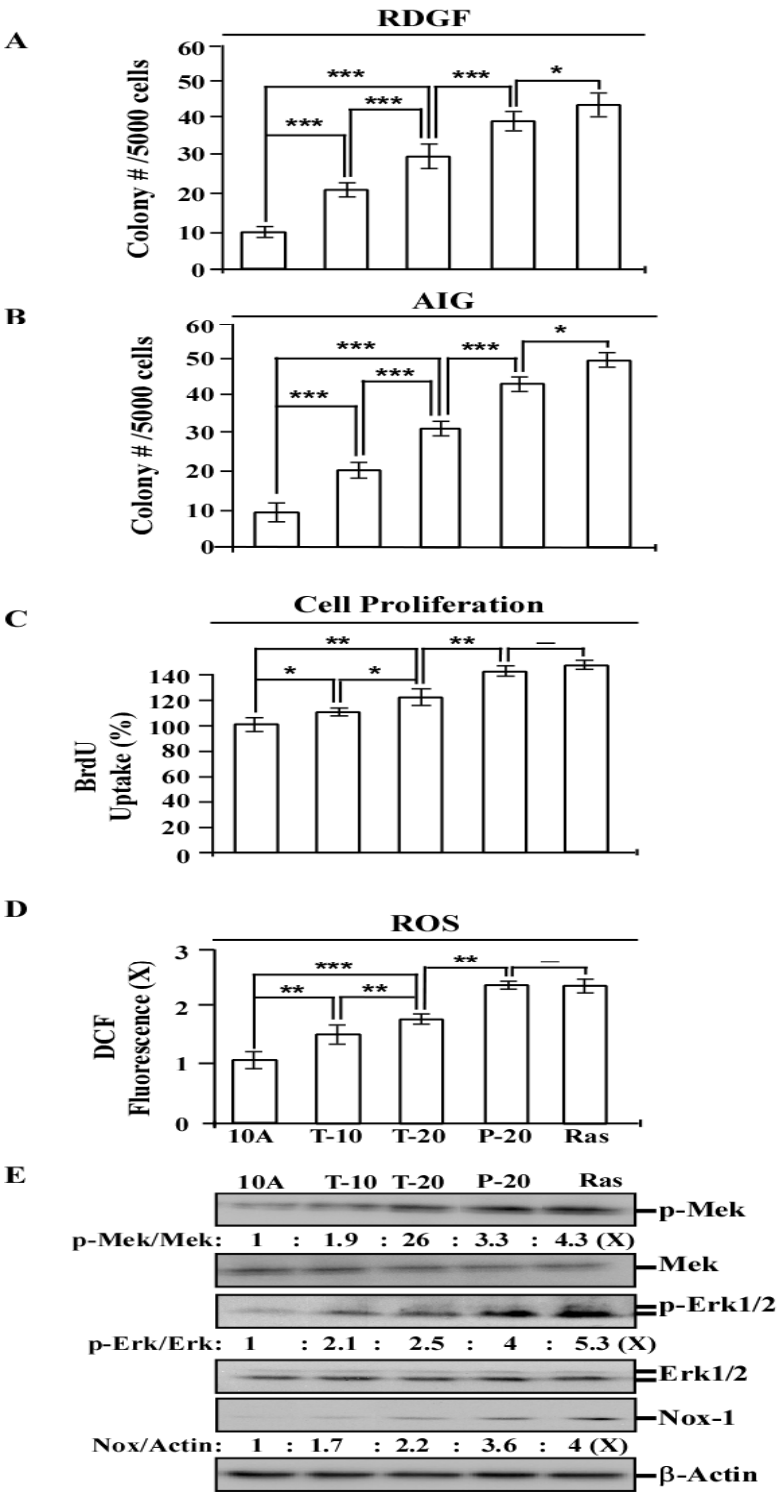

Figure 2.3: Constitutive endpoints induced by exposure to TCC.

2.4.4 Intervention of TCC-induced Carcinogenesis by Curcumin

Early intervention of cellular carcinogenesis from a non-cancerous stage to pre-malignant stages conceivably contributes to effective cancer prevention [2]. To identify preventive agents effective in intervention of cellular pre-malignancy induced by chronic exposure to TCC, we pursued agents, at non-cytotoxic levels, capable of blocking TCC-induced transient and constitutive endpoints in MCF10A cells. As shown in Figure 4A, treatment of cells with curcumin at 10 and 20 μmol/L for 72 h resulted in significant reduction of cell viability, but 0.1 or 1 μmol/L did not result in any detectable reduction of cell viability. We used curcumin at the non-cytotoxic concentration of 1 μmol/L in the following studies.

In studying if curcumin was able to block TCC-induced transient endpoints, we detected that co-exposure MCF10A cells to 200 nmol/L TCC ane1 μmol/L curcumin reduced TCC-induced activation of the Erk-Nox pathway (Figure 2.4B1). Curcumin treatment also significantly reduced TCC-induced transient endpoints of ROS elevation (Figure B2), increased cell proliferation (B3), and DNA damage (B4). These results indicate that co-exposure to curcumin interfered in TCC-induced initiation of cellular carcinogenesis in a single exposure. After 10 cycles of exposure, we detected that co-exposure to curcumin significantly reduced TCC-induced constitutive endpoints of reduced dependence on growth factors (Figure 2.4C1), anchorage-independent growth (C2), increased cell proliferation (C3), and ROS elevation (C4), as well as activation of the Erk-Nox pathway (C5); these constitutive endpoints were increased in T-10 cells but reversed in curcumin-protected, T/C-10 cells. Accordingly, curcumin was effective in the intervention of transient and constitutive endpoints induced by TCC in single and

accumulated exposures, respectively. Thus, curcumin should be considered for early intervention of TCC-induced, cellular pre-malignancy.

Legend for Figure 2.4. Intervention of TCC-induced carcinogenesis by curcumin. (A) MCF10A (10A) cells were treated with 0, 0.1, 10, and 20 μmol/L (μM) curcumin for 72 h. Quantification of cell viability in these cultures was determined with an MTT assay kit. Relative cell viability was normalized by the value determined in control cells (0 μM), set as 100%. (B1 to B4) 10A cells were treated with 200 nmol/L TCC (T) in the absence or presence of 1 μmol/L curcumin (C) for 24 h. (B1) Cell lysates were then prepared and analyzed by Western immunoblotting to detect levels of p-Erk1/2, Erk1/2, and Nox-1, with β-Actin as a control, and these levels were quantified by densitometry. The level of specific phosphorylation of Erk1/2 (p-Erk/Erk) was calculated by normalizing the level of p-Erk1/2 with the level of Erk1/2, then the level set in control cells as 1 (X, arbitrary unit). The level of Nox-1 was calculated by normalizing with the level of β-Actin and the level set in control cells as 1 (X, arbitrary unit). (B2) Relative ROS levels were measured with CM-H_2DCF-DA labeling and normalized by the fluorescence intensity determined in control cells, set as 1 (X, arbitrary unit). (B3) Relative cell proliferation was determined and normalized by the value of BrdU detected in control cells, set as 100%. (B4) Relative DNA damage was measured by a comet assay and normalized by the value of average tail moment determined in control cells, set as 1 (X, arbitrary unit). (C1 to C4) 10A cells were repeatedly exposed to 200 nmol/L TCC (T) in the absence and presence of 1 μmol/L of curcumin (C) for 10 cycles, resulting in 10A, C-10, T-10, and T/C-10 cell lines. To determine levels of cellular acquisition of reduced dependence on growth factors (RDGF) (C1) and anchorage-independent growth (AIG) (C2), cells were maintained in LM medium and seeded in soft-agar, respectively. Cell colonies grown in LM medium and in soft-agar were

counted. (C3) Relative cell proliferation was determined and normalized by the value of BrdU detected in parental control cells, set as 100%. (C4) Relative ROS levels were measured with CM-H_2DCF-DA labeling and normalized by the fluorescence intensity determined in parental control cells, set as 1 (X, arbitrary unit). (C5) Cell lysates were prepared and analyzed by Western immunoblotting to detect levels of p-Erk1/2, Erk1/2, and Nox-1, with β-Actin as a control, and these levels were quantified by densitometry. The level of specific phosphorylation of Erk1/2 (p-Erk/Erk) was calculated by normalizing the level of p-Erk1/2 with the level of Erk1/2, then the level set in parental control cells as 1 (X, arbitrary unit). The level of Nox-1 was calculated by normalizing with the level of β-Actin and the level set in control cells as 1 (X, arbitrary unit).). *Columns*, mean of triplicates; *bars*, SD. Statistical significance is indicated by ** $P <$ 0.01, *** $P < 0.001$. All results are representative of three independent experiments.

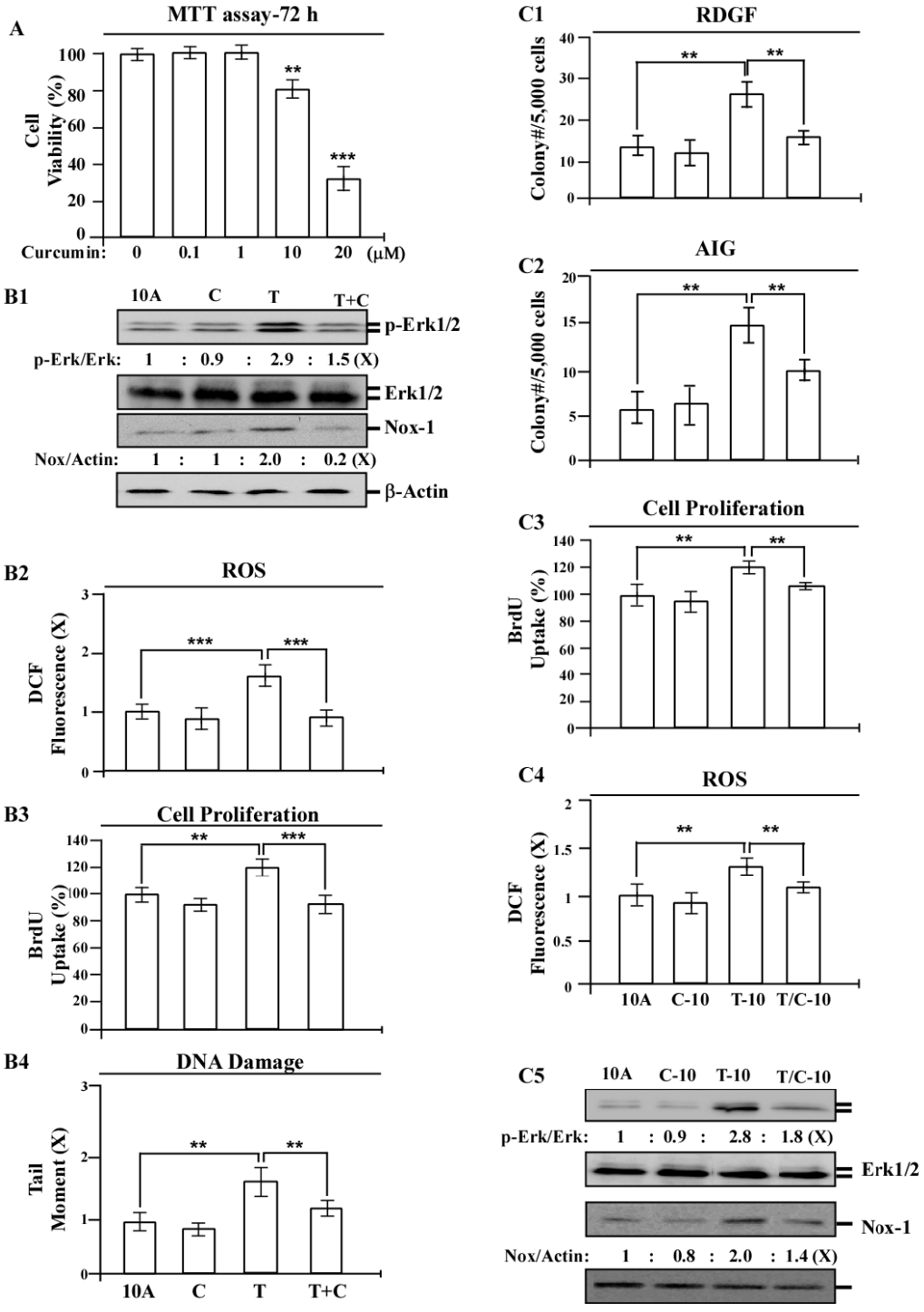

Figure 2.4. Intervention of TCC-induced carcinogenesis by curcumin

2.5 Conclusion

American lifestyles involve wide uses of cleaning agents containing TCC and consumption of TCC-contaminated agricultural products. The role of TCC in increasing breast cancer risk has been a concern; however, it has not yet been fully addressed. Our model system revealed, for the first time, that cumulative exposures of human breast cells to TCC at physiologically-achievable, nanomolar concentrations induced progressive carcinogenesis from a non-cancerous stage to pre-malignant stages in a dose- and exposure-dependent manner, suggesting the novel ability of TCC, as a co-carcinogen, to induce breast cell pre-malignancy. Thus, the co-carcinogenic potential of low-dose TCC should be seriously considered in epidemiological studies to reveal the significance of TCC in the development of sporadic breast cancer. Using TCC-induced transient and constitutive endpoints as targets will enable us to identify additional non-cytotoxic preventive agents, such as curcumin, effective in suppressing TCC-induced cellular pre-malignancy. However, whether TCC is able to enhance malignancy with other carcinogenic agents remains to be clarified.

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Chapter-III

TCC (3,4,4'- trichlorocarbanilide) enhances PhIP-induced breast cell carcinogenesis

Research described in this chapter is slightly modified version of an article that will be submitted to Molecular Carcinogenesis.

TCC (3,4,4'- trichlorocarbanilide) enhances PhIP-induced breast cell carcinogenesis by Shilpa Sood, Shambhunath Choudhary, and Hwa-Chain Robert Wang

In this paper "our" and "we" refers to me and co-authors. My contribution in the paper includes the following:

(1) Selection of the topic (2) Compiling and interpretation of the literature (3) Designing experiments (4) understanding the literature and interpretation of the results (5) providing comprehensive structure to the paper (6) Preparation of the graphs and figures (7) Writing and editing

3.1 Abstract

More than 85% of breast cancers are sporadic and attributable to long-term exposure to environmental carcinogens and co-carcinogens. Few studies have been conducted to study the role of co-carcinogens in potentiating the ability of environmental carcinogens known to cause sporadic breast cancer. We sought to determine the activity of triclocarban (TCC), an antimicrobial agent commonly used in household and personal care products in enhancing PhIP-induced breast cell carcinogenesis. We have shown for the first time that repeated co-exposures to both TCC and PhIP at bio-achievable nanomolar levels effectively induced breast cell carcinogenesis of human breast epithelial MCF10A cells from a non-cancerous stage to cancerous state in an *in vitro* model. The degree of cellular carcinogenesis induced by chronic co-exposures to PhIP and TCC was significantly higher than that induced upon chronic exposure to PhIP alone. Cellular carcinogenesis upon co-treatment with TCC and PhIP was characterized by significant increases in acquisition of cancer-associated properties of reduced dependence on growth factors, anchorage-independent growth, increased cell proliferation, invasion and migration as compared to chronic exposure to PhIP alone. These biological changes were accompanied by biochemical and molecular changes, including the activation of the Erk-Nox pathway, and reactive oxygen species (ROS) elevation which were also significantly higher than treatment with PhIP alone. Moreover, single simultaneous exposure of TCC and PhIP also significantly induced transient induction of the Erk-Nox pathway, ROS elevation, increased cell proliferation, and DNA damage which were higher as compare to PhIP treatment alone. These constitutively- and transiently-induced endpoints were effectively blocked by non-cytotoxic doses of mimosine and ergosterol. Our data

suggests that TCC can act as a co-carcinogen to potentiate PhIP-induced breast cell carcinogenesis in *in vitro* study models. In addition, mimosine and ergosterol showed a lot of potential as novel agents useful in the intervention of sporadic breast cancer as was evidenced by suppression of transient and constitutive end points induced upon co-treatment with TCC and PhIP**.**

3.2 Introduction

Breast cancer is the most common type of cancer among women in Europe and North America and more than 85% of cases of breast cancer are sporadic in nature [1]. Exposure to environmental carcinogens is an important factor in the development of sporadic breast cancer which is a multistep, multiyear, multipath disease process that involves genetic and epigenetic alterations and progression from a non-cancerous stage to a precancerous stage and ultimately leading to malignant breast cancer [2,3,4,5]. More than 200 chemical carcinogens have been found to be capable of inducing acute mammary carcinogenesis at high dosages in vitro and in vivo studies[5,6]. While carcinogens induce cellular malignancy, co-carcinogens can either induce a state of cell pre-malignancy or are capable to enhancing cellular malignancy. However, there is limited amount of work on the role of co-carcinogens at their physiologically achievable doses in enhancing the level of carcinogenesis induced upon chronic exposure to low levels of environmental carcinogens, in an event of a co-exposure.

Our lab has developed a cellular model that helps to identify potential carcinogens and co-carcinogens with the ability to induce breast cell carcinogenesis at their physiologically relevant doses [7,8,9,10,11,12,13,14,15]. Our model mimics everyday exposure of breast epithelial cells to environmental carcinogens and co-carcinogens at physiologically relevant doses by identifying cellular, biochemical and molecular transient and constitutive endpoints that are major drivers of initiation and progression of breast cell carcinogenesis [11,12,14,15]. Our model also aids in identification of dietary preventive agents that can effectively target these transient and constitutive endpoints [9,10,11,12,13,14,15].

Triclocarban (TCC), or 3,4,4'-trichlorocarbanilide, is an antibacterial agent commonly used in personal care products such as disinfectants, soaps, body washes deodorants, detergents, cleansing lotions, wipes etc [16]. TCC is an environmental pollutant and a potential co-carcinogen (unpublished results) whose wide spread poses a great risk for human health. Recent research has shown that TCC can act as an endocrine disrupter and can augment the activities of certain steroid hormones [17, 18].

2-amino-1-methyl-6-phenylimidazo [4, 5-*b*] pyridine (PhIP) is heterocyclic amine (HCAs) abundantly found in high temperature cooked meats particularly produced during grilling and barbequeing procedures [19, 20, 21]. Consumption PhIP via such meats has been associated with an enhanced risk of breast neoplasia by epidemiological studies [22; 23]. Several studies have reported that PhIP is genotoxic to human cells and a concentration of PhIP as low as 450 nmol/L induced DNA adduct formation [21, 22, 23, 24]. Transient exposure of MCF10A cells at nanomolar doses of PhIP caused increased cellular proliferation and activated the ERK pathway [25]. Recently, it was reported that long-term exposure of breast cells to PhIP at physiologically-achievable, picomolar to low nanomolar doses induced carcinogenesis and tumorigenicity [12]. Step wise induction of carcinogenesis was accompanied by acquisition of cancer-associated properties of reduced dependence on growth factors, anchorage-independent growth, increased cellular proliferation, migration, invasion and carcinogenesis in mouse models [12]. In addition an upregulated H-Ras gene expression, ERK pathway activation with downstream elevation of Nox-1 expression, and elevated reactive oxygen species production was noted. Co-exposure of environmental carcinogens at picomolar to nanomolar doses can have an additive effect in the induction of breast cell carcinogenesis

[11]. Whether simultaneous co-exposure of breast cells to PhIP and TCC can enhance PhIP-induced breast cell carcinogenesis has not been addressed. Various polyphenolic dietary components found to be present in a wide variety of fruits, vegetables, fruits have been shown to possess anti-oxidant and antitumor activities such as suppressing proliferation, induction of apoptosis. Moreover a lot many more naturally occurring component with a potential to inhibit tumor formation need to explored. Ergosterol and mimosine are two such promising compounds on the horizon whose anti tumorigenic potential needs to evaluated.

Ergosterol forms a major constituent of fungal cell membranes and stimulates fungal growth. It has been shown to possess antitumor activity attributed to its ability to inhibit of angiogenesis in solid tumors [26]. Ergosterol extracted from Japanese edible mushroom *Hypsizigus marmoreus* inhibited 12-O-tetradecanoylphorbol-13-acetate (TPA) induced ear inflammation and tumor promotion in carcinogenesis mice models (27). Various derivatives of ergosterol viz Ergosterol Peroxide (EP) found in lichens and mushrooms possess immunosuppressive anti-viral, anti-inflammatory and anti-tumor activities [28, 29, 30, 31]. However, the role of ergosterol and mimosine in sporadic breast cancer prevention has not been previously determined.

Mimosine or leucenol is an alkaloid, β-3-hydroxy-4 pyridone amino acid, initially isolated from *Mimosa pudica.* is also found to occur in some members of genus *Mimosa* and all members of the related genus *Leucaena* [32]. Mimosine is an iron chelator and that inhibits DNA synthesis and mammalian cell proliferation by blocking cell cycle at the late G_1 phase [33, 34, 35, 36]. It has been shown to inhibit cell proliferation in prostate carcinoma cells [37]. In vitro and vivo studies have demonstrated that it has an

anticancer effect on human lung cancer cells and xenografts [38, 39].Studies have been conducted that gave an evidence of the ability of mimosine to suppress the growth of human pancreatic cancer xenografts that were subcutaneously transplanted in nude mice and a flow cytometric analysis of the tumor cells yielded a significantly increased sub-G1 fraction indicating occurrence of apoptosis [40].

Even though recent experimental data suggests that mimosine and ergosterol have a potential anticancer activity, their role in the prevention of progressive cellular carcinogenesis especially of breast tissue has not been addressed so far. So, this work was undertaken with the objective of establishing the role of TCC as a co-carcinogen in enhancing PhIP-induced breast cell carcinogenesis and to determine if mimosine and ergosterol could suppress breast cell carcinogenesis induced by co-exposure to TCC and PhIP.

3.3 Materials and methods

3.3.1 Cell Cultures and Reagents

MCF10A (American Type Culture Collection [ATCC], Rockville, MD) and derived cell lines were maintained in complete (CM) medium (1:1 mixture of DMEM and HAM's F12, supplemented with 100 ng/mL cholera enterotoxin, 10 µg/mL insulin, 0.5 µg/mL hydrocortisol, 20 ng/mL epidermal growth factor, and 5% horse serum) [7, 8 , 9, 10, 11, 12, 13, 14, 15]. Human breast cancer MCF7 and urinary bladder carcinoma J82 cells (ATCC) were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum [lab ref]. All cultures were maintained in medium supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin in 5% CO_2 at 37°C. Stock aqueous solutions of TCC (Sigma-Aldrich, St. Louis, MO), chloromethyl-dichlorodihydrofluorescein-diacetate (CM-H_2DCF-DA) (Invitrogen, Carlsbad, CA), U0126 (Cell Signaling, Beverly, MA), and curcumin (MP Biomedicals, Solon, Ohio) were prepared in DMSO and diluted in culture medium. Stock aqueous solutions of *N*-acetyl-*L*-cysteine (NAC) (Alexis, San Diego, CA) were prepared in H_2O and diluted in culture medium for assays.

3.3.2 Chronic Induction of Cellular Carcinogenesis

Twenty-four hours after each subculturing, MCF10A cells were exposed to TCC for 48 h, as one cycle of exposure for 10 to 20 cycles; cultures were subcultured every 3 d [7, 8 , 9, 10, 11, 12, 13, 14, 15]. After exposures to TCC, cells were assayed to detect acquired cancer-associated properties.

3.3.3 Reduced dependence on growth factors

Five × 10^3 cells were seeded in 60-mm culture dishes and maintained in low-mitogen (LM) medium containing reduced total serum and mitogenic additives to 2% of the concentration formulated in CM medium. Cell colonies (≥ 0.5 mm diameter) grown in LM medium were counted microscopically [7, 8 , 9, 10, 11, 12, 13, 14, 15].

3.3.4 Anchorage-independent Growth

Five x 10^3 cells were mixed with soft-agar consisting of 0.4% SeaPlaque agarose (Sigma-Aldrich) in a mixture (1:1) of CM medium with 3 d conditioned medium prepared from MCF10A cultures, plated on top of the 2% SeaPlaque agarose base layer in a 60-mm culture dish, and maintained for 20 d to develop cell clones. Cell colonies (≥ 0.1 mm diameter) grown in soft-agar were counted microscopically [7, 8 , 9, 10, 11, 12, 13, 14, 15].

3.3.5 Cell Viability Assay

A Methyl Thiazolyl Tetrazolium (MTT) assay kit (ATCC) was used to measure cell survivability in cultures. Five x 10^3 cells were seeded into each well of 96-well culture plates for 24 h. After indicated treatments, cells were incubated with MTT Reagent for 4 h, followed by incubation with detergent reagent for 24 h. Reduced MTT reagent in cultures was quantified with an ELISA reader (Bio-Tek, Winooski, VT) [41, 42].

3.3.6 Cell Proliferation

Cell proliferation was determined by of 5-bromo-2'-deoxyuridine (BrdU) incorporation into cellular DNA using the BrdU cell proliferation ELISA kit (Roche, Indianapolis, IN). Five x 10^3 cells were seeded into each well of 96-well culture plates for 24 h. After treatment, cells were labeled with BrdU for 12 h, fixed, incubated with peroxidase-conjugated BrdU-specific antibodies, and stained with peroxidase substrate. Quantification of BrdU-labeled cells was determined with an ELISA reader (Bio-Tek) [41, 42, 43].

3.3.7 Intracellular ROS Measurement

As performed previously [11,12,44], cells were incubated with 5 μmol/L CM-H_2DCF-DA for 1 h to detect ROS level by flow cytometry; the mean fluorescence intensity of DCF was quantified using Multicycle software (Phoenix).

3.3.8 In vitro cell invasion and migration

The cell invasion assay was performed using 24-well Transwell insert-chambers with a polycarbonate filter with a pore size of 8.0 μm (Costar, Corning, NY). Two x 10^4 cells in serum-free medium were seeded on top of a Matrigel-coated filter (BD Biosciences) in each insert-chamber. Then, insert-chambers were placed into wells on top of culture medium containing 10% horse serum as a chemoattractant. The migration assay was performed using 24-well Transwell insert-chambers with a polycarbonate filter without Matrigel. The invasive or migratory ability of cells was determined by the number of cells translocated to the lower side of filters [45, 11, 12].

3.3.9 DNA Damage

DNA damage was measured by a comet assay [11, 12, 46]. Cells were mixed with 1% low-melting agarose and placed on agarose-coated slides. Slides were then immersed in lysis solution (1.2 mol/L NaCl, 100 mmol/L Na_2EDTA, 1% Triton X-100, and 0.3 nmol/L NaOH, pH 13) at 25°C for 1 h and rinsed three times with alkaline buffer (2 mmol/L Na_2EDTA and 300 mmol/L NaOH) for 20 min each. After electrophoresis in the same alkaline buffer at 20V for 30 min, slides were stained with 2.5 μg/mL of propidium iodide for 20 min and examined with a Zeiss fluorescence microscope (Thornwood, NY) equipped with an excitation filter of 546 nm and barrier filter of 590 nm. Fifty nuclei per slide were scored for tail moment (% of DNA in the tail × tail length) using CometScore software (Tritek, USA).

3.3.10 Western Immunoblotting

Equal amounts of cellular proteins were resolved by electrophoresis in 10% SDS-polyacrylamide gels and transferred to nitrocellulose filters for immunoblotting [47, 48, 49]. Specific antibodies were used to detect H-Ras, phosphorylated Mek (p-Mek), Mek, phosphorylated Erk1/2 (p-Erk1/2), Erk1/2, Nox-1, and β-Actin (Santa Cruz Biotechnology, Santa Cruz, CA). Antigen-antibody complexes on filters were detected by the Supersignal chemiluminescence kit (Pierce, Rockford, IL).

3.3.11 Statistical Analysis

The Student *t* test was used to analyze statistical significance, indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; a P value of ≤ 0.05 was considered significant.

3.4 Results

3.4.1 TCC-enances PhIP-induced breast cell carcinogenesis

Normal cells require growth factors for their survival and growth. In the absence of growth factors cells undergo apoptosis [50, 51]. Normal cells also require cell adhesion to extracellular matrix for survival and in its absence they die by a process of anoikis [50]. Cells that progressively acquire abilities to grow under the limiting conditions of reduced amounts of growth factors and absence of adhesion to extracellular matrix are thought to have acquired tumorigenic potential. Moreover, anchorage–independent growth and reduced dependence on growth factors are two important cancer-associated properties and have been used to measure progressive carcinogenesis of non-cancerous cells in response to exposure to environmental carcinogens [7, 8, 9, 10, 11, 12]. We used these two cancer-associated properties as long-term targeted endpoints to determine whether co-exposure of TCC and PhIP enhanced PhIP-induced breast cell carcinogenesis. We repeatedly exposed MCF10A cells to either individual or combined TCC or PhIP treatments at their physiologically achievable doses of 200 nM and 10 nM respectively for 10 and 20 cycles (Fig 3.1). We detected that individual and combined treatments to TCC and PhIP resulted in significant increase in number of surviving cell clones under the conditions of reduced dependence of growth factors and anchorage independence with increasing exposures (Fig 3.1A1-A2). Our results validated the previous published data that PhIP at physiologically achievable doses of 10 nM induced breast cell carcinogenesis in MCF 10 cells.

Legend for Figure 3.1. TCC enhances PhIP-induced breast cell carcinogenesis

MCF10A cells were repeatedly exposed to individual (T, P) or combined TCC and PhIP (TP) at 200 nmol/L and 10 nM/L respectively for 10, and 20 cycles. MCF10A cells were stably transfected to ectopically express oncogenic H-Ras, resulting in the MCF10A-Ras cell line (Ras). (A1) To determine acquisition of the cancer-associated property of reduced dependence on growth factors (RDGF), cells were maintained in LM medium. (A2) To determine acquisition of the cancer-associated property of anchorage-independent growth (AIG), cells were seeded in soft-agar. Cell colonies grown in LM medium and soft-agar were counted microscopically. (B1) Relative ROS levels were measured with CM-H2DCF-DA labeling and normalized by the fluorescence intensity determined in control (10A) cells, set as 1 (X, arbitrary unit). (B2) Relative cell proliferation was determined and normalized by the value of BrdU detected in MCF10A cells, set as 100%. (B3) Migratory and (B4) invasive activities were determined by counting the numbers of cells translocated through a polycarbonate filter without or with coated Matrigel, respectively, in 10 arbitrary visual fields. *Columns*, mean of triplicates; *bars*, standard deviation. The Student's *t* test was used to analyze statistical significance, indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

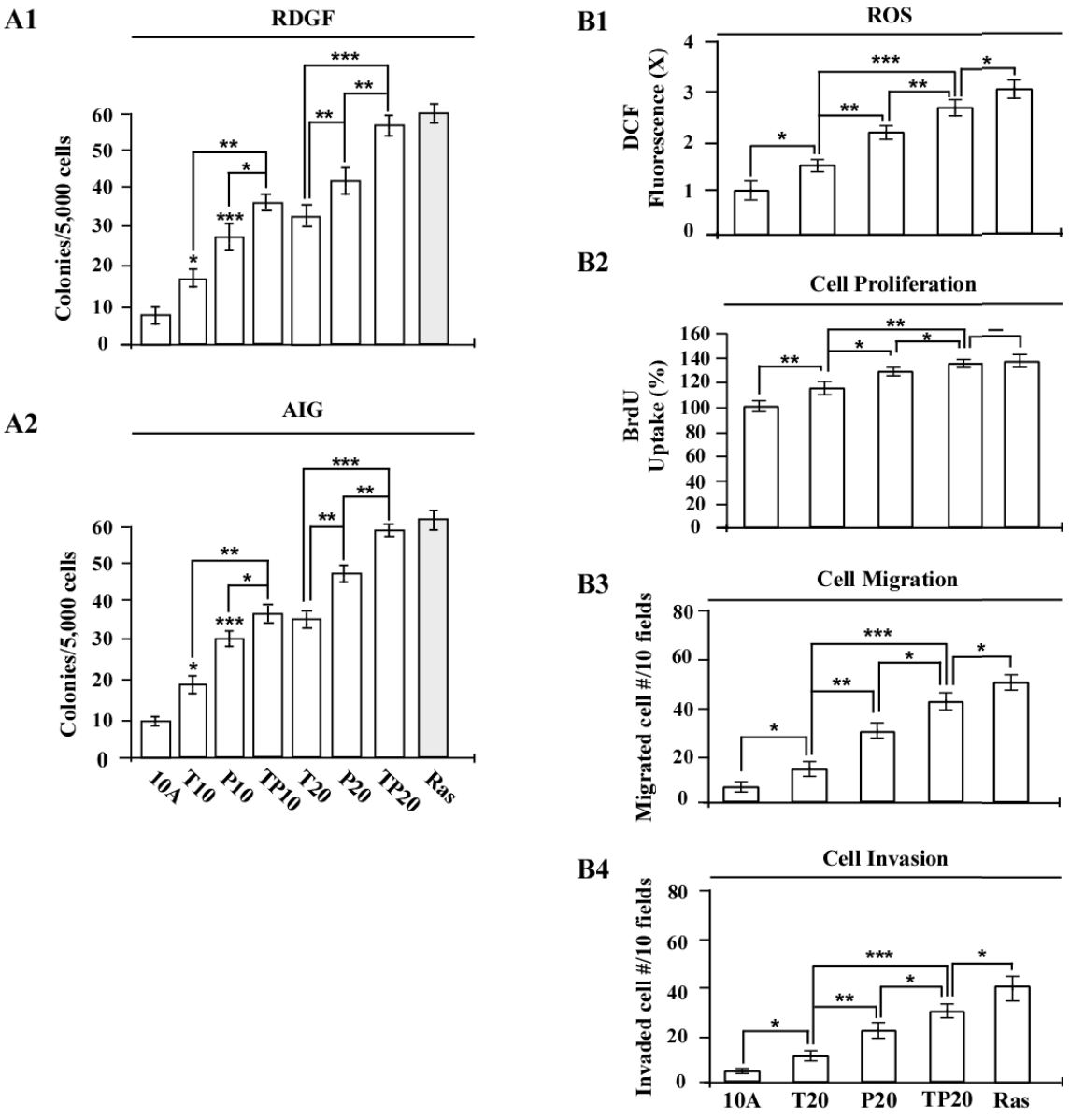

Figure 3.1. TCC enhances PhIP-induced breast cell carcinogenesis.

More significantly, accumulated exposures to combined treatments additively increased number of cell clones formed under the conditions of reduced dependence of growth factors and anchorage–independent growth as compared to those formed after cumulative exposures to PhIP or TCC alone (Fig 3.1A1-A2).

Since exposures of MCF10A cells to PhIP after 20 cycles resulted in fifty percent of mice forming tumors in our earlier studies [12], we used twenty cycle exposed cells for our further studies to determine the potential of TCC to confer additional carcinogenic abilities to PhIP cells. We named MCF10A cells treated either individually or together with TCC and PhIP as T20, P20 and TP20 cells respectively. We used MCF10A-Ras cells, which are MCF10A cells with ectopic expression of oncogenic H-Ras, as positive control for our studies. Our previous data had shown that P20 cells produced ROS, proliferated at a higher degree and had higher abilities of invasion and migration as compared to the MCF10A cells. We wanted to see if twenty cycles of co-exposure to TCC and PhIP could enhance ROS production, cell proliferation, migration and invasion potential of P20 cells. As shown in Figure 3.1B1-B4, TP20 cells produced higher amounts of ROS, proliferated at higher degrees and had significantly higher abilities of invasion and migration as compared to P20 cells but either comparable or significantly less degrees as compared to MCF10A-Ras cells that served as a positive control. Hence, our results showed that cumulative exposures to TCC could enhance breast cell carcinogenesis induced by PhIP.

3.4.2 Role of ERK pathway in TP-induced cancer associated properties

P20 cells have been shown to have higher expression of Ras, p-ERK, p-MEK and Nox-1 levels [12] and activation of Ras-Erk Nox pathway drove the progressive carcinogensis of MCF10A cells from nonmalignant to premalignant to a malignant stage. We wanted to see if co-exposure to TCC and PhIP would result in a higher activation of Ras ERK Nox pathway. Twenty cycles of co-exposure to TCC and PhIP didn't induce higher protein levels of Ras than P20 cells (Fig 3.2A). However, TP20 cells had a higher expression of p-Mek, p-Erk, Nox-1 protein as compared to P20 cells. So, TP20 cells had a higher degree of activated Mek-Erk-Nox pathway as compared to P20 cells (Fig 3.2A). To further validate the involvement of ERK pathway in inducing cancer associated properties in TP20 cells, we found that inhibiting ERK pathway with U0 not only blocked the activation of ERK pathway but also repressed elevated Nox-1 protein levels (Fig 3.2B). U0 also blocked ROS and cell proliferation in TP20 cells (Fig 3.2B2-B3). These results prove that ERK pathway played an important role in the enhanced carcinogenic potential of TP20 cells as compared to P20 cells.

Legend for Figure 3.2. Role of ERK Pathway in modulation of TP-induced cancer-associated properties. MCF10A cells were repeatedly exposed to individual (T, P) or combined TCC and PhIP (TP) at 200 nmol/L and 10 nM/L respectively for 20 cycles. MCF10A cells were stably transfected to ectopically express oncogenic H-Ras, resulting in the MCF10A-Ras cell line (Ras). (A) Lysates from cells exposed to TCC or PhIP individually or together for 20 cycles were prepared and analyzed by immunoblotting using specific antibodies to detect levels of phosphorylated Mek1/2 (p-Mek1/2), Mek1/2, phosphorylated Erk1/2 (p-Erk1/2), Erk1/2, Nox-1, with β-Actin as a control. The level of specific phosphorylation of Mek1/2 (p-Mek1/2) was calculated by normalizing the level of p-Mek1/2 with the level of Mek1/2, then the level set in MCF10A cells as 1 (X, arbitrary unit). The level of specific phosphorylation of Erk1/2 (p-Erk1/2) was calculated by normalizing the level of p-Erk1/2 with the level of Erk1/2, then the level set in MCF10A cells as 1 (X, arbitrary unit). The levels of Nox-1 were calculated by normalizing with the level of β-Actin, the level set in MCF10A cells as 1 (X, arbitrary unit). All results are representative of three independent experiments. (B1-B3) MCF10A andTP20 cells were treated without or with 10 μM/L U0126 (U) for 48 h. (B1) Cell lysates were prepared and analyzed by immunoblotting to detect levels of phosphorylated Erk1/2 (p-Erk1/2), Erk1/2, and Nox-1, with β-Actin as a control, and these levels were quantified by densitometry. The levels of Nox-1 were calculated by normalizing with the level of β-Actin and the level set in untreated control cells as 1 (X, arbitrary unit). The levels of specific phosphorylation of Erk1/2 (p-Erk1/2) were calculated by normalizing the levels of p-Erk1/2 with the levels of Erk1/2 , then the level set in control cells as 1 (X, arbitrary unit). (B2) ROS levels were measured with CM-H_2DCF-DA labeling; relative

level of ROS, as fold induction (X, arbitrary unit), was normalized by the level determined in MCF10A cells, set as 1. (B3) Cell proliferation was determined; relative cell growth rate was normalized by the value of BrdU detected in MCF10A cells, set as 100%. *Columns*, mean of triplicates; *bars*, standard deviation. The Student's *t* test was used to analyze statistical significance, indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. All results are representative of three independent experiments.

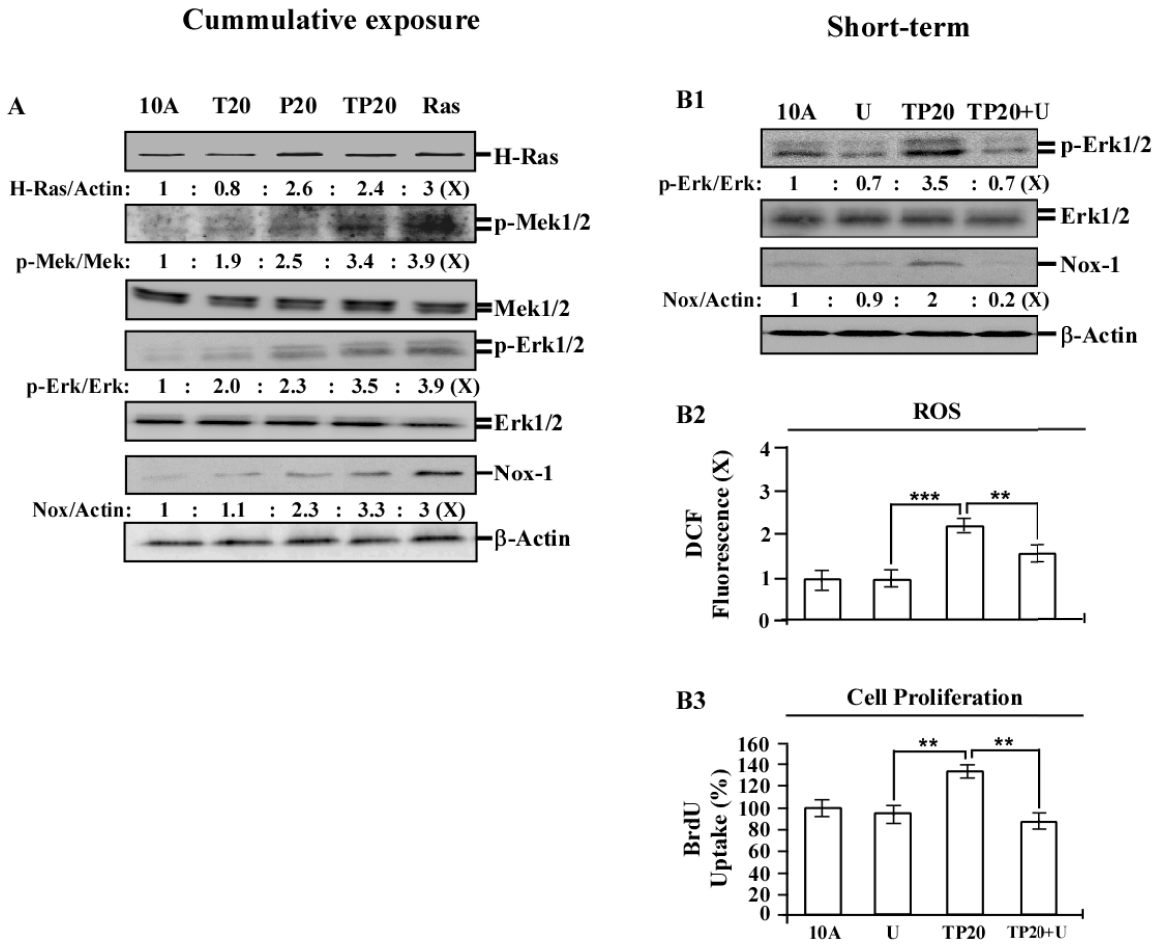

Figure 3.2 Role of ERK Pathway in modulation of TP-induced cancer-associated properties

3.4.3 TCC enhances PhIP-induced transient endpoints

To investigate the mechanisms underlying chronic carcinogenesis induced by simultaneous treatments with PhIP and TCC in MCF10A cells, and to determine if short term treatment with TCC also enhanced, PhIP-induced transient endpoints in MCF10A cells and thus contributed towards enhancement of PhIP-induced cellular carcinogenesis by TCC in TP20 cells, we subjected MCF10A cells to single exposure of TCC and PhIP at their bio-achievable doses**.** First of all, we wanted to determine the time of maximum activation of ERK pathway after co-treatment with TCC and PhIP. PhIP has been shown to induce maximum p-ERK levels in 24 hours. As can be seen in Figure 3.3A, combined treatment of PhIP and TCC induced maximal levels of p-ERK after 24 hours of the co-treatment. We chose this time interval for our further experiments. We have also shown earlier that 24 h of PhIP treatment leads to a transient upregulation of HRas protein and also induced downstream ERK-Nox pathway [12]. We wanted to investigate whether co-exposure of TCC and PhIP would elevate the protein expression of HRas and activation of downstream ERK-Nox pathway. As we can see in Figure 3.3B, short-term treatment of MCF10A cells with PhIP and TCC didn't cause any changes in HRas expression levels induced by PhIP. However, we found that co-treatment of TCC and PhIP were able to additively enhance protein expression of phosphorylated Erk1/2 levels and Nox-1 levels induced by PhIP. Moreover, upon blocking ERK pathway by use of inhibitors like U0, ERK-Nox activation was blocked. In our previous study, we had shown that PhIP-induced transient activation of ERK pathway leading to ROS elevation which increased cell proliferation in MCF10A cells [12].

Legend for Figure 3.3. Short-term targeted endpoints: TCC enhances ROS elevation, ERK pathway, cell proliferation, and DNA damage transiently induced by short term exposure to PhIP. (A) MCF10A (10A) cells were coexposed to 200 nmol/L TCC (T) and 10 nM PhIP (P) for the indicated periods. (B) MCF10A cells were treated either individually (T, P) or together (TP) with 200 nmol/L TCC (T) and 10 nM PhIP (P) for 24 h. (C) MCF10A cells were treated either individually (T, P) or together (TP) with 200 nmol/L TCC (T) and 10 nM PhIP (P) for 24 h in the absence or presence 10 μmol/L U0126 (U). (A-C) Cell lysates were prepared and analyzed by Western immunoblotting to detect levels of p-Erk1/2, Erk1/2, and Nox-1, with β-Actin as a control, and these levels were quantified by densitometry. The levels of Nox-1 were calculated by normalizing with the level of β-Actin and the level set in untreated control cells as 1 (X, arbitrary unit). The levels of specific phosphorylation of Erk1/2 (p-Erk1/2) were calculated by normalizing the levels of p-Erk1/2 with the levels of Erk1/2, then the level set in control cells as 1 (X, arbitrary unit). (D1-D3) MCF10A (10A) cells were treated either individually (T, P) or together (TP) with 200 nmol/L TCC (T) and 10 nM PhIP (P) in the absence or presence of 10 μmol/L U0126 (U), 5 mmol/L NAC (N) for 24 h. (D1) Relative ROS levels were measured with CM-H_2DCF-DA labeling and normalized by the fluorescence intensity determined in untreated cells, set as 1 (X, arbitrary unit). (D2) Relative cell proliferation was determined and normalized by the value of BrdU detected in untreated cells, set as 100%. (D3) Relative DNA damage was measured by a comet assay and normalized by the value of average tail moment determined in untreated counterpart cells, set as 1 (X, arbitrary unit). (E1) To determine reduced dependence on growth factors (RDGF) cells were maintained in LM medium. (E2) To determine

anchorage-independent growth (AIG), cells were seeded in soft-agar. Representative images of DNA damage in the comet assay are shown. (E1-E2) MCF10A (10A) cells were repeatedly exposed to 200 nM TCC and 10 nmol/L PhIP in the absence and presence of 2 μmol/L U0126 (U), for 5 cycles, resulting in 10A, TP5, TP5/U5 cell lines respectively. *Columns*, mean of triplicates; *bars*, standard deviation. The Student's *t* test was used to analyze statistical significance, indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. All results are representative of three independent experiments.

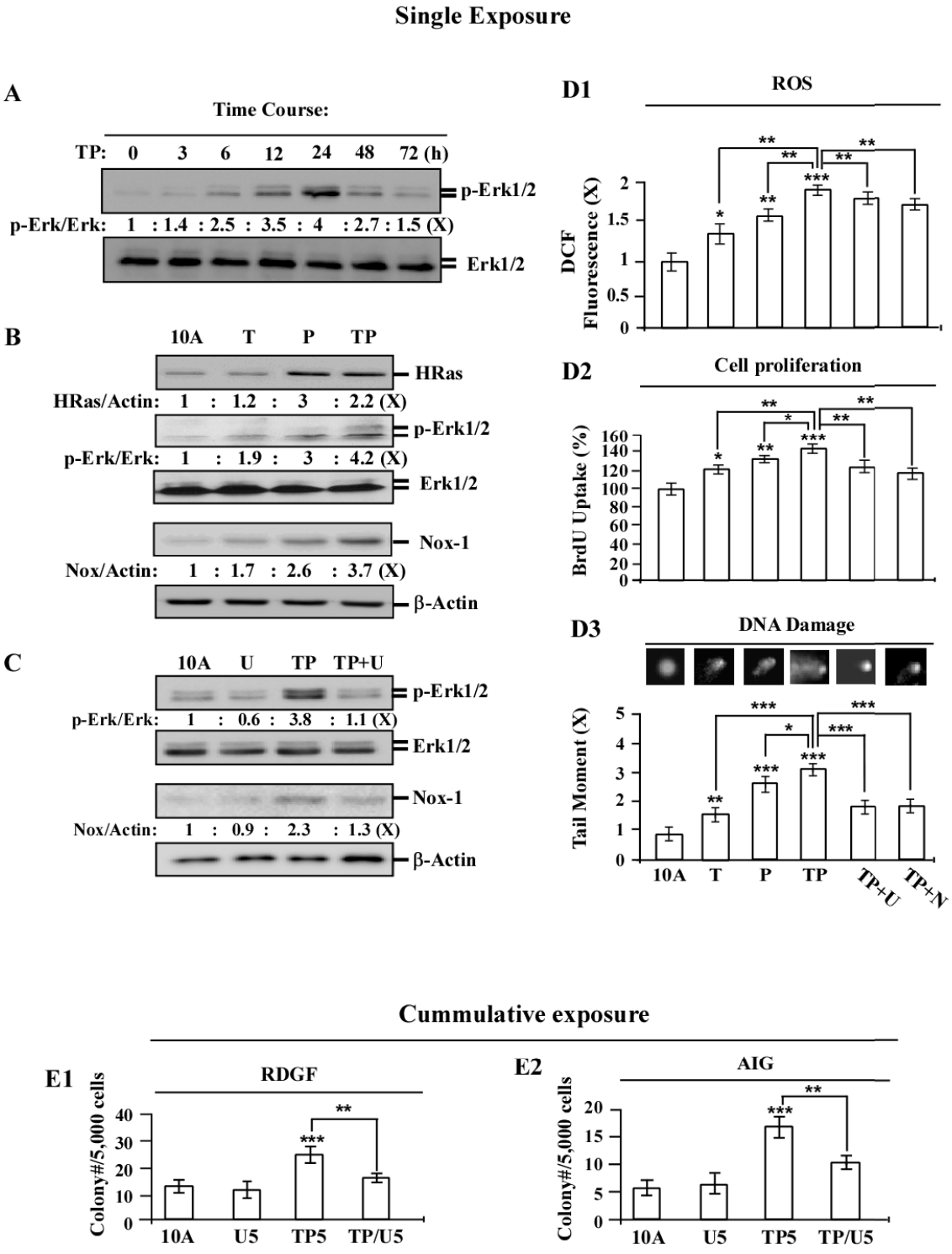

Figure 3.3. Short-term targeted endpoints: TCC enhances ROS elevation, ERK pathway, cell proliferation, and DNA damage transiently induced by short term exposure to PhIP

Increased ROS production was responsible for inducing DNA damage. We wanted to determine whether TCC can potentiate PhIP's ability to cause ROS production, cell proliferation and induce DNA damage after transient co-exposure with TCC and PhIP. As shown in figure 3.3D1-3.3D3, TCC significantly enhanced PhIP-induced ROS production, cell proliferation and DNA damage which was suppressed by blocking ERK Pathway and ROS production using U0 and NAC respectively. These results indicate that ERK pathway and ROS played important role in TCC's ability to enhance PhIP-induced transient targeted endpoints.

This proves that TCC increased transient induction of the Erk-Nox pathway to cause ROS elevation and cellular proliferation, induced by PhIP treatment with each exposure cycle. Hence, cumulative co-exposures to TCC and PhIP increased the accumulation of DNA damage caused by PhIP treatment alone leading to an enhanced level of progressive carcinogenesis in PhIP treated cells.

To further validate the role of ERK pathway in TP-induced carcinogenesis, we treated MCF10A cells with TCC and PhIP in the presence and absence of U0(U) for five consecutive exposure cycles to generate 10A, U5, TP5 and TP/U5 cells. We found that U0 was able to repress number of colonies formed under the conditions of reduced dependence of growth factors and anchorage-independent growth in MCF10A cells co-treated with TCC and PhIP for 5 cycles(Fig 3.3E). These data show that co-exposure to TCC and PhIP potentiated the degree of transient as well as constitutive endpoints induced by treatment with PhIP alone and that activated ERK pathway played an important role in potentiating initiation and progression of PhIP-induced carcinogenesis by co-treatment with TCC and PhIP.

3.4.4 TCC enhanced PhIP-induced transient endpoints in breast carcinoma and bladder cancer cells.

To investigate if TCC's ability to enhance the degree of PhIP-induced transient endpoints was not limited to MCF10A cells, we subjected breast carcinoma MCF7 cells and bladder cancer J82 cells to a single exposure of physiologically achievable doses of TCC and PhIP. Co-exposure to TCC and PhIP was able to induce Erk-Nox pathway (Fig3 4A) leading to ROS elevation (Fig 3.4B) and DNA damage (Fig 3.4C) in MCF7 and J82 cells. More importantly, combined exposure significantly enhanced the ability of PhIP to activate ERK Nox pathway, ROS production and cell proliferation. This shows that co-treatment with TCC has the similar effect of potentiating PhIP-induced activation of the ERK Pathway in other types of cell lines. These results also show that TCC induced transient endpoints not only in non-cancerous, ER-negative MCF10A cells but also in the ER-positive cancerous MCF7 cells. Therefore, TCC –induced potentiation of cellular carcinogenesis due to PhIP exposure is independent of ER- status of the cells.

Legend for Figure 3.4. TCC enhances PhIP-induced ERK Pathway activation, ROS elevation, and DNA damage in breast carcinoma and bladder cancer cells. (A-C) MCF7 and J82 cells were exposed to individual (T, P) or combined TCC and PhIP (TP) at 200 nmol/L and 10 nM/L respectively for 24 h. (A) Cell lysates were prepared and analyzed by immunoblotting to detect levels of p-Erk1/2, Erk1/2, and Nox-1, with β-Actin as a control, and these levels were quantified by densitometry. The level of specific phosphorylation of Erk1/2 (p-Erk1/2) was calculated by normalizing the level of p-Erk1/2 with the level of Erk1/2, then the level set in control cells as 1 (X, arbitrary unit). The level of Nox-1 (Nox/Actin) was calculated by normalizing with the level of β-Actin and the level set in control cells as 1 (X, arbitrary unit). (B) Relative ROS levels were measured with CM-H_2DCF-DA labeling and normalized by the fluorescence intensity determined in control cells, set as 1 (X, arbitrary unit). (C) Relative DNA damage was measured by a comet assay and normalized by the value of average tail moment determined in control cells, set as 1 (X, arbitrary unit). *Columns*, mean of triplicates; *bars*, SD. Statistical significance is indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. All results are representative of three independent experiments.

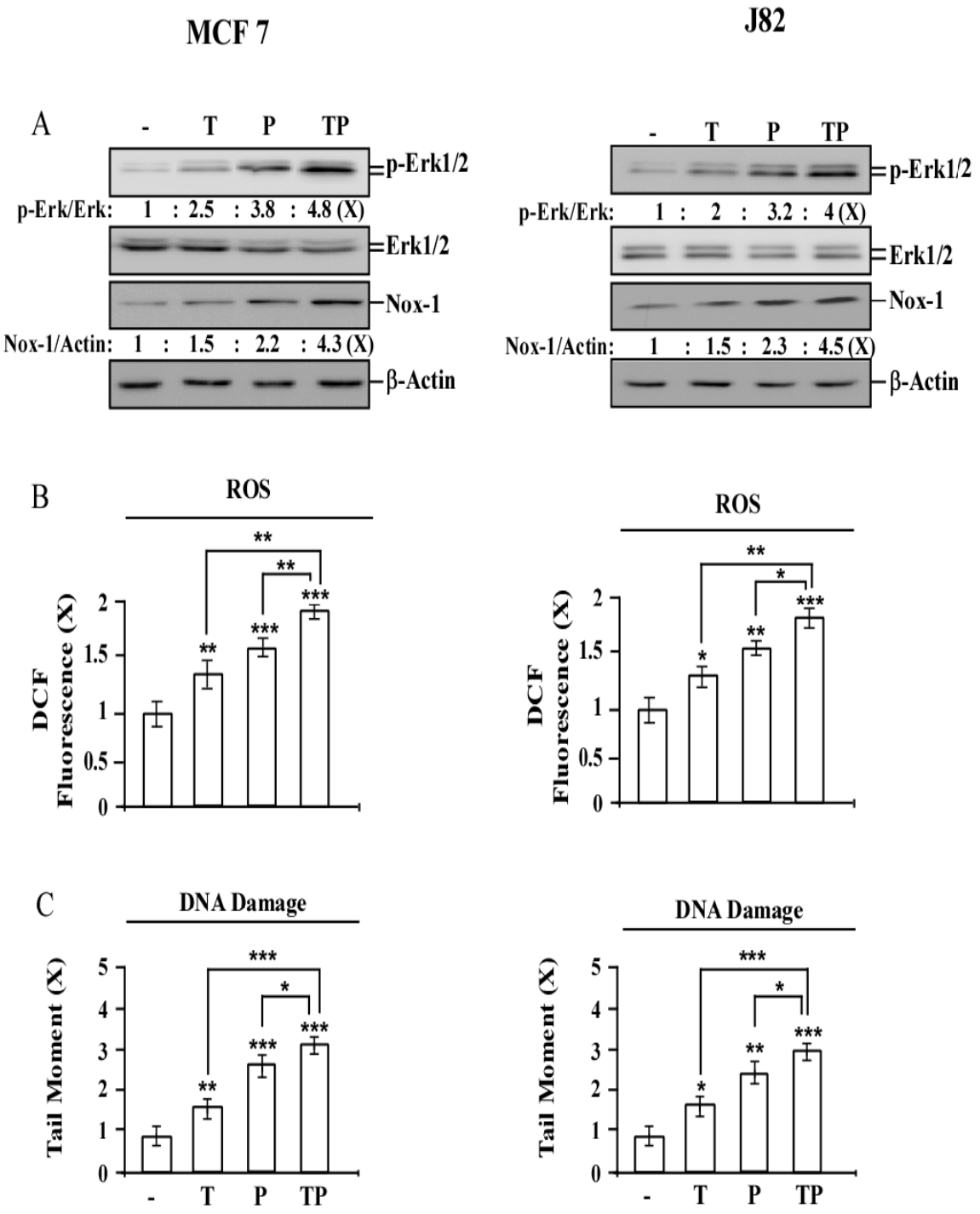

Figure 3. TCC enhances PhIP-induced ERK Pathway activation, ROS elevation, and DNA damage in breast carcinoma and bladder cancer cells.

3.4.5 Mimosine and ergosterol suppression of TP-induced short-term targeted endpoints

To first determine the non-cytotoxic doses of ergosterol and mimosine, we studied cytotoxicity of these two compounds in MCF10A cells. As shown in the figure 3.5A1 and3 5A2, treatment of MCF10A cells with either ergosterol or mimosine at concentrations of 100 nM or more significantly reduced cell viability. However, treatment with neither mimosine nor ergosterol at 10 nM induced any detectable reduction in cell viability. So, we used 10 nM ergosterol or mimosine in our subsequent studies. To study whether ergosterol or mimosine could block combined TCC and PhIP-induced initiation of carcinogenesis, we exposed MCF10A cells to either DMSO or TCC and PhIP together in the presence and absence of these two compounds for 24 h. As shown (Fig3.5B1-B4), both ergosterol and mimosine were able to suppress simultaneous TCC and PhIP treatment induced transient phosphorylation of Erk1/2, induction of Nox-1, ROS elevation, increased cell proliferation, and DNA damage. These results indicate that ergosterol and mimosine at non cytotoxic concentrations could block combined TCC and PhIP-induced transient endpoints of breast cell carcinogenesis.

Legend for Figure 3.5. Mimosine and ergosterol suppression of TP-induced short term targeted endpoints. (A1) MCF10A cells were treated with 0, 100 pM, 1 nM, 10 nM, 100 nM, 1 μM, 10 μM and 20 μmol/L ergosterol (E) for 72 h. Quantification of cell viability in these cultures was done with an MTT assay kit. Relative cell viability was normalized by the value determined in untreated cells, set as 100%. *Columns*, mean of triplicates; *bars*, SD. (A2) MCF10A (10A) cells were treated with 0, 100 pM, 1 nM, 10 nM, 100 nM, 1 μM, 10 μM and 20 μmol/L mimosine (M) for 72 h. Cell viability in these cultures was determined with an MTT assay kit. Relative cell viability was normalized by the value determined in untreated cells, set as 100%. *Columns*, mean of triplicates; *bars*, SD. (B1-B4) MCF10A (10A) cells were treated with 200 nmol/L TCC (T) and 10 nmol/L PhIP (P) together (TP) in the absence or presence of 10 nmol/L ergosterol (E) or 10 nmol/L mimosine (M) for 24 h. (B1) Cell lysates were then prepared and analyzed by Western immunoblotting to detect levels of specific phosphorylation of Erk1/2 (p-Erk1/2), Erk1/2, and Nox-1, with β-Actin as a control, and these levels were quantified by densitometry. The level of Nox-1 were calculated by normalizing with the level of β-Actin and the level set in control MCF10A cells as 1 (X, arbitrary unit). The level of specific phosphorylation of Erk1/2 (p-Erk1/2) was calculated by normalizing the level of p-Erk1/2 with the level of Erk1/2, then the level set in MCF10A cells as 1 (X, arbitrary unit). (B2) Relative ROS levels were measured with CM-H2DCF-DA labeling and normalized by the fluorescence intensity determined in MCF10A cells, set as 1 (X, arbitrary unit). (B3) Relative cell proliferation was determined and normalized by the value of BrdU detected in MCF10A cells, set as 100%. (B4) Relative DNA damage was measured by a comet assay and normalized by the value of average tail moment

determined in untreated counterpart cells, set as 1 (X, arbitrary unit). *Columns*, mean of triplicates; *bars*, standard deviation. The Student's *t* test was used to analyze statistical significance, indicated by ** $P < 0.01$, *** $P < 0.001$. All results are representative of three independent experiments.

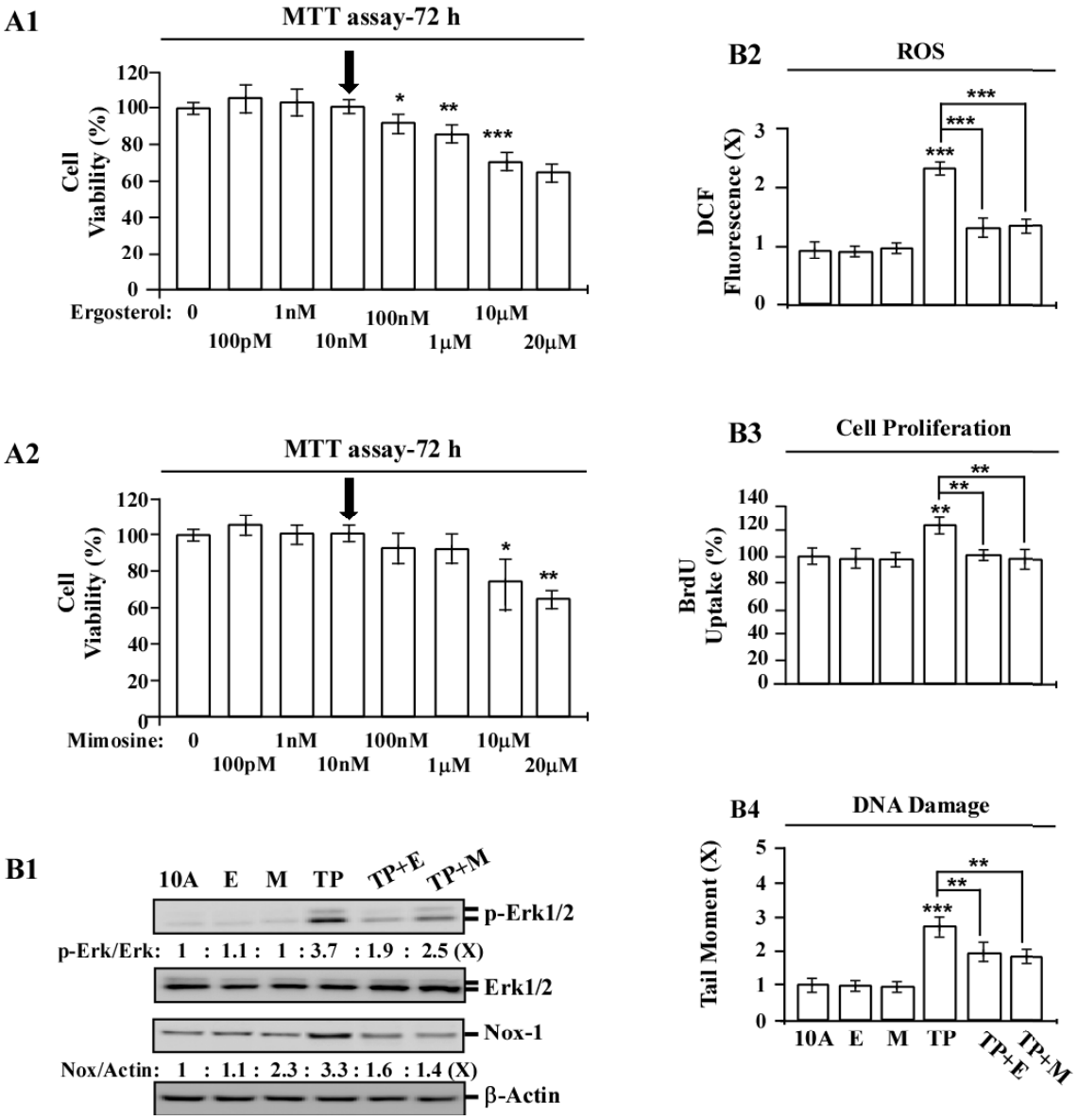

Figure 3.5. Mimosine and ergosterol suppression of TP-induced short term targeted endpoints

3.4.6 Intervention of TP-induced chronic cell carcinogenesis by mimosine and ergosterol

To determine whether mimosine and ergosterol could suppress chronic cellular carcinogenesis induced by co-exposure to TCC and PhIP, MCF10A cells were exposed to either DMSO or PhIP and TCC together, in the absence and presence of ergosterol and mimosine for 10 cycles to yield 10A, E10, M10, TP10, TP/E10 and TP/M10 cells respectively. Cells co-exposed to TCC and PhIP after 10 cycles exhibited higher degrees of cancer-associated properties of anchorage-independent growth and reduced dependence on growth factors which were significantly reduced by mimosine and ergosterol co-treatment (Figure 3.6A and 3.6B). Moreover, mimosine and ergosterol reduced combined PhIP and TCC-induced constitutively increased ROS production and cell proliferation in TP10 cells (Figure 3.6C-3.6D). We also found that chronically induced Erk-Nox pathway in TP10 cells was suppressed by both ergosterol and mimosine (Figure 3.6E). These results indicate that mimosine and ergosterol were able to block TCC and PhIP-induced chronic cellular carcinogenesis. So, they are new promising agents that can be useful in the prevention of sporadic breast cancer.

Legend for Figure 3.6. Intervention of TP-induced chronic breast cell carcinogenesis by ergosterol and mimosine. (A-E) MCF10A (10A) cultures were repeatedly exposed to either DMSO or 200 nmol/L TCC and 10 nmol/L PhIP together (TP) in the absence and presence of 10nM/L ergosterol (E)or 10 nM/L mimosine (M) for 10 cycles, resulting in 10A, E10, M10, TP10, TP/E10 and TP/M10 cell lines, respectively. To determine reduced dependence on growth factors (RDGF) (A) and anchorage-independent growth (AIG) (B), cells were maintained in LM medium and seeded in soft-agar respectively. Cell colonies grown in LM medium and soft-agar were counted. (C) Relative ROS levels were measured with CM-H_2DCF-DA labeling and normalized by the fluorescence intensity determined in MCF10A cells, set as 1 (X, arbitrary unit). (D) Relative cell proliferation was determined and normalized by the value of BrdU detected in MCF10A cells, set as 100%. (E) Cell lysates were prepared and analyzed by Western immunoblotting to detect levels of Erk1/2 phosphorylation (p-Erk1/2), Erk1/2, and Nox-1, with β-Actin as a control, and these levels were quantified by densitometry. The level of Nox-1 were calculated by normalizing with the level of β-Actin and the level set in control MCF10A cells as 1 (X, arbitrary unit). The level of specific phosphorylation of Erk1/2 (p-Erk1/2) was calculated by normalizing the level of p-Erk1/2 with the level of Erk1/2, then the level set in MCF10A cells as 1 (X, arbitrary unit). *Columns*, mean of triplicates; *bars*, standard deviation. The Student's *t* test was used to analyze statistical significance, indicated by ** $P < 0.01$, *** $P < 0.001$. All results are representative of three independent experiments.

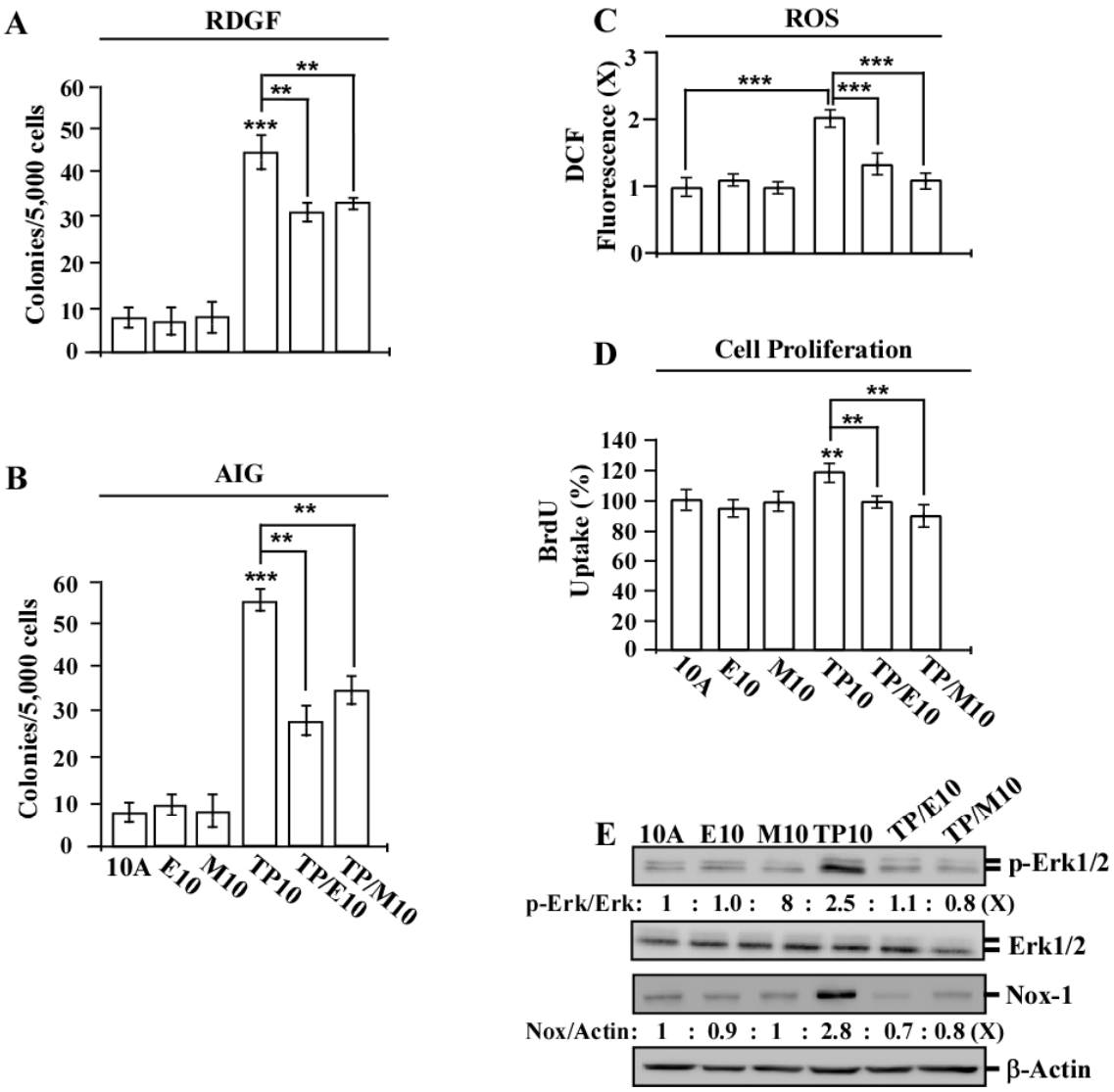

Figure 3.6. Intervention of TP-induced chronic breast cellular carcinogenesis by ergosterol and mimosine.

3.5 Discussion

Human body is constantly exposed to different kinds of pollutants on a day to day basis through a variety of ways for example, through diet and use of personal care products. Exposure to some of the known carcinogens like consumption of PhIP through over cooked meat has been shown to have the potential to cause breast carcinogenesis. Many of the constituents used in the manufacture of personal care products like TCC are under the scanner for their capability to cause breast cancer or act as a co-carcinogen whwereby they enhance the potential of other carcinogens like PhIP in causing breast cancer. In this study we wanted to determine whether TCC has the ability to enhance the initiation and progression of malignant breast cell carcinogenesis caused by PhIP. We have developed a model system in our lab which has shown a remarkable ability to detect induction and progression of chronic breast cell carcinogenesis by repeated exposures to physiologically relevant doses of environmental carcinogens like NNK, B[a]P and PhIP [11,12]. In contrast to other cell systems that utilize high dose regimens in the micromolar to millimolar ranges to acutely induce carcinogenesis, our model system relies on bio-achievable nanomolar to picomolar concentrations of carcinogens to study progressive cellular carcinogenesis [8,9,10,11,12]. In addition, our model system has shown the potential to identify naturally occurring agents that are easily consumed through daily diet at their non-cytotoxic doses, which have the capability to intervene breast cell carcinogenesis induced by repeated exposures to environmental agents. In the present study, we utilized our unique model system to identify the roles of mimosine and ergosterol in suppressing chronic breast cellular carcinogenesis induced by co-treatment of TCC and PhIP. Our model system also revealed the mechanisms behind the ability of

mimosine and ergosterol to target the process of chronic carcinogenesis induced by co-exposure to TCC and PhIP at biological, biochemical and molecular levels. Hence our model system will help in identification of additional carcinogens, co-carcinogens and preventive agents capable of induction and intervention of sporadic breast cancer.

Using certain biological, biochemical and molecular transient and constitutive targeted endpoints, we determined that TCC potentiated the ability of PhIP to initiate and progress the process of cellular carcinogenesis in MCF10A cells. MCF10A cells co-exposed to PhIP and TCC (TP20) acquired higher levels of cancer-associated properties, ROS generation potential, proliferation, migration and invasion capabilities as compared to P20 cells and these properties were comparable to that of MCF10A-Ras cells. Co-treatment with TCC and PhIP for 20 cycles did not increase Ras protein expression in TP20 cells when compared to Ras expression level in P20 cells. However, phosphorylated Mek, phosphorylated Erk and Nox-1 levels were enhanced leading to significantly higher ROS production and cell proliferation. Also, co-exposure of PhIP and TCC was able to induce ROS in a Ras-independent manner. Therefore, ROS generated via a Ras-independent manner was responsible for enhancing PhIP-induced activation of ERK pathway, ROS production, cell proliferation and DNA damage in cells simultaneously treated with TCC and PhIP. However, the mechanism behind Ras-independent induction of ROS that contributed towards enhancement of TP-induced carcinogenesis remains to be studied. Moreover, our study has shown that mimosine and ergosterol were able to block breast cell carcinogenesis induced by simultaneous treatment with PhIP and TCC. This study reveals for the first time the role of TCC as a co-carcinogen in enhancing PhIP-induced breast cell carcinogenesis in an in-vitro model.

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Chapter 4

4.1 Summary

An overwhelming majority of breast cancers are sporadic in nature attributable to chronic exposure to environmental factors, through a multi-year, multi-step, and multi-path process involving genetic and epigenetic alterations leading to progressive development from a non cancerous stage to premalignant and ultimately malignant breast cancer. Most current studies for induction of carcinogenesis involve use of high doses of carcinogens in their micromolar to millimolar doses to acutely induce cancers. However, this does not truly reflect the physiological process of carcinogenesis which involves low dose exposure to carcinogens. In the present study, I used a model that mirrors the actual carcinogenesis process by using a low dose exposure strategy in which normal breast epithelial MCFA cells are exposed to physiologically achievable doses of co-carcinogens and carcinogens over multiple passages (cycles). The experimental scheme which I used for my work is shown in Figure 4.1. MCF10A cells were exposed to either TCC, PhIP alone or together, in the absence or presence of preventive agents like curcumin, ergosterol or mimosine for 10-20 cycles. After the multiple exposures to either TCC or PhIP individually or together, the cells were analysed for acquisition of cancer associated properties of reduced dependence on growth factors and anchorage independent growth.

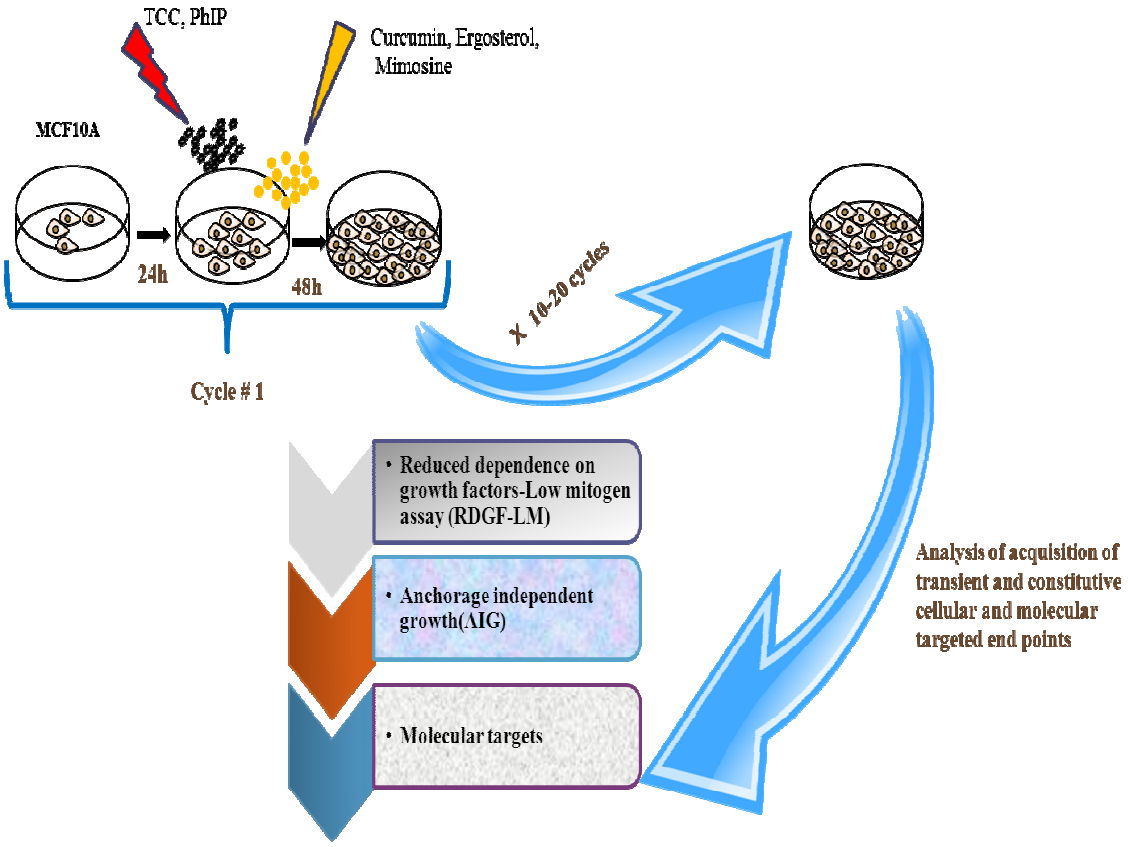

Figure 4.1 Experimental Scheme of induction of premalignant breast cellular carciniogenesis and intervention

Cells were also evaluated for acquisition of cetain molecular endpoints.as shown in Figure 4.2. These endpoints included activation of the Erk pathway with increased phospho-Erk and down stream Nox-1 protein levels, increased cell proliferation and ROS production. My hypothesis was that long-term exposure to TCC can induce breast cell carcinogenesis and co-exposure to TCC and PhIP can enhance PhIP-induced breast cell carcinogenesis which can be prevented using curcumin, ergosterol and mimosine**.** First of all, I wanted to determine if chronic exposure to physiologically achievable doses of TCC would cause breast epithelial cells to acquire the cancer-associated properties and also wanted to evaluate the potential of curcumin in the intervention of TCC-induced breast cell carcinogenesis. Working towards these objectives, I treated MCF10A cells with TCC for 10-20 cycles with three different doses of TCC including the bioachievable dose of 200 nM. It was observed that there was induction of acquisition of cancer-associated properties of reduced dependence on growth factors and anchorage–independent growth in cells treated with TCC in a dose and exposure dependent manner with activation of Erk-Nox pathway. These changes were constitutively induced in response to chronic TCC treatment. Even though physiologically achievable 200 nM was found to induce significant premalignant breast cell carcinogenesis related changes in MCF10A cells after 20 cycle of exposure, these T20 cells were not found to be tumorigenic in nude mice. Single exposure of TCC to MCF10A cells was also found to induce transient endpoints of Erk pathway activation, enhanced ROS production, cellular proliferation and DNA damge. Inhibiting Erk pathway and ROS suppressed transient as well as constitutive endpoints induced by TCC. Curcumin at noncytotoxic dose of 1uM

was tested for its ability to impede TCC-induced transient and constitutive endpoints in MCF10A cells.

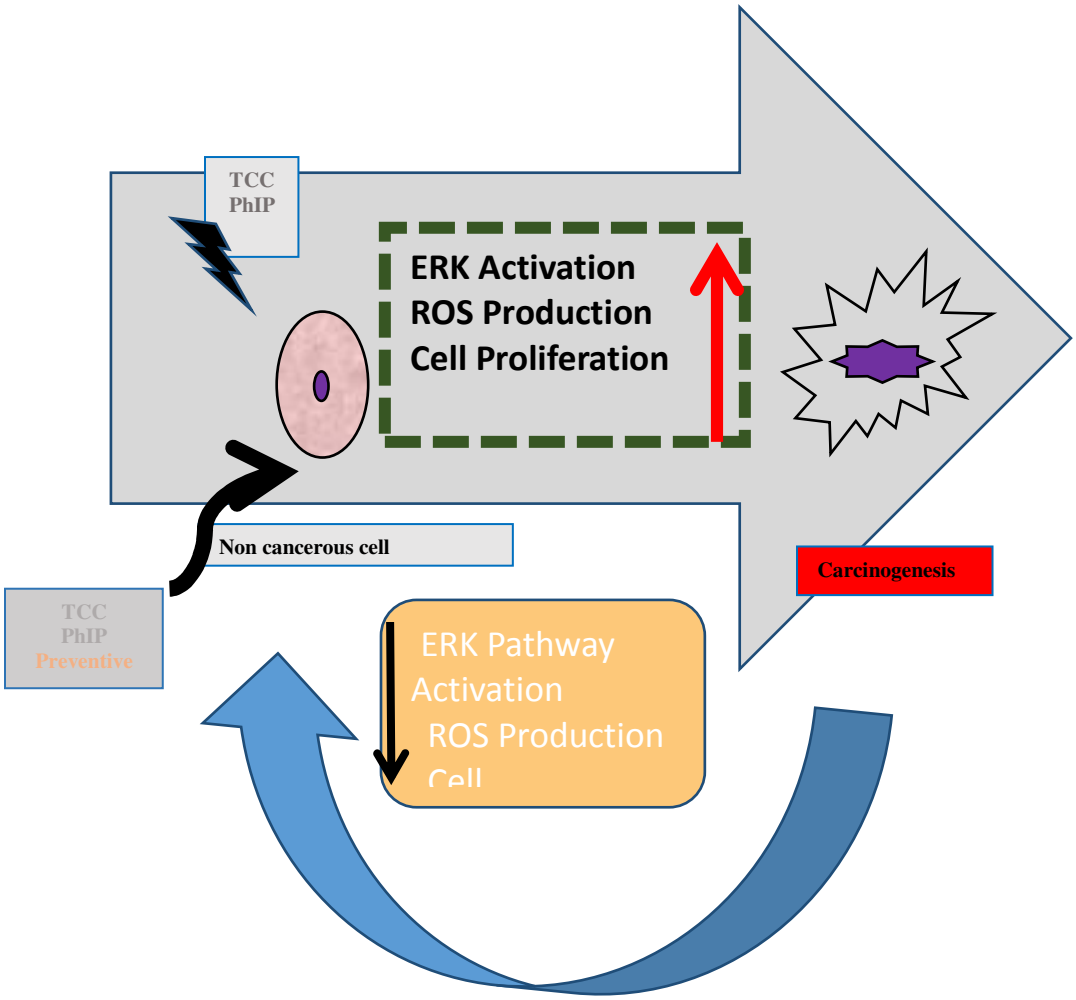

Figure 4.2 Targeted Molecular endpoints in breast cell carcinogenesis

It was observed that curcumin suppressed TCC-induced cancer associated properties of reduced dependence on growth factors, anchorage independent growth and also suppressed constitutive and transiently induced cell proliferation and ROS production, erk pathway activation. Co-treatment with curcumin also caused significant reduction in TCC induced DNA damage in MCF 10A cells.

To further study the potential of TCC to act as a co-carcinogen, I sought to determine if TCC could enhance PhIP-induced breast cell carcinogenesis and also wanted to determine whether mimosine and ergosterol could block breast cell carcinogenesis induced by co-treatment with TCC and PhIP. MCF10A cells were treated with bioachievable doses of PhIP and TCC either individually or together for 10 and 20 cycles. Validating previous results, I found that cells treated with either PhIP or TCC induced formation of significantly higher number of cell clones when compared to the untreated control cells. More importantly however, co-treating cells with PhIP and TCC significantly increased the level of cancer-associated properties acquired by these cells when compared to the cells treated with PhIP or TCC alone. Similarly, simultaneous TCC and PhIP treatment for twenty cycles was found to enhance migration, invasion, cell proliferation and ROS generation capabilities of cells treated for 20 cycles with PhIP alone. Chronic TCC and PhIP co-treatment was able to activate Erk-Nox pathway to a higher level as compared to cells treated with PhIP alone. These results indicated that TCC was able to significantly enhance breast cell carcinogenesis induced by PhIP alone. Erk pathway was found to play an important role in chronic breast cell carcinogenesis induced by co-exposure to TCC and PhIP since its inhibition blocked breast cell carcinogenesis induced by treatment with TCC and PhIP. To find the underlying

mechanism of breast cell carcinogenesis induced by the co-exposure to TCC and PhIP, cells were treated with TCC and PhIP for 24 h to see if transient end points of carcinogenesis were induced. The results indicated that co-treatment caused activation of Erk-Nox pathway in MCF10A cells to a higher level as compared to that induced by individual treatment with PhIP. In the similar manner, co-treatmet with both the compounds also induced significanty higher levels of ROS, cell proliferation and DNA damage as compared to cells treated with PhIP alone. These results proved that co-treatment with TCC and PhIP significantly potentiated the ability of PhIP to induce transient end points of breast cell carcinogenesis in MCF10A cells and thus contributed towards enhancing its ability to potentiate chronic breast cell carciniogenesis caused by co-exposure to TCC and PhIP. Similar changes were also seen when MCF7 and J82 cells were subjected to TCC and PhIP together for a single exposure. These results indicated that TCC was able to augment PhIP-induced constitutive and transient endpoints of breast cell carcinogenesis. Next, in order to evaluate the ability of mimosine and ergosterol to suppress breast cell carcinogenesis induced by co-exposure to TCC and PhIP, MCF10A cells were co-treated with TCC and PhIP in the absence and presence of non cytotoxic concentrations of ergosterol and mimosine in long term exposure for 10 cycles as well as short-term exposure for a single cycle. Results indicated that both mimosine and ergosterol inhibited acquisition of cancer-associated properties of anchorage-independent growth, reduced dependence on growth factors induced by co-exposure to TCC and PhIP. Simultaneous treatment with preventive agents also suppressed chronic increase in cell proliferation, ROS production and Erk-Nox activation induced upon co-treatment with TCC and PhIP. Transient endpoints of Erk pathway activation, ROS production, cell

proliferation and DNA damage were also suppressed by the two preventive agents. These results prove that ergosterol and mimosine were effective in suppressing breast cell carcinogensis induced by co-treatment with TCC and PhIP.

To conclude, this study has demonstrated that cumulative exposures of non cancerous human breast epithelial cells MCF10A, with physiologically achievable doses of triclocarban induced cancer-associated properties of reduced dependence on growth factors, anchorage-independent growth in MCF10A cells. The premalignant breast cellular carcinogenesis induced by TCC was characterized by activation of ERK pathway, ROS production, DNA damage and increased cellular proliferation. Curcumin, a dietary component of turmeric, easily ingested in the form of curry powder at non cytotoxic concentrations inhibited acquisition of cancer-associated properties in MCF10A cells and blocked pre-malignant carcinogenesis induced by cumulative exposures to TCC. TCC also enhanced PhIP–induced breast cell carcinogenesis by significantly increasing the degree of cancer-associated properties attained by cells treated with PhIP alone. Erk pathway activation played a major role in breast cell carcinogenesis induced by co-exposure to TCC and PhIP in MCF10A cells. Ergosterol and mimosine at their low nanomolar noncytotoxic concentrations intervened with this TCC and PhIP co-exposure induced breast cell carcinogenesis in a significant manner. The mechanism of breast cell carcinogenesis and prevention is summarized in the scheme shown in Figure 4.3. Exposure to TCC and PhIP induces increased expression of phospho-Erk in a Ras independent manner. Erk activation causes increased expression of downstream Nox-1 which leads to increase in ROS production.

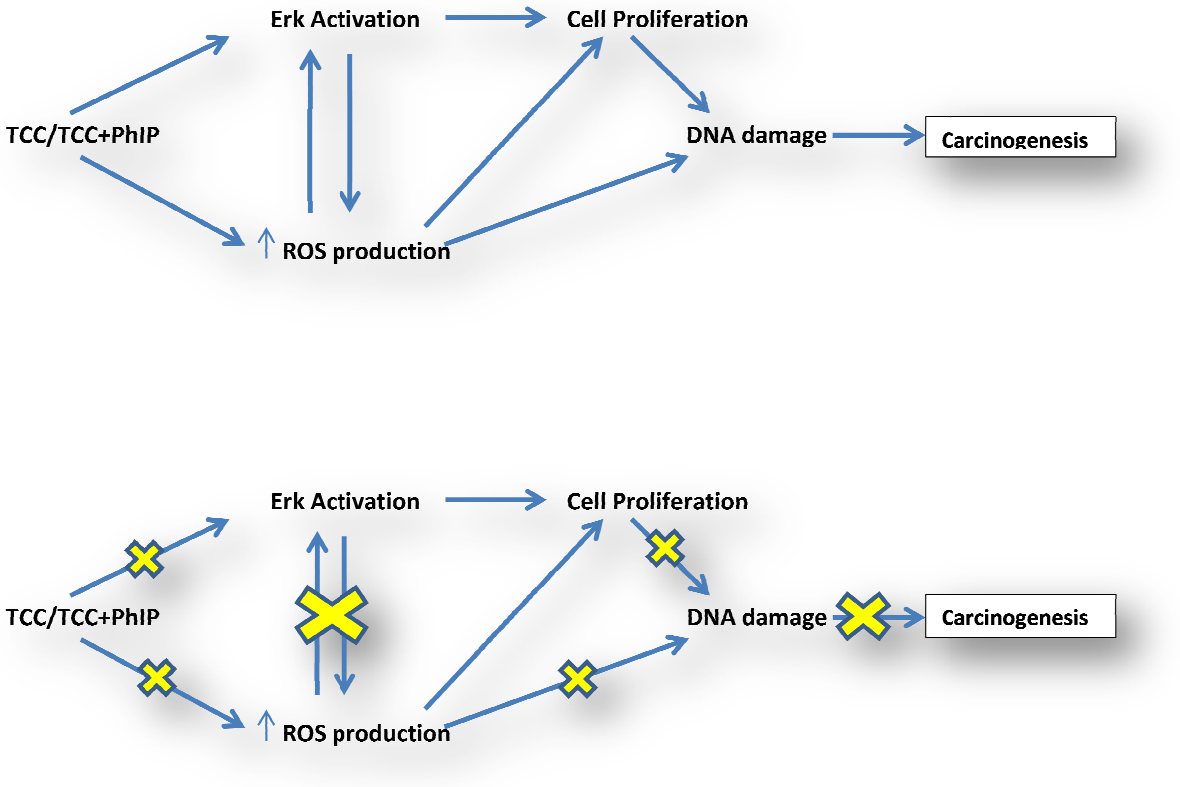

Figure 4.3 Schematic summary of breast carcinogenesis induced by TCC and PhIPand its intervention

Treatment with these compounds also causes increased ROS production in a Nox-1 independent manner possibly via mitochondriawhich also contributes towards activation of Ras independent Erk pathway. Finally, ROS production and Erk pathway activation causes increased cell proliferation leading to DNA damage and breast cell carcinogenesis. Use of preventive agents in the form of curcumin, ergosterol and mimosine intervened with Erk pathway activation, ROS production, increased cell proliferation, DNA damage, ultimately leading to suppression of breast cell carcinogenesis.

4.2 Future directions

Our study shows that TCC elevated ROS production in breast epithelial cells and induced the ERK pathway in a Ras independent manner. However, how this portion of ROS was induced by TCC in MCF10A and MCF7 cells and how ROS was able to induce Ras-independent activation of Mek1/2 and Erk1/2 remain to be determined. We have shown the co-exposure of MCF10A cells to TCC and PhIP at physiological concentrations for 20 cycles can enhance various invitro cancer-associated properties, but this could not potentiate tumorigenic potential of PhIP in mice models, therefore it is important to identify if further co-treatments with TCC and or treatment with other carcinogens will make cells significantly more tumorigenic than P20 cells. Also, it needs to be determined whether TCC can potentite PhIP-induced EMT (epithelial to mesenchymal transition) in MCF10A cells. For example, whether TCC can cause an increase in Aldehyde dehydrogenase (ADLH)-positive and mammosphere populations induced by PhIP treatment of MCF10A cells and alter other markers of metastasis induced by PhIP treatment like causing changes in expression of E-cadherin, Epithelial cell adhesion

molecule (EpCAM) , matrix metallo proteinase (MMP)-9, and vimentin. Also, it needs to be studied, whether there are other dietary agents that can intervene with TCC-induced or TP-induced breast cellular carcinogenesis.

VITA

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