INVOLVEMENT OF THE 5-LIPOXYGENASE PATHWAY IN ORO-ESOPHAGEAL CARCINOGENESIS AND CHEMOPREVENTIVE EFFECTS OF ZILEUTON AND GREEN TEA CATECHINS

by

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ABSTRACT OF THE DISSERTATION

Involvement of the 5-lipoxygenase pathway in oro-esophageal carcinogenesis and chemopreventive effects of zileuton and green tea catechins

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Oral and esophageal squamous cell carcinomas (SCC) are common neoplasms worldwide, especially in developing countries. Aberrant arachidonic acid (AA) metabolism, involving both the cyclooxygenase (Cox) and lipoxygenase (Lox) pathways has been suggested to play an important role in the development of inflammation and associated carcinogenesis.

The primary aim of this study was to evaluate the role of 5-Lox in oro-esophageal carcinogenesis. Zileuton, a 5-Lox inhibitor, was evaluated for its effect, in short- and long-term studies on oral carcinogenesis using the 7,12-dimethylbenz[*a*]anthracene (DMBA) induced hamster cheek pouch model. In the short-term study, zileuton

significantly inhibited aberrant AA metabolism and cell hyperproliferation and in the long-term experiment it suppressed the incidence of visible tumors as well as the SCC. Zileuton and green tea catechins, alone and in combination, were also evaluated in 4-nitroquinoline-1-oxide (4-NQO)-induced oro-esophageal carcinogenesis in mice. In the first experiment, 4-NQO was administered to A/J mice (wild-type and $p53^{A135V}$ mutant) in drinking water for 8 weeks, followed by zileuton (1000 ppm in diet) or polyphenon E (PPE, 0.6% in drink) for another 16 weeks. Zileuton significantly reduced the overall incidence of SCC in tongue, esophagus and forestomach in wild-type A/J mice. PPE only significantly reduced carcinogenesis in the tongue of $p53^{A135V}$ mutant mice. Both agents suppressed cell proliferation and induced apoptosis, while only zileuton significantly reduced the mast cells density in cancer tissues of tongue and esophagus. In the second experiment, 4-NQO was administered to C57BL/6J mice in drinking water for 12 weeks, followed by zileuton (500, 1000 and 2000 ppm in diet) and epigallocatechin-3-gallate (EGCG, 0.16% and 0.32% in drink) alone or in combination (500 ppm zileuton and 0.16% EGCG) for another 16 weeks. Both agents were able to significantly inhibit tongue cancer. Zileuton and EGCG inhibited overall carcinogenesis, though only the effect of the former was dose-dependent. An additive effect was observed in the combination group in inhibiting the tongue and overall cancer incidence. In summary, we report here that 5-Lox is involved in the pathogenesis of oro-esophageal carcinogenesis and its inhibitors, zileuton and green tea catechins, elicit chemopreventive activity in animal models.

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Introduction

Cancer

Cancer can be defined as “a pathological breakdown in the processes which control proliferation, differentiation and death of particular cells” [1]. It affects all communities worldwide with marked regional differences with respect to type and incidence. It is estimated that globally about 10 million people are diagnosed and 6 million die of cancer each year [1].

Cancer, a multifactorial disease, is caused by several factors acting alone or in combination with each other over an extended period of time. The etiology of cancer involves various factors such as personal habits (smoking, alcohol, sedentary lifestyle) [2,3], chronic infections (e.g. *Helicobacter pylori* increases the risk of stomach cancer) [4], environmental factors (occupational exposure, pollution, ultraviolet radiation, etc.) [5] and food contaminants such as mycotoxins [6]. Diet and nutrition also plays an important role in the development and progression of cancer. It has been shown that consumptions of a diet rich in fruits and vegetables can lower the incidence of various cancers [7].

Carcinogenesis is a multi-step process involving many molecular alterations. The four hallmarks of cancer are mutations, DNA lesions, impaired cell division and impaired defense system [8]. In the normal cell, a fine balance exists between growth promoting and inhibiting signals. This may be disrupted by various factors (chemicals, viruses and

radiation), leading to initiation of the carcinogenesis process. A schematic representation of multistage carcinogenesis model has been described in a recent publication [9].

Oral cancer

Oral cancer mainly includes cancers of the lip, tongue and buccal mucosa. It is usually included in the category of “Head and Neck” cancers. Globally it is the sixth most common cancer with about 500,000 new cases and 200,000 deaths reported each year [10,11] . More than 90% of oral cancer cases are squamous cell carcinoma (SCC) [12]. There is great regional disparity among the target population and it is more common in developing countries such as Sri Lanka, India, Brazil and Philippines, where it constitutes up to 25% of all cancers [13]. Tongue is the most common cancer site in Europe and Western countries, while in the Asian countries it is more prevalent in the buccal mucosa [14]. In the United States about 34,360 new cases and 7,550 deaths are expected in 2007 [15]. Despite improvements in radiotherapy and chemotherapy, the 5-year survival rate has not improved significantly over the past decade and remains around 50% [14].

There are several risk factors that contribute to development of oral SCC:

1) Tobacco: Tobacco use, both smoking and chewing, is the leading cause of oral cancer. Tobacco has more than 300 carcinogens and procarcinogens, of which nicotine is the most abundant [16]. These chemicals belong to various classes of compounds like polycyclic aromatic hydrocarbons, *N*-nitrosocompounds, *N*-nitrosamines, etc. Upon activation, these compounds can form DNA adducts, ultimately leading to tumor formation in the tissues. Almost 30% of all types of cancers, excluding skin cancer, have been attributed to the use of tobacco [2].

2) Alcohol: Alcohol, alone and in conjunction with tobacco, has been implicated in carcinogenesis of the upper aero-digestive tract [16]. Alcohol consumption together with tobacco accounts for about 20%-80% of cancers of oral cavity, pharynx and esophagus [17]. Alcohol, mostly in the form of ethanol, is metabolized by alcohol dehydrogenase (ADH) leading to the formation of acetaldehyde which results in the formation of free radicals, hydroxylation of DNA bases and cytotoxicity [16]. Acetaldehyde has been shown to be mutagenic in experimental model systems [18].
3) Betel quid chewing: This habit is mainly prevalent in Asian countries such as India, Taiwan, and Philippines. Betel quid consists of a mixture of areca nut, catechu, lime and tobacco which can cause chronic inflammation and oxidative stress leading to the formation of DNA adducts, cell proliferation and ultimately oral cancer. Ingredients like catechu have also been shown to be mutagenic and clastogenic [19].
4) Viruses: Strong evidence has shown that human papilloma virus, serotype 16 and 18 (HPV-16, 18) have been implicated in the pathogenesis of oral SCC. HPV-16 and -18 been have been detected in 22% and 14% of oral cancers cases respectively [12].
5) Diet: A diet rich in fats and oil and low in fruits and vegetable have been linked to a majority of cancers including oral and esophageal cancer. A diet rich in β-carotene, vitamin C and vitamin E have been shown to have a protective effect against oral cancer [16].

Esophageal cancer

Globally this cancer is ranked eighth among all the cancers and consists of two histological types, squamous cell carcinoma and adenocarcinoma. Worldwide about 400,000 cases occur each year of which almost 90% are squamous cell carcinoma [20]. The five-year survival rate is very poor and ranges around 15% [21]. In the United States (US), it is estimated that 15,560 cases and 13,940 deaths will occur in 2007 [15]. Esophageal SCC is mainly found in Asian countries while adenocarcinoma is known to occur more in the Western world. In the US, the rate of esophageal SCC has declined by 30% while the adenocarcinoma has seen a four-fold increase in the past few decades [22].

Esophageal and oral SCC share similar etiology, which includes tobacco and alcohol consumption and low intake of fruits and vegetables. Other factors may be consumption of food toxins, hot food and pickled vegetables [23]. The major cause for development of esophageal adenocarcinoma is believed to be persistent gastric reflux. The constant irritation, due to the reflux, in the distal part of the esophagus leads to the process of metaplasia in which the normal squamous epithelium gets converted to columnar type. This condition is called as Barrett’s esophagus and is known to be a precursor to esophageal adenocarcinoma [22].

Histopathological progression of oral and esophageal cancer

The epithelium of oral cavity and the esophagus is comprised of squamous cell. Cancers arising from the epithelium are known as carcinomas and hence this disease is known as oral or esophageal squamous cell carcinoma. The histopathological progression of oral and esophageal cancer is similar (Figure 1), and involves progression through multiple steps (hyperplasia, dysplasia, carcinoma). Hyperplasia can be defined as the increase in number of basal cells in the epithelium. Dysplasia can be determined by the morphology and structural arrangement of the cells in the tissue. It is characterized by irregular epithelial stratification, pleomorphism, increase in nuclear to cytoplasmic ratio and loss of polarity of the basal cells. When the dysplastic features occur in the entire thickness of the epithelium, the lesion is referred to as carcinoma *in situ* (CIS) and is known to be a preinvasive stage of cancer. Cancer formation is further characterized by rapid invasion of epithelial cells into the surrounding tissues and metastasis [24].

Molecular alterations in oral and esophageal SCC

Tumorigenesis is a complex process that is controlled by various genes, which affect several critical functions of the cells like cell division, differentiation and death. Proto-oncogenes and tumor suppressor genes are two types of genes whose activation or suppression can lead to tumor formation. Proto-oncogenes can be activated to give rise to oncogenes by gene mutation, deletion, amplification and translocation [25]. Proto-oncogene activation is known to be a dominant mechanism as its dysregulation, even in a single allele, can lead to gain of a specific function. Various oncogenes are implicated in carcinogenesis, such as *Ras* and *Myc*. Tumor suppressor genes act complementary to oncogenes through regulation of the cell cycle. These genes can be inactivated by point mutation, deletions or insertions and promoter methylation. Inactivation of these genes is known to be a recessive mechanism as alteration in both the alleles is required for the loss of its functional properties. Various tumor suppressor genes are implicated in carcinogenesis, including retinoblastoma (*Rb*), *p53* and *p16*.

There are several genes which are activated and deactivated during oral and esophageal carcinogenesis [23,25-28]. The most important oncogenes which are activated include:

1) Cyclin D1: This gene is involved in the cell cycle regulation and has been shown to be amplified in 18% to 58% of head and neck squamous cell carcinoma (HNSCC).
2) Epidermal growth factor receptor (*EGFR*): It is a trans-membrane glycoprotein belonging to the class of receptor tyrosine kinase. It regulates the cell growth

when stimulated by epidermal growth factor (EGF) and transforming growth factor α (TGF-α). EGFR has been shown to be overexpressed in about 43% to 90% of HNSCC cases [29].

3) *Ras*: This family includes three closely related genes: H-, K-, N- *ras*. In its normal function, *ras* proteins are activated by binding to guanosine triphosphate (GTP) and are inactivated following its hydrolysis to guanosine diphosphate (GDP). When the gene becomes mutated at specific sites, the mutated *ras* protein is always activated and hence sends uninterrupted signals to the nucleus. A high incidence, about 35%, of H-*ras* mutations in oral cancer has been found in Asian countries, especially India.
4) *Myc*: There are different members of the *myc* family (c-, n- and l-*myc*). Their overexpression have been linked to malignant growth in HNSCC in about 9 to 48% of the cases.
5) *Bcl-2* and *Bax*: These genes play an important role in the regulation of apoptosis. *Bcl-2* and *Bax* are known to inhibit and promote apoptosis, respectively. Data showing their involvement in HNSCC are conflicting. Some reports have suggested that their expression had no prognostic value in terms of clinical outcome. Other reports have indicated that a better prognosis was seen in patients, which showed high apoptotic index in tongue carcinoma.

Tumor suppressor genes which are involved in the progression of oral and esophageal cancers include:

1) *p53*: This is one of the most important genes involved in the development many types of cancers and its mutation is known to be an early event in oral and esophageal cancer. It is mainly involved in cell cycle regulation, DNA repair and apoptosis. It can be altered by mutations, deletions or insertions and is known to occur in about 60% HNSCC cases.
2) *p16*: This gene is involved in cell cycle regulation. It binds with cyclin dependent kinases 4 and 6 to form a complex, thus interfering with and inhibiting the cell cycle progression. Its inactivation leads to unchecked cell proliferation. The loss of expression of *p*16 has been detected in up to 67% of the HNSCC cases and is known to be an early event in oral and esophageal cancer.
3) Retinoblastoma (*Rb*): It is an important gene involved in the regulation of cell cycle progression. Mutation or inactivation of *Rb* can lead to its unchecked phosphorylation, thus aiding in cell cycle progression. It has been shown to be downregulated in about 6% to 74% of HNSCC cases in various studies. Loss of *Rb* expression has been associated with *p*53 alterations in human esophageal cancer [26].

Inflammation and cancer

Inflammation is the local reaction or response of the body to tissue injury or damage [30]. Cell or tissue damage may be caused by several agents such as heat, chemicals, radiation, microbial infection, trauma as well as certain immunological processes. To counteract the tissue damage, body has two types of defense, the primary defense system (immediate; such as inflammation) and the secondary defense system (controlled by the immunological system) [31]. Inflammation is commonly characterized by four cardinalsymptoms: redness, swelling, pain and heat, which is due to the "extravasation of plasma and infiltration of leukocytes into the site of inflammation" [30]. Upon tissue injury, temporary vasoconstriction of blood vessels takes place followed by local vasodilatation and increased capillary permeability, which can increase blood flow through the area leading to redness and heat. During this process, due to the increased permeability, fluid can accumulate in the interstitial space leading to edema. Also, leukocytes move through the walls of the blood vessels into the site of injury through a process of chemotaxis to attack the antigen. These processes are referred to as acute inflammation, during which release of various agents such as vasoactive peptide mediators [e.g., histamine, cytokines, chemokines and upregulation of enzymes such as cyclooxygenase 2 (Cox-2) and 5 lipoxygenase (5-Lox)] occurs [30]. Acute inflammation, if left unresolved over an extended period of time, due to dysregulation of various events, may lead to development of chronic inflammation which is characterized by tissue damage, hyperproliferation, pre-dominance of inflammatory cells in the vicinity of the injury, fibrosis, necrosis, etc. All the above events lead to the formation of a microenvironment which forms reactive oxygen and nitrogen species which damage the

DNA, various proteins and cell membrane ultimately leading to cancer development [32-34]. A proposed mechanism showing the histopathological progression from a normal tissue to carcinoma is shown in Figure 2 to elucidate the multi-stage carcinogenesis progression during a chronic inflammatory response.

Eicosanoids in cancer

Eicosanoids (which means having 20 carbons) are compounds derived from fatty acids such as arachidonic acid (AA). AA, a polyunsaturated fatty acid (PUFA), is not formed naturally in humans and thus has to be provided in the diet. It is mainly found esterified to the phospholipids in the cell membrane. It has four double bonds, which makes it highly susceptible to oxidation reactions. Its structure is shown in Figure 3.

Upon activation, cellular phospholipases such as phospholipase A_2 [through the phosphorylation of mitogen activated protein kinase (MAPK)] act on membrane phospholipids to release free arachidonic acid. This can then either be reincorporated into phospholipids or undergo metabolism through several pathways to generate a wide variety of mediators such as prostaglandins (by Cox pathway) and leukotrienes (by Lox pathway), which serve as important intra- and intercellular messengers. AA can also undergo metabolism through the cytochrome P-450s to give rise to epoxyeicosatrienoic acids (EETs) [35]. Among the various metabolic pathways of AA, the cyclooxygenase and lipoxygenase are the best studied and will be discussed in the following sections.

Cyclooxygenase pathway

Cyclooxygenase is an important bifunctional enzyme discovered in 1971 by Vane *et al* [36]. It incorporates two oxygen molecules, through its oxidase activity, in free arachidonic acid and leads to the formation of hydroperoxy endoperoxide (PGG2). Through its peroxide activity it then converts the PGG2 into an unstable hydroxy endoperoxide H2 (PGH2). PGH2 can be acted upon by the various synthases and isomerases to form biologically active prostanoids and thromboxane A2 (TXA2). Prostanoids are of different types depending upon the type of synthases that lead to their formation, e.g. prostaglandin E2 (PGE2) by PGE synthase. The cyclooxygenase pathway is shown in the Figure 5 [36]. Among the Cox pathway, PGE2 is the most important and best studied metabolite. Its structure is shown in Figure 5.

Different metabolites of the cyclooxygenase pathway have different functions. For example, TXA2 is a potent vasoconstrictor and stimulator of platelet aggregation while PGE2 is a vasodialator and a potent mediator of inflammation [37]. Presently, the cyclooxygenase enzyme is known to occur in three isoforms: Cox-1, -2, -3. Cox-1 is mainly expressed constitutively in the kidneys, endothelium, platelets, vascular smooth muscle and the gastrointestinal tract. Cox-2 is usually undetectable in most tissues and hence is known as an inducible form as it is up-regulated during the process of inflammation due to the production of cytokines and growth factors [38]. However, recent data have shown Cox-1 to be inducible in some specific cells and Cox-2 to be constitutively expressed in the kidneys and brain [38]. Cox-3 has also been identified recently in central nervous system and its inhibition by therapeutic doses of

acetaminophen has been shown to decrease the levels of PGE2 both *in vitro* and *in vivo* [39].

The role of Cox enzymes in the process of inflammation has been established. PGE2 is the most abundant mediator formed by the action of Cox enzyme on AA and can exert both homeostatic and inflammatory responses in the body [39]. It is known to cause various signs of inflammation like pain and fever, and because of this, it has been recognized as a target for pharmaceutical and dietary agents for several decades. Aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) have been used as analgesic and antipyretics for many years as they have the ability to decrease the production of PGE2 by inhibiting the Cox enzyme [40]. However, long-term use of these drugs may lead to gastrointestinal bleeding and renal toxicity, as they not only inhibit the Cox-2 enzyme but also Cox-1 which has been shown to possess protective functions [40]. The above adverse effects have led to the development of various Cox-2 selective inhibitors such as celecoxib and rofecoxib which can alleviate pain and reduce the risk of various cancers with minimal toxicity [38]. But recently, it was shown that this class of inhibitors, if used for long time, can lead to cardiovascular side effects [41]. Celecoxib was the most commonly used drug among the class of Cox-2 inhibitors, and its structure is shown in Figure 6.

There is a large body of evidence, both *in vitro* and *in vivo,* which has shown a link between the Cox-2 enzyme and various types of cancers. Cox-2 has been shown to be up-regulated in various types of cancers including oral, esophageal, colon, liver,

pancreas, breast, lung and skin [42]. The role of Cox-2 in carcinogenesis has been evaluated in numerous animal and cell culture studies. Its overexpression has been associated with cell hyperproliferation, metastasis, angiogenesis, resistance to apoptosis, immunosupression and increased invasiveness. [43-46].

Lipoxygenase pathway

Lipoxygenases (Lox) are a group of iron containing enzymes whose activity was first observed in 1975 in rabbit reticulocytes [47]. They are also called dioxygenases because they can insert two molecules of oxygen into various PUFAs like arachidonic and linoleic acid, leading to the formation of different metabolites depending on the position of insertion of the oxygen molecule. In the mammalian cells, arachidonic acid is the major substrate which gives rise to various metabolites such as leukotrienes and cysteinyl leukotienes. When linoleic acid is the substrate it can be only acted upon by 15-Lox, the end product being 13-hydroxyoctadecadienoic acid (13-HODE) [48]. It has been shown in various studies that 5-, 8-, 12- Lox have procarcinogenic functions while 15-Lox has anti-carcinogenic activity [48].

Among all the lipoxygenases 5-Lox is the best studied. It converts AA to 5-hydroperoxyeicosatetraenoic acid (5-HPETE), which further gives rise to leukotriene A4 (LTA4). As this intermediate is unstable; it can undergo enzymatic hydrolysis by LTA4 hydrolase (LTA4H) to give rise to leukotriene B4 (LTB4) or it can conjugate with glutathione in the presence of leukotriene C4 synthase (LTC4S) to form leukotriene C4 (LTC4). LTC4 can undergo successive eliminations of glutamic acid and glycine by the action of γ-glutamyl tranferase and dipeptidase to give leukotriene D4 (LTD4) and leukotriene E4 (LTE4), respectively. The HPETE generated from 5-Lox can also be converted to 5-hydroxyeicosatetraenoic acid (5-HETE), which can further be dehydrogenated to form 5-oxo-ETE. By the action of 12- and 15-Lox LTA4 can also be converted into lipoxin A4 (LXA4) and lipoxin B4 (LXB4) respectively. LTB4 and

cysteinyl LTs elicits their physiological functions via specific receptors (e.g. BLT1, BLT2 and CysLT1, CysLT2 respectively) [38]. The general scheme of lipoxygenase pathway is shown in Figure 7. Among the various metabolites of lipoxygenase pathway, LTB4 is the most potent agent and best characterized. Its structure is shown in Figure 8.

LTB4 is a strong chemotactic agent involved in the recruitment and activation of neutrophils, macrophages and eosinophils. It also assists in the migration of leukocytes to the site of inflammation by promoting their adhesion to the vascular endothelium. By activating eosinophils, it may also lead to oxidative stress by releasing singlet oxygen and hydrogen peroxide. LTB4 also contributes to the immune reaction by stimulating the release of pro-inflammatory cytokines [36,49]. The CysLTs play an important role in diseases like asthma and allergic rhinitis. They are potent bronchoconstrictors and induce airway edema during an asthma attack. They can also lead to mucus production and bronchial smooth muscle cell proliferation [38]. 5-HETE and 5-oxo-ETE were also shown to possess pro-inflammatory activity *in vivo* [50]. Lipoxins e.g. LXA4 and LXB4 are compounds which have been also shown to possess anti-inflammatory activity [51].

A link between the enzymes, metabolites and receptors involved in the 5-Lox pathway has been established in inflammation and a variety of cancers. Various cancer cell lines such as lung and prostrate have shown overexpression of 5-Lox [52-54]. Also, many *in vivo* studies have shown 5-Lox to be overexpressed in a variety of cancers including colon, pancreas, testis, urinary bladder and prostrate [55-59]. In colon cancer, 5-Lox over-expression was negatively associated with clinical prognosis [57]. In

pancreatic cancer cells, 5-Lox played an important role in mediating oxidative stress and cell survival induced by extracellular matrix [60]. Inhibitors of the 5-Lox pathway were shown to possess anti-proliferative and pro-apoptotic activities in various cancer cells [54,61-64]. *In vivo*, these inhibitors suppressed carcinogenesis in animal models of lung [65], skin [66], and pancreas [67,68]. In the oral cavity, LTB4 was found to be 10- to 30-fold higher in hamster and human squamous cell carcinoma (SCC) than in normal tissues [69]. Various inhibitors of 5-Lox pathway such as zileuton, have been shown to be chemopreventive *in vitro* and *in vivo* studies, further supporting its relationship with carcinogenesis [59,65-68,70-75]. LTA4H was also shown to be overexpressed in cancers of colon, lung and esophagus and its inhibitor, bestatin, showed chemopreventive activity in an *in vivo* study [76]. The above data suggest an important role of the 5-Lox pathway in the pathogenesis of various cancers.

The physiological functions of 5-Lox and LTA4H are less critical than Cox-2. The only change observed in 5-Lox knockout mice was increased resistance to inflammation and certain inflammation associated diseases, but there was increased susceptibility to some infections [77-79]. However, Cox-2 knockout mice have shown to die early of renal abnormalities [80,81]. Thus it might be reasonable to use 5-Lox inhibitors, due to fewer side effects, instead of Cox-2 inhibitors as the former will not only inhibit inflammation but also the associated carcinogenesis.

Zileuton

As 5-Lox has been implicated in a variety of inflammatory disorders and associated carcinogenesis, its inhibition might be a good target for preventing many diseases. 5-Lox is a non-heme iron containing monomeric protein of 75-80 kD and has about 673 amino acids. It is present in many inflammatory cells like polymorphonuclear leukocytes, monocytes and macrophages. In the resting phase it is localized in the cytosol and in some cell types in the nucleus. Irrespective of its location in unstimulated cells, it is translocated to the nuclear envelope upon activation. There are number of important steps involved in the activation of 5-Lox, including binding with calcium ions, stimulation by ATP in the presence of calcium and oxidation of the inactive ferrous form of iron to the active ferric state in the active site of the enzyme [82]. Once 5-Lox translocates to the nuclear envelope, it interacts with a protein known as 5-Lox activating protein (FLAP) which helps to transfer the substrate AA to 5-Lox for synthesis of variety of mediators [36].

Various approaches have been designed to inhibit the 5-Lox pathway. They are divided into three categories according to how 5-Lox is activated [36].

1) Redox inhibitors or anti-oxidants: This type of inhibitors act by reducing the iron in the active site, thereby disturbing the catalytic cycle of this enzyme. These agents are generally small lipophilic molecules such as phenols and caffeic acid which show inhibitory activity in various experiments, e.g. AA861. But due to the lack of specificity they led to many side effects, e.g. formation of methaemoglobin and genotoxicity.

2) Non-redox competitive inhibitors: This type of inhibitors (e.g. methoxyalkylthiazoles) competes with AA for binding to 5-Lox. Due to their pharmacokinetic profile such as poor solubility and short half-life, this class of inhibitors was not developed further.
3) Iron-chelating agents: This class of inhibitors contains hydroxamic acid or *N*-hydroxy urea functional groups. They act by chelating the iron in the active site of 5-Lox so as to make it unavailable for catalytic reactions. The inhibitors that contained hydroxamic acid were found to be effective *in vitro* but due to their rapid inactivation to form carboxylate group and production of nitroxide radicle *in vivo*, this group was not developed further. The compounds containing N-hydroxy urea were found to be more stable and had less glucoronidation. The most prominent of this class of compounds was zileuton structure of which is shown in Figure 9.

Zileuton was shown to inhibit LTB4 production in various *in vitro*, *ex vivo* and *in vivo* experiments [83]. It inhibited LTB4 production from rat and human polymorphonuclear leukocytes at IC_{50} values of 0.4 μM. Doses of 0.5 to 5 mg/kg and 2 mg/kg (p.o), in dogs and rats respectively, led to decrease in synthesis of LTB4 in blood *ex vivo*. It was also shown to inhibit AA induced edema in a mouse ear inflammatory model. In a Phase I trial, the blood samples collected from patients dosed with zileuton showed a dose-dependent inhibition of LTB4 production *ex vivo* [84]. A single dose of 800 mg showed inhibition of greater than 80% in LTB4 biosynthesis in this study. Zileuton also showed inhibitory activity by decreasing the

nasal LTB4 levels in patients with allergic rhinitis. In ulcerative colitis patients, a single dose of zileuton led to reduction in the levels of LTB4.

Zileuton was marketed under the trade name of *Zyflo* for the treatment of asthma. Although it has been shown to ameliorate the symptoms of asthma effectively, it has not been of much benefit in other diseases such as rheumatoid arthritis. Apart from its beneficial effects this compound also has some adverse effects such as hepatotoxicity and adverse drug interactions [85-87]. Zileuton has also been shown to be chemopreventive in animal models of esophagus, pancreas and lung cancers [65,67,68,75]. Zileuton is mainly metabolized in the liver by glucoronidation. It has a half-life of 2.3 hours with about 80% of drug being eventually excreted in the form of glucoronide metabolites in urine [88,89].

Tea

Tea, obtained from the dried leaves of the plant *Camellia sinensis*, is widely consumed throughout the world. It can be of different varieties depending upon the manufacturing process; e.g. green, black and oolong tea. The major chemical constituents of fresh tea leaves that make up to 30-40% of its dry weight are polyphenols such as flavanoids, flavandiols and phenolic acid. Catechins, which belong to the class of flavan-3-ols or flavanols, are the major flavanoid present in tea and constitute 20% of its dry weight. Flavonols such as quercetin and myricitin and their glycosides are also present in tea. Alkaloids such as caffeine, theobromine and theophylline constitute 5% while amino acids (arginine, γ-amino butyric acid, theanine) constitute 4% of the dry weight [90].

Green tea is mostly consumed in Asian countries, such as China and Japan, and constitutes about 20% of the tea manufactured worldwide. Its manufacturing involves rapid steaming or pan frying of freshly picked tea leaves so as to inactivate the enzyme polyphenol oxidase, thus preventing oxidation or fermentation and producing the dry final product having a moisture content of about 5% [91]. The composition of green tea is similar to that of fresh tea leaves in which catechins represent the majority of the polyphenolic content. The structures of green tea catechins are shown in Figure 10.

Green tea has been proposed to have numerous beneficial effects. This topic has been reviewed extensively in many research articles and books. Green tea has been proposed to inhibit hypertension, hyperglycemia, hypercholesterolemia, bacterial and viral activity, mutagenesis and carcinogenesis [92]. Epigallocatechin gallate (EGCG) is

the major catechin that has been widely studied for its biological activities. EGCG has only limited bioavailability, which is attributed to its high molecular weight and presence of many hydroxyl groups. EGCG and other catechins are known to be metabolized by methylation, sulfation and glucoronidation reactions [93]. It was shown in a human study that after the consumption of catechins (Polyphenon E and EGCG), EGC and EC were mainly present in the conjugated form while EGCG was found in the free form in plasma [94]. EGCG has been shown to be excreted through the bile while EGC and EC are excreted via the bile and urine mostly in the form of glucoronide and sulfate conjugates [95,96]. The metabolites of EGCG with methylation at different positions have been found in the bile of rats. The conversion of EGCG to EGC and ECG to EC may take place in the intestine by microorganisms, which are known to produce ring fusion products valerolactones of EGC and EC [97]. These products have been detected in urine and plasma, and in some cases their level was even higher than their precursor parent compounds [96].

Pharmacokinetic studies on catechins have been conducted in mice, rats and humans. In one study in mice and rats, 0.6% of green tea polyphenol preparation was administered as drinking fluid [98]. In rats, after the administration of the polyphenol preparation, the levels of EGC and EC increased over time in plasma but decreased with prolonged exposure. Low and high levels of EGCG were found in plasma and feaces respectively, when compared to other catechins. In mice also, an initial increase and the subsequent decrease of catechins was also observed but the levels of EGCG were much higher when compared to rats. The peak plasma level in mice was 2.3 μM. In a study

involving consumption of a tea preparation by humans, the plasma levels of EGCG were slightly higher than EGC and the pharmacokinetics were similar to mice rather than the rats [98]. In a separate human study, after consumption of 1.5 gms of decaffeinated green tea solids in 500 ml of water, the peak plasma concentration of EGCG, EGC and EC were 326 ng/ml, 550 ng/ml and 190 ng/ml, respectively. These peak values were attained within 1.5-2.4 hrs of tea consumption, with the half-life of EGCG (5 hrs) reported to be higher than those of other catechins [99]. It has also been reported that by holding green tea leaves in the mouth for 2-5 min, high levels of catechins (2.2-131 μM) are observed in saliva with a half-life of 25-44 minutes [100]. These catechin levels are higher when compared to those in plasma after oral administration. In the oral cavity EGCG can be converted to EGC by esterases. Both these catechins can be absorbed through the oral mucosa and may account in some part to the total levels of plasma catechins [101].

Green tea and cancer

Green tea catechins have been shown in numerous *in vitro* and *in vivo* studies to possess chemopreventive activity. Tea has been shown to act on the initiation, promotion and progression phases of cancer development. Green tea has inhibitory effects on carcinogenesis in animal models of various types of cancers such as skin, esophagus, oral cavity, lung, fore-stomach, colon, liver and small intestine [102]. It is generally believed that since the tea constituents have direct contact with the gastrointestinal tract, they may show better chemopreventive activity in organs like the oral cavity, esophagus and intestine when compared to other sites. Epigallocatechin-3-gallate (EGCG) and polyphenon E (PPE, a mixture of various green tea catechins having approximately 65%

EGCG) are the two most well studied preparations among the green tea constituents. In a DMBA-induced oral carcinogenesis study in hamsters, various green tea preparations were shown to inhibit the mean tumor burden, and the incidence of dysplasia and oral SCC [103]. Tea preparations led to a reduction in number of micronucleated and proliferative cells. In another study, 0.6% green tea led to a significant reduction in number and volume of visible tumors in the buccal pouch of hamsters [104]. In addition, tea treatment led to an increase in apoptosis and decrease in cell proliferation. In a study by Mohan *et al*, PPE led to inhibition of both, the incidence of visible tumors and SCC in the buccal pouch of hamsters [105]. In a 4-NQO-induced oral carcinogenesis study in rats, supplementation of green tea polyphenols, led to increase in cellular status of certain anti-oxidants such as reduced glutathione, thereby reducing the chance of cancer development [106]. Green tea was also shown to be effective in inhibiting nitrosomethylbenznelamine-induced esophageal carcinogenesis in rats [107].

Various mechanisms have been proposed for the chemopreventive activity of green tea catechins, such as inhibition of arachidonic acid metabolism, anti-oxidant activity, modulation of enzymes and induction of apoptosis. In addition, green tea has also been shown to inhibit enzymes such as MAP kinases, DNA methyltransferases, dihydrofolate reductases and telomerases. Recently, this topic has been extensively reviewed in several articles [108,109].

The results of epidemiology data on tea consumption in human have been conflicting. Various studies conducted in Japan and China showed a beneficial effect of

tea consumption on esophageal and gastric cancer. But these results were not consistent with other reports. One such report on the data collected from a review of 21 epidemiological studies revealed that green tea had no beneficial effect on stomach and intestinal cancer [110]. In another study, women of Asian origin living in Los Angeles, who consumed green tea had a significant reduced risk of breast cancer [111]. Consistent with this study, women in Japan who drank more than ten cups of tea per day had a lower risk of breast cancer [112]. However, it was reported recently that there was no reduction in risk of breast cancer among women who drank five cups of green tea per day when compared to those who drank one cup per day [113]. Studies have also been conducted to determine the association between green tea consumption and prostate cancer risk. A case-controlled study in China showed that green tea consumption had a lower risk of prostate cancer development in patients with prostatic adenocarcinoma [114]. However, a recently conducted study in Japanese men found no association between green tea consumption and prostate cancer risk [115].

Epidermal growth factor receptor (EGFR) pathway

Tyrosine kinases are enzymes, which transfer phosphate groups from ATP to the tyrosine residues in the polypeptide chain. They consist of a subclass of transmembranous proteins which includes four members namely, epidermal growth factor receptor (EGFR or ErbB1), ErbB2, ErbB3 and ErbB4 which are known to regulate cell proliferation, differentiation, migration, angiogenesis and apoptosis [29,116,117]. When activated by various ligands, these receptors homo- or hetro-dimerize to form active tyrosine kinases, and activate various downstream signaling molecules, such as MAPKs, PI3K and STATs [118]. Targeted disruption of EGFR inhibited the development of intestinal adenoma in $Apc^{Min/+}$ mice and the development of human papillomavirus-immortalized keratinocytes [119,120].

EGFR is expressed in normal oral squamous epithelial cells of humans and hamsters [103,121]. Amplification and overexpression of EGFR were shown to be early events during oral carcinogenesis, and paralleled the progression of the disease [122]. Expression levels of EGFR, ErbB2 and ErbB3 together have stronger prognostic value than any one of them alone [123-125]. Among the four EGFR family members, EGFR and ErbB2 are most frequently co-expressed in the same cell layer of neoplastic epithelium [126]. Patients who exhibit overexpression of both EGFR and ErbB2 tend to have more aggressive tumors and poorer clinical prognosis when compared to those expressing either one of them [123], suggesting an important role of the EGFR/ErbB2 heterodimer in oral carcinogenesis. In addition, it was shown recently that hamsters vaccinated in the buccal pouch with a DNA coding for the ErbB2 receptor elicited an

antibody response that suppressed the development and progression of SCC [127]. Both *in vitro* and *in vivo* data have suggested that the dual inhibition of both EGFR and ErbB2 is more effective in cancer therapy than agents targeting individual receptors [128].

There is ample evidence in the literature to suggest cross-talk between the EGFR signaling cascade and AA metabolism. EGFR mediated activation of MAPK has been shown to upregulate Cox-2 gene transcription, while Cox-2 derived PGE2 can stimulate cell proliferation through EGFR transactivation [42]. It has been reported earlier that AA metabolites act as secondary messengers during EGF-induced cytoskeletal changes in rat fibroblasts and HeLa cells [129]. Also in another study, an anti-EGF monoclonal antibody was able to reduce the levels of PGE2 in human amnion cells [130]. Moreover, combined use of both EGFR and Cox-2 inhibitors has shown synergistic effects in cancer therapy [131,132].

Topical delivery of agents for oral cancer chemoprevention

Oral mucosa consists of an outer sheet of stratified squamous epithelium (40-50 cell layers thick) resting on a basement membrane. There are three histological types of oral epithelium: keratinizing squamous epithelium (e.g., gingiva, hard palate), non-keratinizing squamous epithelium (e.g., floor of mouth, buccal wall), and specialized epithelium (dorsum of tongue) [133]. The permeability to topically applied agents differs according to the thickness of the epithelium and the extent of keratinization (sublingual < buccal < palatal). As compared to skin, buccal mucosa is 4-4000 times more permeable [134]. There are two routes of drug transport into the oral mucosa, paracellular and transcellular, with the paracellular route as the major one. Lipophilic drugs are more likely to be absorbed through the transcellular route than hydrophilic drugs. However, only a small amount of the topically applied agent can be absorbed directly into oral mucosa, while the majority is absorbed through the gastrointestinal tract when washed down by saliva [135,136]. In theory, the transcellular route is preferred over the paracellular route to reach an intracellular target for oral cancer prevention, even though the agents absorbed through the paracellular route may also be effective after reaching the blood stream through the vasculature.

Topical delivery of chemopreventive agents avoids the first pass effect and problems related to gastrointestinal absorption. However, a high concentration may be required for a topically applied agent to exert its specific biochemical effect, and chemopreventive effect thereafter. Pharmacokinetics of the topically applied agent may become less predictable. Some drugs may not be suitable for topical delivery at all. Here

we describe a theoretical approach for choosing topically applied non-steroidal anti-inflammatory drugs (NSAIDs) to target cyclooxygenase 2 (Cox2) for oral cancer chemoprevention. For the high-risk patients on long-term treatment with a Cox-2 inhibitor, topical delivery appears very attractive in order to minimize the gastrointestinal, cardiovascular and renal toxicities, due to which rofecoxib has been recently withdrawn from the market by Merck & Co., Inc.

Absorption of the topically applied agents into the oral mucosa is a process of passive diffusion. In theory, the total flux through the oral mucosa (J_{max}) can be estimated by adding the transcellular flux (J_{TC}) and the paracellular flux (J_{PC}). We modify the formulas of Zhang and Robinson's as follows [137].

$$J_{max} = J_{TC} + J_{PC}$$

$$J_{TC} = \frac{(1-e)}{h_{TC}} \cdot D_{TC} \cdot \log P \cdot C$$

$$J_{PC} = \frac{e}{h_{PC}} \cdot D_{PC} \cdot C$$

e: the faction of surface area of the paracellular route; D_{TC}: the diffusion coefficient through the transcellular route; log*P*: the partition coefficient between the lipophilic and hydrophilic region; C: the concentration of the topically applied agent on oral mucosa; h_{TC}: the path length of the transcellular route; D_{PC}: the diffusion coefficient in the intercellular space; h_{PC}: the path length of the paracellular route.

Hypothesis

Aberrant AA metabolism has been associated with inflammation and the associated carcinogenesis. However, most studies have focused on the Cox2 pathway. The objective of my research was to investigate the role of 5-Lox in the development of oral and esophageal SCC and to determine whether its inhibition would lead to a chemopreventive effect. My hypothesis is as follows:

"5-*Lox is overexpressed and plays an important role in the pathogenesis of oral and esophageal SCC. Inhibition of 5-Lox activity by zileuton and green tea catechins can elicit chemopreventive activity in animal models"*.

In order to test the above hypothesis, following experiments will be conducted:

1) A short- and long-term DMBA-induced hamster oral cancer experiment will be conducted to study the expression of 5-Lox and test the efficacy of its inhibitor, zileuton, alone and in combination with a Cox2 inhibitor, celecoxib.
2) Long-term studies in 4-NQO-induced mouse oro-esophageal cancer model will be undertaken to study the expression of 5-Lox. Both, zileuton and green tea catechins would be evaluated alone and in combination for their chemopreventive activity.

Materials and Methods

Chemicals

DMBA, 4-NQO and GW2974 were purchased from Sigma, St. Louis, MO. Zileuton was purchased from Abbott Laboratories, Abbott Park, IL. Celecoxib was purchased from LTK Laboratories, Inc., St. Paul, MN. AA861 was purchased from Biomol International (Plymouth Meeting, PA). Zileuton and celecoxib were mixed with AIN-93M diet at pre-desired concentrations. All diets were made at Research Diets, New Brunswick, NJ. Polyphenon E and EGCG were obtained as gifts from Mitsui Norin Co. Ltd. (Tokyo, Japan). PPE preparation contained w/w of 65.6 % EGCG, 9% epicatechin, 3.2% epigallocatechin, 7.6% epicatechingallate, 4.3% gallocatechin gallate, 0.6% caffine and other catechins in minor concentrations, as per supplier's instructions.

Cell line

Tongue SCC cell line (CAL27) was purchased from American Type Culture Collection (ATCC, Manassas, VA). The cells were maintained in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 0.1 mg/ml streptomycin in a 5% carbon dioxide atmosphere at 37 ºC and 95% relative humidity. The cells were sub-cultured every 48 hours.

Hamster oral cancer model

This model has been used extensively over the past five decades to study the pathogenesis of oral cancer [138]. The chemical used to induce carcinogenesis is 7, 12-dimethylbenz[*a*]anthracene (DMBA), a lipid soluble synthetic compound belonging to the class of polycyclic aromatic hydrocarbons. This compound is known to be oxidized by the cytochrome P450 enzyme system to produce DMBA-3,4-epoxide. This is hydrolyzed by epoxide hydrolases to the corresponding diol, which is further oxidized by CYP1B1 and CYP1A1 to DMBA-3,4-diol-1,2-epoxide. This compound is the ultimate carcinogen and has been shown to produce DNA adducts. The general scheme of DMBA activation is given in Figure 11 [139].

The hamster oral cancer model was developed in 1954 by Sally and co-workers [140]. They reported that application of DMBA three times per week for sixteen weeks, led to the development of tumors in their buccal cavity. Since its introduction, numerous researchers have modified this model to suit their respective research goals. This model initially was of two types: a complete model and a two-stage model [141]. As mentioned earlier, the duration of the complete model was sixteen weeks and only DMBA (0.5% 3 times per week) was used to induce cancer development. The two-stage model was for 45 weeks, in which DMBA was applied for the first 2 weeks and then a promoter like 12-O-tetradecanoylphorbol-13-acetate (TPA) or benzoyl peroxide for another 43 weeks (40 mg/200 µl acetone) [141,142]. Recently, we modified the protocol to establish a post-initiation model which can mimic former smokers [104]. DMBA (0.5%, 3 times per week, 6 weeks) was topically applied leading to the development of hyperplasia and

dysplasia in 100% and 75% of the animals, respectively, at the end of six weeks. Without further DMBA treatment, 77% of the animals developed SCC by Week 24.

The DMBA induced hamster oral cancer model has advantages and disadvantages [138,143]. The major advantage of this model is its similarity in histopathological, molecular and biochemical alterations to human oral cancer, such as changes in p53 and ras genes, expression of γ-glutamyl transpeptidase and overexpression of growth factors like epidermal growth factor. [143]. The other advantages include easy accessibility to the cheek pouch, lack of a requirement for anesthesia during animal treatment, simplicity of application and large amount of sample available for various analysis. It has also been used to test the chemopreventive activity of various agents. The major disadvantages are lack of similar anatomical structure in humans, reduced vascular drainage in the buccal pouch, non-occurrence of metastasis and excessive handling of the carcinogen.

Mouse oral and esophageal cancer model

The mouse model for oral cancer was developed as an alternative to the hamster oral cancer model. This model uses 4-nitroquinoline-1-oxide (4-NQO) as the chemical carcinogen for inducing the tumors. 4-NQO is a water-soluble, synthetic compound belonging to the class of quinoline derivatives, first synthesized by Ochiai and co-workers in 1945 [144]. This carcinogen can be administered either by painting or in drinking fluid, and undergoes activation by reduction through nitro- and quinone reductases to form 4-hydroxylamino-quinoline-1-oxide (4HAQO). This is considered the ultimate carcinogen and can form various DNA adducts [145,146]. 4-NQO can be

detoxified by conjugation with glutathione-S-transferase (GSTP1). The general scheme of its activation is shown in Figure 12.

4-NQO induces tumor formation through multiple pathways [147]. It can undergo redox cycling leading to oxidative stress, forms DNA adducts specifically at the guanine residue, causes adenosine substitution for guanosine and DNA strand breaks. The first successful use of 4-NQO to induce oral cancer was conducted in rats by Wallenius *et al* in 1973 [148]. They painted 0.5% 4-NQO in propylene glycol 3 times per week to the rat palate to induce tumors. Since then various models have been established and have been modified. The first mouse model using 4-NQO to study oral cancer was established by Hawkins *el al* in 1994. They administered 4-NQO by painting the palate three times a week for sixteen weeks and reported that a variety of lesions are formed, the severity of which increases with the increase in duration of carcinogen treatment [149]. Recently, Tang *et al* established an oro-esophageal cancer model in which 4-NQO was administered by painting or drinking method in C57 and CB mice [150]. They reported that the drinking method was more successful in inducing tumors, and that a dose of 100 μg/ml for 16 weeks could induce carcinoma of tongue and esophagus in 100% of the animals.

The 4-NQO model also has advantages and disadvantages. 4-NQO is known to induce the various stages of cancer development, e.g., hyperplasia, dysplasia, carcinoma *in situ*, invasive carcinoma, and thus resembles the human situation more closely than the DMBA-induced oral cancer model [149,151]. Most of the histopathological changes

occur after the end of carcinogen treatment and thus helps ascertain whether the lesion is truely neoplastic or transient one [143,152]. This situation resembles the humans in that the tumors develop after contact with the carcinogen for extended periods of time. 4-NQO has also been shown to produce similar molecular alterations to humans such as alteration of biomarkers like H-ras, p53, Bcl-2, Bax, p16 and cyclin D1 during its treatment [143,147]. In a recent study, it was shown that 4-NQO could induce genomic instability in the non-neoplastic cells of oral mucosa. These stimulated non-neoplastic cells may eventually lead to cancer development in the later stages, thus resembling the process of carcinogenesis [153]. Finally, this model has been used to study various putative chemopreventive targets and agents, which has been reviewed in a recent publication [147]. Despite all the above advantages, 4-NQO model has drawbacks. First, the tumor produced by this model tends to be highly differentiated and less aggressive when compared to comparable human lesions. Second, the tumors rarely metastasize in this model while in humans metastasis is known to occur to regional lymph nodes [138].

Animals

Male Syrian golden hamsters were purchased from Harlan, Indianapolis, IN. A/J mice, both wild type and $p53^{A135V}$ mutant, were obtained as a gift from Dr. Ming You, Washington University School of Medicine. These mice were bred in our lab and genotyped by Ms. Jing Hao according to the protocol of the original developer [154]. The $p53^{A135V}$ mutant mice were developed by microinjection of FVB/J mouse oocytes with a BALB/c mouse genomic clone of the *p53* gene containing a point mutation in codon 135

(Ala-to-Val) at exon 5. C57BL/6J wild type mice were purchased from the Jackson Laboratory (Bar Harbor, ME).

Prediction of physico-chemical and permeability properties of NSAIDs for oral cancer chemoprevention

Skin J_{max} is calculated using aqueous solubility and log*P*, which are predicted by the ACD Suite (Version 8.0, Advanced Chemistry Development Inc., Toronto, Canada). Theoretical activity indices ($J_{max}/IC_{50\text{-}Cox\text{-}2}$) were calculated based on the IC_{50} values in the literature (Table1).

Animal experiments

Short-term effects of topical zileuton, celecoxib and GW2974 on aberrant AA metabolism and cell proliferation in DMBA-treated hamster cheek pouch

This study was conducted at Rutgers University under Protocol number 91-024 as per the experimental scheme shown in Figure 13. Male Syrian golden hamsters (6 weeks old) weighing 60–80 g were housed 4 per cage in a room with controlled temperature and humidity with 12 h light: dark cycles. All animals were given AIN-93M diet and water *ad libitum*. After 1 week of acclimatization, the animals were divided into 2 groups, with Group A serving as the negative control (9 animals). The left cheek pouch of the remaining 48 hamsters was topically treated with 0.5% DMBA in 100 µl mineral oil using a paintbrush 3 times per week for 3 weeks. They were then randomly divided into 7 groups with Group B (12 animals) serving as the positive control and receiving no

further treatment. Groups C (3% zileuton), D (6% zileuton), E (3% celecoxib), F (6% celecoxib), G (3% zileuton and 3% celecoxib) and H (160 µM GW2974) were treated with the respective chemopreventive agents topically in 100 µl mineral oil 3 times per week for 1 week (6 animals each). Six animals were sacrificed from the negative control (Group A) and the positive control (Group B) at the end of DMBA treatment (Week 3). The rest of the animals were sacrificed at the end of the experiment (Week 4), 6 hours after the last treatment. The animals were injected with bromodeoxyuridine (BrdU) *i.p.* at 50 mg/kg body weight 2 hours prior to sacrificing. The cheek pouch was harvested, one half being snap frozen in liquid nitrogen for analysis of AA metabolites, and the other half being fixed in 10% PBS-buffered formalin for histopathology.

Long-term effects of topical zileuton and celecoxib on DMBA-induced hamster cheek pouch carcinogenesis

This animal study was conducted by our collaborators in China. The histopathological, immunohistochemistry and biochemical analysis was conducted at the Lab for Cancer Research at Rutgers University. The experimental scheme is shown in Figure 14. Male Syrian golden hamsters (6 weeks old) weighing 60–80 g were housed, 4 per cage in a room with controlled temperature and humidity with 12 h light: dark cycles. All animals were given AIN-93M diet and water *ad libitum.* After 1 week of acclimatization, the animals (158 in number) were divided into various groups as shown in Figure 14. The left cheek pouch of all the hamsters (except the negative control) was topically treated with 0.5% DMBA in 100 µl mineral oil using a paintbrush 3 times per week for 6 weeks. The DMBA treated animals were then divided into six groups with

Group B serving as the positive control and receiving no further treatment. Groups C (3% zileuton), D (6% zileuton), E (3% celecoxib), F (6% celecoxib) and G (3% zileuton and 3% celecoxib) were treated with the respective chemopreventive agents topically in 100 µl mineral oil 3 times per week for 18 weeks. All animals were sacrificed at week 24.

Long-term effects of topical GW2974 on DMBA-induced carcinogenesis in hamster cheek pouch carcinogenesis

This animal study was conducted by our collaborators in China. The experimental scheme is shown in Figure 15. Male Syrian golden hamsters (6 weeks old) weighing 60–80 g were housed, 4 per cage in a room with controlled temperature and humidity with 12 h light: dark cycles. All animals were given AIN-93M diet and water *ad libitum.* After 1 week of acclimatization, the animals were divided into various groups. Group A (10 animals) served as the negative control. Rests of the animals were treated topically with 100 µl of 0.5% DMBA in mineral oil using a paintbrush 3 times per week for 6 weeks. The animals were then randomly divided into 3 groups (30 animals each), with Group B receiving no further treatment. Groups C and D were treated with 4 mM and 8 mM of GW2974 respectively, 3 times per week for another 18 weeks. At the end of Week 24, all animals were sacrificed and the left hamster cheek pouch was harvested and fixed in 10% buffered formalin for histopathology.

Long-term effects of zileuton and Polyphenon E on 4-NQO induced mouse oro-esophageal cancer in A/J mice

This study was conducted at Rutgers University under Protocol # 91-024. Male and female A/J mice, both wild type and *p*53^{A135V} mutant, were housed 10 per cage in a room with controlled temperature and humidity with 12 h light: dark cycles. All animals were given AIN-93M diet and water *ad libitum*. They were then divided into different groups as shown in Figure 16. The carcinogen (4-NQO) was given in drinking water at a concentration of 100 μg/ml for 8 weeks to all the groups, excluding the negative control. The carcinogen containing drink was changed once every week. Five animals were sacrificed at week 8 from the positive control for histopathological purposes. Zileuton (1000 ppm in diet) and polyphenon E (0.6% in drink, solution changed 3 times per week) were administered for another 16 weeks to the respective groups. All the animals were sacrificed at the end of the experiment, week 24. Tongue, esophagus and fore-stomach were collected and fixed in 10% buffered formalin for histopathology.

Long-term dose-dependent effects of zileuton and EGCG on 4-NQO induced mouse oro-esophageal cancer in C57BL/6J mice

This study was conducted at Rutgers University under Protocol number 91-024. Male and female black C57 wild type mice were housed 10 per cage in a room with controlled temperature and humidity with 12 h light: dark cycles. All animals were given AIN-93M diet and water *ad libitum*. They were then divided into different groups as shown in Figure 17. The carcinogen (4-NQO) was given as a drink in water at a concentration of 100 μg/ml for 12 weeks to all the groups, excluding the negative control.

The carcinogen containing drink was changed once every week. Five animals were sacrificed from the positive control to study the histopathology at the end of carcinogen treatment. Zileuton (500 ppm, 1000 ppm and 2000 ppm in diet) and EGCG (0.16% and 0.32% in drink) were administered for another 16 weeks to various groups. All the animals were sacrificed at the end of the experiment, week 28. Tongue and esophagus were collected and fixed in 10% buffered formalin for histopathology. Fore-stomach samples were snap frozen in liquid nitrogen.

Profiling of AA metabolites with LC/MS/MS

Profiling for AA metabolites was conducted in the buccal mucosa from the short-term hamster experiment. This analysis was undertaken by our collaborators at the M.D. Anderson Cancer Center, Houston, TX. After homogenization in a buffer containing 10 μM zileuton and indomethacin, tissues were extracted with hexane:ethyl acetate under controlled light and temperature conditions. PGE2-d4 was used as an internal standard. The samples were dried under nitrogen, reconstituted in methanol:2mM ammonium acetate, and analyzed using the established method [155,156]. The levels of LTB4, PGE2, 5-HETE, 12-HETE, and 15-HETE were determined by HPLC and expressed as nanograms per milligram protein.

Enzyme immunoassay for LTB4 and PGE2

The level of LTB4 and PGE2 was determined in the buccal mucosa from the long-term hamster experiment by using the ELISA method. Frozen samples of the hamster oral mucosa were analyzed immediately after being taken out of -80 ºC freezer.

After pulverization and homogenization in a buffer containing 10 μM of zileuton and indomethacin, a part of the sample was used for analyzing the protein concentration while the other was extracted with an organic solvent. The organic extract was dried under nitrogen and reconstituted in the enzyme immunoassay buffer for analysis with a kit according to the manufacturer's instructions (Cayman Chemical Co., Ann Arbor, MI). The levels of LTB4 and PGE2 were expressed as picogram per milligram protein.

Histopathological analysis

For the hamster studies, the whole cheek pouch was excised and spread on a transparent sheet for counting the number of visible tumors. The length, width and height of each tumor was measured with a caliper and the tumor volume calculated using the formula: volume=$4/3\pi r^3$ (where r was the average radius of the three diameter measurements in mm). Formalin-fixed pouches were cut into 4–6 pieces of approximately equal width, Swiss-rolled, processed and embedded in paraffin. Thirty sections (5 μm) of each sample were cut and the 1^{st}, 15^{th} and 30^{th} slides were stained with hematoxylin and eosin (H&E) for histopathological analysis. For the mouse studies, the tongue, esophagus and the fore-stomach were harvested and all the tissues fixed in 10% buffered formalin. Routine histology procedures were followed as mentioned above. Basal cell hyperplasia, dysplasia, squamous cell carcinoma and papillomas were diagnosed with established criteria [157,158].

Immunohistochemistry

5-Lox imunohistochemistry was performed on hamster, mouse and human samples using avidin-biotin-peroxidase system (ABC kit, Vector labs, Burlingame, CA). 5-Lox monoclonal antibody was purchased from RDI Inc., Parsippany, NJ and was used at a dilution of 1:50 on formalin fixed, paraffin embedded tissues. The paraffin sections were pretreated with antigen unmaking solution (BD PharMingen, San Diego, CA) before being incubated with the primary antibody.

Semi-quantification of 5-lox

Semi-quantification of 5-lox was performed in the study involving A/J and C57BL/6J mice with help of our pathologist (Dr. X. Hao). 5-Lox IHC was conducted as described in the previous section. In brief, the staining pattern for 5-lox was observed carefully under light microscopy in the 4-NQO treated group only (positive control). The staining intensity was graded as weak, moderate or strong in the different pathology stages for both tongue and esophagus.

Cell proliferation analysis

To evaluate cell proliferation in hamster and mouse tissue (buccal pouch, and tongue and esophagus respectively) bromodeoxyuridine (BrdU) immunostaining was performed on formalin-fixed, paraffin-embedded tissue sections. The avidin-biotin peroxidase method was used with a rat monoclonal antibody (Serotec, Raleigh, NC) at a dilution of 1:100. For analysis in the short-term hamster study, three and six cases were analyzed from the negative control and various treatment groups respectively. Three

noncontiguous, randomly selected fields under 400x were photographed per sample. In the study involving A/J mice the analysis was conducted only in the cancer tissue of tongue and esophagus. Ten cancer cases were evaluated from each group (except Group C which had only four cancer cases). One, two or three (depending on the cancer area available on the tissue) noncontiguous, randomly selected fields under 400x were photographed per sample. The slides were counterstained with heamatoxylin. The sum of all positive cells was divided by total number of cells to calculate the percentage positive cells (BrdU labeling index). During entire analysis the slides were blinded so that identity of various groups was unknown. Image-Pro Plus 4.5 software (Media Cybernetics, Silver Spring, MD) was used for cell counting.

Apoptosis analysis

To evaluate the extent of apoptosis in the cancer tissue of tongue and esophagus in A/J mouse, cleaved caspase 3 immunostaining was performed on formalin-fixed, paraffin-embedded tissue sections. The avidin-biotin peroxidase method was used with a rabbit monoclonal primary antibody (Cell Signalling, Danvers, MA) at a dilution of 1:100. Ten cancer cases, including tongue and esophagus, were evaluated from each group (except Group C which had only four cancer cases). One, two or three (depending on the cancer area available on the tissue) noncontiguous, randomly selected fields under 400x were photographed per sample. The slides were counterstained with hematoxylin. The sum of all positive cells was divided by total number of cells to calculate the percentage positive apoptotic cells (Apoptotic index). During entire analysis the slides

were blinded so that identity of various groups was unknown. Image-Pro Plus 4.5 software (Media Cybernetics, Silver Spring, MD) was used for cell counting.

Mast cell analysis

To evaluate the number of mast cell in the cancer tissues of tongue and esophagus, in A/J mouse, staining with toluidine blue was performed on formalin-fixed, paraffin-embedded tissue sections, as described previously [159]. Ten cancer cases, including tongue and esophagus, were evaluated from each group (except Group C which had only four cancer cases). One, two or three (depending on the cancer area available on the tissue) noncontiguous, randomly selected fields under 200x were photographed per sample. During entire analysis the slides were blinded so that identity of various groups was unknown. Number of mast cell was counted per mm^2 for each sample using AxioVision imaging software version 4.1 (Thornwood, NY). The average number of mast cells was counted for each group to give the mast cell density per mm^2.

Assessment of growth inhibitory activity of zileuton, AA861 and EGCG on tongue squamous cell carcinoma cell line

The effect of zileuton, AA861 and EGCG on viability of tongue carcinoma cells (CAL27) was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously [160]. In brief, CAL27 cells were plated at a density of 3×10^3 cells/well in a 96 well plates and incubated at 37 ºC in a 5% carbon dioxide atmosphere. The cells were allowed to attach for 24 hours. The cells were then incubated with various agents for another 48 and 72 hours. At the end of the treatment the

cells were treated with 0.5 mg/ml MTT solution and incubated for 2-3 hours. The resulting formazan product was solubilized in dimethlysulfoxide (DMSO). The absorbance was measured at 570 nm.

Statistical analysis

Short- and long-term hamster studies

The tumor incidence was compared by the *Chi-square* test. One-way ANOVA test was used to compare the number of visible tumors and oral lesions. The tumor volume was analyzed using the Wilcoxon signed rank test. The statistical significance between the levels of AA metabolites among the various groups was analyzed using the Student's *t* test.

A/J and C57BL/6J mouse studies

The effect of various treatments, alone or in combination, on cancer incidence was analyzed by the *Chi-square* test. The dose-dependent effects of zileuton and EGCG were evaluated using GraphPad Prism Version 4 (San Deigo, CA). The statistical significance for evaluating the cell proliferation, apoptosis and mast cell density among the various groups was analyzed using the Student's *t* test.

Cell line study

Student's *t* test was used for the evaluation of statistical significance among various groups.

Results

Prediction of physico-chemical and permeability properties of NSAIDs for oral cancer chemoprevention

The aim of this analysis is to theoretically predict the most effective NSAID for oral cancer chemoprevention. Skin J_{max} is calculated with aqueous solubility and log*P*, which are predicted by the ACD Suite. Comparing the predicted and experimentally measured values (aqueous solubility and log*P*), we find that this software gives very good predictions for the partition coefficient (log*P*). Theoretical activity indices ($J_{max}/IC_{50\text{-}Cox\text{-}2}$) were calculated based on the IC_{50} values in the literature (Table1). Among the 12 commonly used NSAIDs, celecoxib, nimesulide and ibuprofen have the highest theoretical activity index, and may be the agents of choice to target Cox-2 in oral epithelial cells through topical application.

Short-term effects of topical zileuton, celecoxib and GW2974 on aberrant AA metabolism and cell proliferation in DMBA-treated hamster cheek pouch

Hamsters were generally healthy throughout the study. The carcinogen treated animals weighed 5-10% less when compared to the negative control, probably due to the metabolic stress induced by DMBA. The aim of this study was to examine the effects of 5-Lox inhibitor zileuton, Cox2 inhibitor celecoxib and EGFR/ErbB2 kinase inhibitor GW2974 on DMBA-induced aberrations of AA metabolism in the hamster cheek pouch. The tissue levels of various AA metabolites are shown in Table 2. The levels of both LTB4 and PGE2 were significantly higher after DMBA treatment for 3 weeks. The levels of LTB4 increased by ~58 fold as compared to the negative control (Group A),

while the level of PGE2 increased by only ~3 fold. Even 1 week after DMBA was discontinued (at week 4), the levels of LTB4 and PGE2 were still much higher than the control group (~20 and ~7 fold respectively). This suggests that the 5-Lox pathway may be the major AA-metabolizing pathway activated by application of DMBA.

Topical treatment with zileuton (3% and 6% in Groups C and D, respectively) significantly reduced the levels of LTB4, PGE2, 5-HETE, 12-HETE and 15-HETE; whereas, only 6% celecoxib significantly reduced the levels of PGE2, but not those of other AA metabolites. These results suggested that suppression of 5-Lox pathway by zileuton was effective in ameliorating the aberrant AA metabolism. Topical application of 160 μM GW2974 (Group H) also significantly reduced the levels of the above metabolites (Table 2).

DMBA treatment for 3 weeks produced hyperproliferation in hamster cheek pouch (Figure 18). One week after discontinuation of DMBA (Week 4), the rate of cell proliferation reduced dramatically. Treatment with zileuton (3% and 6%) for 1 week significantly inhibited cell proliferation. Combination of zileuton and celecoxib (3% each) had similar effects on AA metabolism and cell proliferation as zileuton alone. No synergy between zileuton and celecoxib could be demonstrated based on our data. The increase in the cell proliferation was also significantly inhibited by 160 μM GW2974 (Figure 18).

Long-term effects of topical zileuton and celecoxib on DMBA-induced hamster cheek pouch carcinogenesis

Hamsters were generally healthy throughout the study. The carcinogen treated animals weighed 5-10% less when compared to the negative control, probably due to the metabolic stress induced by DMBA. The aim of this experiment was to study the long-term effects of a 5-Lox and Cox-2 inhibitor on oral carcinogenesis. At the end of the study (week 24), 76.9% of animals in the positive control (group B) developed SCC. The incidence and volume of visible tumors, as well as oral SCC was reduced by both zileuton and celecoxib (Table 3). The effect of zileuton seemed to be more pronounced in this study. The incidence of SCC decreased to 36% in the combination group. This effect seemed to be additive and not synergistic.

The concentration of both LTB4 and PGE2 was measured by using the commercially available ELISA kits. The levels of LTB4 (Figure 19) and PGE2 (Figure 20) increased significantly in the positive control (Group B) when compared with the negative control (Group A). Zileuton, (3% and 6%) decreased the levels of LTB4 significantly while only 3% celecoxib reduced the PGE2 concentration in the hamster oral mucosa (Figure 19 and 20, respectively). The combination group decreased the levels of both LTB4 and PGE2 significantly.

5-Lox immunohistochemistry was performed in both hamster and human samples to study its expression during different stages of carcinogenesis. In the hamster normal mucosa, 5-Lox was only expressed in few stromal cells (Figure 21a). However, once

treated with DMBA, 5-Lox was upregulated in the epithelial as well as stromal cells of histologically normal mucosa (Figure 21b). This expression further increased in hyperplasia, dysplasia and SCC suggesting that 5-Lox overexpression may be an early event in oral carcinogensis (Figure 21c-e). 5-Lox overexpression was also observed in different stages of the human oral cancer samples (Figure 21f-i).

Long-term effects of topical GW2974 on DMBA-induced oral carcinogenesis in hamster cheek pouch

Hamsters were generally healthy throughout the study. The carcinogen treated animals weighed 5-10% less when compared to the negative control, probably due to the metabolic stress induced by DMBA. The aim of this experiment was to study the chemopreventive effects of GW2974 on DMBA-induced oral carcinogenesis in hamster cheek pouch. Consistent with our previous studies [74,104,161], topical application of DMBA (100 µl, 0.5% in mineral oil, 3/week for 6 weeks) led to the development of oral lesions in the hamster cheek pouch at the end of 24 weeks. Topical application of GW2974 (4 mM and 8 mM, 100 µl, 3/week for 18 weeks) at the post-initiation stage significantly inhibited the incidence, number and size of DMBA-induced visible tumors. Under the microscope, GW2974 led to a significant decrease in the number of oral lesions (hyperplasia, dysplasia, and SCC) (Table 4).

Long-term effects of zileuton and Polyphenon E on 4-NQO induced mouse oro-esophageal cancer in A/J mice

The mice were generally healthy throughout the study and did not show any obvious signs of toxicity. The carcinogen treated animals weighed 5-10% less when compared to the negative control, probably due to the metabolic stress induced by 4-NQO. The aim of this study was to investigate the chemopreventive effects of zileuton and PPE on oro-esophageal carcinogenesis. At week 8, the tongue, esophagus and fore-stomach showed no visible tumors or microscopic lesions in the animals from positive control (Group B). All animals were sacrificed at week 24 and the number of visible tumors was counted (Table 5). Only zileuton inhibited number of visible tumors in esophagus and fore-stomach. Overall, 65.4% (17/26) of the animals developed SCC in Group B (positive control) in at least one site (tongue, esophagus or forestomach) (Table 6). Treatment with 1,000 ppm zileuton decreased the overall cancer incidence from 65.4% to 31.3%.

The $p53^{A135V}$ mutant group showed a significant increase in incidence of SCC in tongue and in overall sites when compared to the positive control (Group B). This is consistent with previously reported results that *p*53 transgenic mice are more likely to develop oral SCC when compared to the wild type ones [162]. Treatment with PPE only led to a significant decrease in incidence of SCC in tongue when compared to Group D.

5-Lox immunohistochemistry (IHC) was performed on tongue and esophagus samples (Figure 22). 5-Lox was expressed in epithelia and stromal cells in all the stages

of carcinogenesis (normal mucosa-hyperplasia-dysplasia-SCC). By analysis through semi-quantification (Table 7), we observed that the expression of 5-lox in the tongue increased significantly from 4-NQO treated normal mucosa to dysplasia and then decreased significantly in SCC (Figure 22a-e). In esophagus also, the expression of 5-lox increased significantly from 4-NQO treated normal mucosa to dysplasia and then decreased in SCC, although not statistically significantly (Figure 22f-j).

Cell proliferation analysis was performed in the malignant tissues from tongue and esophagus (Figure 23). Treatment with 4-NQO increased the cell proliferation significantly in the two sites when compared to group A (negative control). Treatment with 1000 ppm zileuton and 0.6% PPE reduced the cell proliferation significantly in both tongue and esophagus in groups C and E when compared to Groups B and D respectively. Representative pictures are shown in Figure 24.

Apoptosis analysis was performed in the malignant tissues from tongue and esophagus (Figure 25). No apoptosis was seen in the epithelium from the negative control (Figure 26a and f). Few positive apoptotic cells were observed in Groups B and D. Treatment with 1000 ppm zileuton and 0.6% PPE led to a significant increase in the number of apoptotic cells in both tongue and esophagus in Groups C and E when compared to Groups B and D respectively. Representative pictures are shown in Figure 26.

Mast cell density was evaluated in malignant tissues from tongue and esophagus (Figure 27). Mast cells were observed primarily scattered in the stromal tissue and along the blood vessels. They were also seen in the peripheral region of the tumor area. Administration of 4-NQO increased the number of mast cells significantly in the two sites when compared to group A (negative control). For our analysis we only took into consideration the mast cells which were located in and around the periphery of the tumor area. Treatment with 1000 ppm zileuton decreased the mast cell density significantly when compared to the positive control (Group B). No decrease in mast cell density was observed in the PPE treated animals (Group E) when compared to Group D. Representative pictures are shown in Figure 28.

Long-term dose-dependent effects of zileuton and EGCG on 4-NQO induced oro-esophageal carcinogenesis in C57 black mice

The mice were generally healthy throughout the study and did not show any obvious signs of toxicity. The carcinogen treated animals weighed 5-10% less when compared to the negative control, probably due to the metabolic stress induced by 4-NQO. The aim of this study was to investigate the dose-dependent effects of zileuton and EGCG in mouse oro-esophageal carcinogenesis. At week 12, the tongue, esophagus and forestomach showed no visible tumors or microscopic lesions. Four out the five animals analyzed from the positive control by hematoxylin and eosin staining showed an inflammatory response, characterized by aggregation of cells like lymphocytes. All animals were sacrificed at the end of the study and the number of visible tumors was counted. A non-significant inhibition of visible tumor incidence was observed (Table 8).

At week 28, 96.5% (28/29) and 55.1% (16/29) of animals developed SCC in tongue and esophagus, respectively (Table 9). The overall cancer incidence (animals having cancer in either tongue, esophagus or both) in the positive control was 100% (29/29). Both zileuton and EGCG were able to inhibit tongue SCC significantly. A dose-dependent effect was observed with zileuton although it was not significant ($p=0.066$ using GraphPad Prism software). The combination of 500 ppm zileuton and 0.16% EGCG had an additive effect in inhibiting tongue carcinogenesis ($p=0.01$ using Chi-square analysis). Both agents, alone and in combination, were not able to significantly inhibit esophageal carcinogenesis.

Zileuton was able to significantly inhibit dose-dependently the overall carcinogenesis in both tongue and esophagus ($p<0.01$ using GraphPad Prism software). Only 1000 and 2000 ppm zileuton significantly inhibited the overall carcinogenesis. The combination of 500 ppm zileuton and 0.16% EGCG showed an additive action on overall carcinogenesis ($p<0.001$ using Chi-square analysis).

5-Lox immunohistochemistry (IHC) was performed on tongue and esophagus samples (Figure 29). 5-Lox was expressed in epithelia and stromal cells in all the stages of carcinogenesis (normal mucosa-hyperplasia-dysplasia-SCC). By analysis through semi-quantification (Table 10), we observed that the expression of 5-lox in the tongue increased significantly from 4-NQO treated normal mucosa to dysplasia. A non-significant decrease was observed from dysplasia to SCC (Figure 29a-e). In esophagus, the expression of 5-lox did not change from normal-dysplasia-SCC (Figure 29f-j).

Assessment of growth inhibitory activity of zileuton, AA861 and EGCG on the CAL27 tongue squamous cell carcinoma cell line

MTT assay was used for the analysis of viable cells on tongue carcinoma cell line (CAL27). MTT is a yellow colored tetrazol product that is reduced to the purple colored formazan product in the mitochondria of the viable cells using the NADH enzyme system. Two 5-lox inhibitors, zileuton and AA861, and EGCG were evaluated for their growth inhibitory activity on CAL27 cells. The inhibitory activity of various agents, alone and in combination is shown in Figures 30 and 31. EGCG showed an IC_{50} value of around 150 μM and 100 μM for 2 and 3 day treatment respectively. Among the two 5-lox inhibitors, AA861 showed a greater inhibitory activity than zileuton. It had an IC_{50} value of around 50 μM for a 3 day treatment. Zileuton only showed an inhibition of around 20%, even at a high dose of 200 μM, for a 3 day treatment.

Discussion

Topical delivery of non-steroidal anti-inflammatory drugs through the oral mucosa has been used for oral cancer chemoprevention. Local permeation of these agents has been one of the major concerns. Here we propose an approach to predict the permeability of topically applied agents for oral cancer chemoprevention. To target the Cox-2 enzyme in oral epithelial cells, it may be desirable to maximize the ratio of J_{TC} and IC_{50} of a Cox-2 inhibitor, $J_{TC}/IC_{50\text{-}Cox\text{-}2}$. $D_{TC,}$ log*P* and $IC_{50\text{-}Cox\text{-}2}$ become the most important factors for selecting an agent for this purpose. Among the 12 commonly used NSAIDs, celecoxib had the highest theoretical activity index, and may be theoretically the agent of choice to target Cox-2 in oral epithelial cells through topical application. However, to validate its biological activity in tissue, well designed *in vivo* studies are required. It should be noted that, *a)* values of the predicted solubility vary according to different studies and solvents used; *b)* substantial differences in the absorption rate may exist between skin and oral mucosa due to the extent of keratinization and the presence of hair follicles in skin. The oral J_{max} should be higher than the skin J_{max} we calculated; *c)* drug metabolism and stability in the oral epithelial cells may affect its biological activity after absorption; *d)* pathology of the oral epithelium may affect drug absorption, for example, oral leukoplakia leads to an increase in its permeability.

Aberrant AA metabolism involving Cox and Lox pathways has been well established as an important factor in inflammation and the associated carcinogenesis, and is involved in both oral and esophageal cancers. Previous studies have focused mainly on

the Cox-2 pathway. Cox-2 was shown to be overexpressed in both pre-malignant and malignant lesions of the oral and esophageal squamous epithelium with increasing levels of expression from hyperplasia to dysplasia and squamous cell carcinoma [163-165]. It has also been shown that overexpression of Cox-2 is an early event in both oral and esophageal SCC [74,163,165]. Consistent with the above observation in oral cancer, other studies have indicated that the PGE2 levels were higher in oral SCC tissue as compared to normal tissue [69]. Cox-2 inhibitors have also been shown to retard the growth of human oral cancer cells in both PGE2-dependent and -independent manners [166] and suppressed 4-nitroquinoline-1-oxide-induced tongue cancer in rats [164] as well as DMBA-induced oral cancer in hamster cheek pouch [74]. Recently other AA pathways such as the 5-Lox have been shown to play important roles in inflammation and associated carcinogenesis. The 5-Lox pathway can lead to the formation of several groups of major metabolites such as leukotriene B4 (LTB4), cysteinyl leukotrienes (LTC4, LTD4 and LTE4), 5- hydroxyeicosatetraenoic acid (5-HETE) and 5-oxo-eicosatetraenoic acids (5-oxoETEs) [167]. These metabolites are known to be potent mediators of inflammation as they recruit and activate inflammatory cells, increase vascular permeability and induce smooth muscles contraction [168].

In our short-term study, topical application of DMBA for 3 weeks induced severe inflammation and aberrant AA metabolism. Subsequent topical treatment with zileuton, celecoxib, or their combination for 1 week significantly suppressed aberrant AA metabolism and cell proliferation in the oral epithelium. Interestingly, zileuton was effective in inhibiting biosynthesis of multiple AA metabolites, including leukotriene B4

(LTB4), 5-, 12-, 15-hydroxyeicosatetraenoic acid and prostaglandin E2 (PGE2), while celecoxib only suppressed PGE2 biosynthesis significantly at a high dose.

In our long-term study of DMBA-induced cancer, chemopreventive effects of zileuton and celecoxib were analyzed. 5-Lox was shown to be expressed in both hamster and human samples in different stages of pathology (Figure 21). Both zileuton and celecoxib, inhibited the incidence and volume of visible tumors, and incidence of oral SCC which correlated with the decrease of LTB4 and PGE2 levels respectively. The effect of zileuton seemed to be more pronounced in this study. An additive chemopreventive effect was observed on the incidence of SCC by zileuton and celecoxib in our animal model. Similar additive effects of the inhibitors of the 5-Lox and Cox pathways have been shown in animal models of lung [169], pancreatic [67], and esophageal cancer [75]. In our study, zileuton (3% and 6%) and celecoxib (3%) reduced the levels of LTB4 and PGE2 in hamster oral tissues, respectively; however 6% celecoxib did not (Figure 19 and 20).

The role of ErbB family of receptors in oral carcinogenesis has been well established. Dual inhibition of EGFR/ErbB2 tyrosine kinases could be an effective approach for chemoprevention and therapy of oral cancer. GW2974 has been shown previously to be highly potent in suppressing the growth of cancer cells *in vitro* and *in vivo* [170]. It was effective in selectively inhibiting the growth of tumor cells in cancer cell lines overexpressing both EGFR and ErbB2. In addition, GW2974 also suppressed the growth of xenograft tumors in a dose-dependent manner due to selective inhibition

of EGFR and ErbB2 receptor phosphorylation [170]. A synergistic effect was observed when GW2974 was treated in combination with Bcl-2 inhibitors in suppressing the growth of various human breast cancer cell lines [171]. In a separate *in vivo* study, GW2974 was more effective than gefitinib in significantly inhibiting the development of gallbladder carcinoma [172].

In our short-term hamster study, GW2974 led to a significant reduction in the levels of pro-inflammatory mediators such as PGE2, LTB4 and 5-, 12-, 15-HETE, which were elevated due to aberrant AA metabolism induced by DMBA treatment (Table 2). In the long-term study, topical application of GW2974 three times a week for 18 weeks to the hamster oral epithelium, not only reduced the number, size, burden and incidence of visible tumors but also modified various microscopic parameters such as the incidence of SCC. Higher dose of GW2974 (8 mM) was more effective in our study (Table 4). GW2974 may inhibit DMBA-induced oral carcinogenesis through modulation of aberrant AA metabolism and cell proliferation. These data are consistent with the observation that EGFR and AA metabolites interact with each other at multiple levels. Activation of EGFR and ErbB2 has been shown to stimulate Cox2 expression and translocation as well as PGE2 synthesis and mitogenesis [173,174].

In both our studies conducted in 4-NQO mouse models, zileuton and green tea catechins were shown to have chemopreventive activity. 5-lox was expressed in all the stages of pathology in both A/J and C57BL/6J mice. Zileuton inhibited the overall cancer incidence in the A/J mice by 50% (Table 6). Both the agents led to decrease in cell

proliferation and increase in apoptosis. Only zileuton was able to inhibit the mast cell density significantly. Mast cells are inflammatory cells which are known to be up-regulated in the oral SCC as the disease progresses [175]. They release wide variety of mediators like tryptase and chymase which have been reported earlier to be involved in process of angiogenesis [176,177]. They have also been shown to be involved in the formation of new vascular tube *in vitro* [178]. Inhibition of mast cells and thus down-regulation of angiogenesis might be one of the mechanisms by which zileuton elicits its chemopreventive activity in our 4-NQO induced oro-esophageal carcinogenesis model in A/J mice. In C57Bl/6J mice, zileuton was able to significantly inhibit dose-dependently the overall carcinogenesis in both tongue and esophagus. The combination of 500 ppm zileuton and 0.16% EGCG showed an additive effect on tongue and overall carcinogenesis.

Green tea preparations have been previously reported to inhibit buccal pouch carcinogenesis in DMBA-induced hamster oral cancer model [103-105]. It was shown have beneficial effects in clinical trials involving patients with oral leukoplakia [179,180]. Green tea was also shown to possess chemopreventive activity in esophageal carcinogenesis [107]. In our study with A/J mice 0.6% PPE was able to reduce the tongue carcinogenesis significantly by about 40% (Table 6). It also led to significant inhibition of cell proliferation and induction of apoptosis in the cancer tissues of both tongue and esophagus. In the study with C57BL/6J mice EGCG was able to inhibit the tongue and overall carcinogenesis significantly. The combination of zileuton and EGCG had additive effect in inhibiting the tongue and overall carcinogenesis. In our studies green

tea catechins were able to show chemopreventive activity specifically in the tongue. This might be due to the fact that the catechins were in direct contact with the oral cavity and stayed for longer time period on the tongue surface. Also, it has been reported by our group that holding green tea leaves in mouth is a convenient way of slow catechin release to elicit their direct effect on the oral cavity [100].

p53^{A135V} is a tumor suppressor gene known to be mutated in majority of the human cancers [181]. It is usually known as the guardian of the cell cycle as it induces cell cycle arrest and apoptosis in the presence of DNA damage. Its mutation leads to loss of these important functions thus rendering the individual more susceptible to carcinogenesis. *p53*^{A135V} mutation has been shown to be involved in pathogenesis of oral SCC [182]. In our study the carcinogenesis in tongue and overall sites increased significantly in the *p53*^{A135V} mutant group when compared to the wild type A/J mice (GroupB, Table 6).

Two 5-lox inhibitors, zileuton and AA861, and EGCG were evaluated for their growth inhibitory activity in tongue squamous cell carcinoma cell line (CAL27). All the test compounds had growth inhibitory effect on CAL27 cancer cells. EGCG and AA861 had an IC_{50} values of around 100 μM and 50 μM respectively, for a three day treatment period. High dose zileuton was only able to achieve 20% inhibition for a three day treatment period. AA861 was found to be more potent in our study. This may be due to the fact that it elicits its 5-lox inhibitory activity through different mechanism. Zileuton chelates the iron in the active site of 5-lox while AA861 acts by reducing the iron, thus

disturbing the catalytic cycle. The anti-proliferative activity of zileuton *in vitro* has not been evaluated extensively. In one study, it showed an IC_{50} values of 43-58 μM in three different mouse colon adenocarcinoma cell lines [169]. However, this IC_{50} values was 10 fold higher than the ones of other 5-lox inhibitors evaluated in the same study. AA861 has been shown to have growth inhibitory activity, IC_{50} between 5-50 μM, in various cancer cell lines such as human leukemia cells and mouse mastocytoma cells [169].

It is known from the literature that green tea catechins are unstable in the neutral and basic pH and leads to oxidative polymerization with production of hydrogen peroxide. This may be in part responsible for the biological effects of green tea catechins in some *in vitro* studies [183]. This H_2O_2-mediated effect can be diminished by addition of superoxide dismutase (SOD) and catalase as they are known to stabilize tea catechins in the cell culture medium. Our lab has previously shown that holding green tea leaves in the oral cavity leads to release of various catechins enabling their direct contact with the oral mucosa [100]. As the oral cavity is exposed to high environmental oxygen partial pressure, the catechins might become unstable and polymerize to produce H_2O_2 [184]. This may be in part responsible for the biological activity of green tea catechins in oral cancer chemoprevention. To mimic the situation in the oral cavity of humans, SOD and catalase were not added in our study to stabilize EGCG in the cell culture medium.

Previously we have reported that combination of two pharmaceutical agents, zileuton and celecoxib, had an additive effect in inhibiting oral and esophageal cancer in a hamster and rat model respectively [74,75]. In our present study in C57BL/6J mice we

have tested a combination of pharmaceutical and dietary agent, zileuton and EGCG, which also showed an additive effect in inhibition of tongue and overall carcinogenesis (Table 9).

LTB4, the metabolite of 5-Lox pathway, may promote carcinogenesis through multiple mechanisms. Both *in vitro* and *in vivo* studies have demonstrated that it stimulates cell proliferation [185-188], regulates the influx of inflammatory cells, and promotes the formation of reactive oxygen species which leads to oxidative stress [189,190]. It has also been shown to inhibit apoptosis in intestinal epithelial cells by regulating the expression of Cox-2, ß-catenin and Bcl-2 [191]. It has been shown recently by our group that topical application of 2 μM LTB4 along with 0.5% DMBA doubled the incidence of SCC in the hamster cheek pouch when compared to treatment with DMBA alone [161]. Similar to LTB4, topical application of PGE2 also promoted carcinogenesis in this study. This is in line with previous studies showing cancer promoting effects of exogenous PGE2 [192,193]. According to an *in vivo* study on the effect of topical LTB4 on human skin, LTB4 quickly recruited polymorphonuclear leukocytes and T lymphocytes, while epidermal proliferation was stimulated thereafter [194]. These observations suggest that the activated 5-Lox pathway promoted oral carcinogenesis by stimulating inflammation and hyperproliferation in DMBA-initiated cells.

In summary, we show here that; a) 5-lox is one of the important AA pathways involved in the pathogenesis of oral and esophageal SCC; b) zileuton, a specific 5-lox inhibitor, has chemopreventive activity in both hamster and mouse carcinogenesis

models; 3) zileuton had an additive effect with both with celecoxib and EGCG; 4) green tea catechins are effective in inhibiting oral and overall carcinogenesis in our mouse model; 5) zileuton, AA861 and EGCG have growth inhibitory activity in CAL27 tongue cancer cells; 6) GW2974, a dual inhibitor of EGFR/ErbB2 kinases showed chemopreventive effect in oral carcinogenesis.

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Table 1

Predicted properties of twelve commonly used NSAIDs

NSAIDs	Physico-chemical Properties							Biochemical Properties [c]		Theoretical Activity Index ($J_{max}/IC_{50\text{-}Cox\text{-}2}$)
	Molecular Weight [a]	Solubility in Pure Water (μg/ml)		Partition Coefficient Log*P*		Permeability Coefficient k_p (cm/h) [b]	Flux (J_{max}) (μg/cm²/h) [b]	$IC_{50\text{-}Cox\text{-}2}$ (μM)	Cox-2 Selectivity $IC_{50(Cox\text{-}1/Cox\text{-}2)}$	
		Predicted [a]	Measured	Predicted [a]	Measured					
Celecoxib	381.37	94.14	7.00 [d]	4.21	-	9.12×10^{-3}	85.85×10^{-2}	0.04 [n]	375 [n]	21.46
Nimesulide	308.31	377.80	26.90 [e]	3.79	2.60 [e]	1.29×10^{-2}	487.46×10^{-2}	1.27 [o]	55.110 [o]	3.83
Ibuprofen	206.28	70.00	96.00 [f]	3.72	3.51 [g]	4.78×10^{-2}	334.60×10^{-2}	1.53 [o]	1.700 [o]	2.19
Ketoprofen	254.28	220.00	294.00 [g]	2.81	3.12 [g]	5.49×10^{-3}	120.78×10^{-2}	2.33 [o]	0.210 [o]	0.52
Diclofenac	296.15	30.00	3.50 [g]	4.06	4.40 [g]	2.34×10^{-2}	70.20×10^{-2}	1.50 [o]	0.600 [o]	0.47
Naproxen	230.26	40.00	14.00 [h]	3.00	3.34 [g]	1.07×10^{-2}	42.80×10^{-2}	2.00 [o]	0.300 [o]	0.21
Rofecoxib	314.36	130.00	9.00 [d]	1.34	-	2.14×10^{-4}	2.78×10^{-2}	0.34 [p]	76.470 [p]	0.08
Aspirin	180.16	6030.00	10000 [i]	1.19	1.19 [j]	1.09×10^{-3}	6.57×10^{-0}	590 [q]	0.007 [q]	1.11×10^{-2}
Ketorolac	255.27	240.00	183.00 [g]	2.08	1.04 [k]	1.66×10^{-3}	39.84×10^{-2}	60.50 [r]	0.520 [r]	6.58×10^{-3}
Indomethacin	357.79	60.00	11.00 [g]	3.10	3.08 [g]	2.08×10^{-3}	12.48×10^{-2}	24.60 [o]	0.070 [o]	5.07×10^{-3}
Sulindac	356.41	20.00	10.00 [i]	3.59	2.86 [l]	4.79×10^{-3}	9.58×10^{-2}	50.70 [r]	0.020 [r]	1.89×10^{-3}
Piroxicam	331.35	390.00	53.30 [g]	1.71	1.80 [m]	3.09×10^{-4}	12.05×10^{-2}	> 500 [r]	< 0.040 [r]	$< 2.41 \times 10^{-4}$

[a] Values provided by the ACD software. Salivary pH varies between 5.5 and 7.0, depending on the flow rate. [b] Values calculated using the Potts and Guy equation [195]; Log k_p (cm/h) = -2.7 + 0.71 x log $P_{predicted}$ - 0.0061 x MW and $J_{max} = k_p$ x solubility$_{predicted}$. [c] IC_{50} is determined by an *in vitro* assay using human recombinant proteins. The values may vary from different sources. [d] Ref. [196]; [e] Ref. [197]; [f] Ref. [198]; [g] Ref. [199]; [h] Ref. [200]; [i] Ref. [201]; [j] Ref. [202]; [k] Ref. [203]; [l] Ref. [204]; [m] Ref. [205]; [n] Ref. [206]; [o] Ref. [207]; [p] Ref. [208]; [q] www.mdsps.com; [r] Ref. [209].

Table 2

Levels of AA metabolites in buccal pouch of hamsters in the DMBA-induced short-term oral carcinogenesis study

AA metabolites	Week 3 Groups		Week 4 Groups							
	A	B	A	B	C	D	E	F	G	H
LTB4	0.01 ± 0.03 **	0.58 ± 0.27	0.02 ± 0.01 **	0.4 ± 0.17	0.1 ± 0.06 **	0.03 ± 0.02 **	0.38 ± 0.17	0.36 ± 0.28	0.05 ± 0.04 **	0.05 ± 0.03 **
PGE2	0.44 ± 0.12 *	1.14 ± 0.49	0.2 ± 0.02 *	1.37 ± 0.56	0.48 ± 0.26 *	0.21 ± 0.09 **	0.7 ± 0.51	0.1 ± 0.06 **	0.24 ± 0.14 **	0.40±0.13 **
5-HETE	0.21 ± 0.09	0.26 ± 0.08	0.08 ± 0.05	0.12 ± 0.05	0.06 ± 0.02 *	0.05 ± 0.01 **	0.12 ± 0.05	0.13 ± 0.06	0.04 ± 0.02 **	0.06 ± 0.01 *
12-HETE	1.56 ± 0.18	1.15 ± 0.41	0.75 ± 0.38 *	1.89 ± 0.66	0.69 ± 0.26 **	0.48 ± 0.21 **	2.27 ± 0.56	1.35 ± 0.8	0.65 ± 0.33 **	1.02± 0.33 *
15-HETE	0.52 ± 0.25	0.64 ± 0.25	0.39 ± 0.14 *	0.95 ± 0.3	0.5 ± 0.14 *	0.26 ± 0.12 **	0.6 ± 0.25	0.67 ± 0.24	0.15 ± 0.06 **	0.46± 0.32 *

The left cheek pouch of hamsters was treated with 0.5% DMBA 3 times per week for 3 weeks. They were then divided into various groups. Group A: Negative control, group B: Positive control (DMBA treated), group C: 3% zileuton, group D: 6% zileuton, group E: 3% celecoxib, group F: 6% celecoxib and group G: 3% zileuton + 3% celecoxib. Tissues from the buccal cavity were harvested. After homogenization in a buffer containing 10 μM zileuton and indomethacin, tissues were extracted with hexane:ethyl acetate under controlled light and temperature conditions. PGE2-d4 was used as an internal standard. The samples were dried under nitrogen, reconstituted in methanol:2mM ammonium acetate, and analyzed using HPLC. The values are expressed in ng/mg protein (average ± SD). Week 3 represents the end of carcinogen treatment. Week 4 represents after one week of treatment with various agents. *$p<0.05$ and **$p<0.01$. Student's *t* test was used for statistical comparison. All groups were compared with group B.

Table 3

Effect of topical zileuton and celecoxib in long-term DMBA-induced oral carcinogenesis in hamster cheek pouch

Group	Treatment		No. of animals	Visible tumors		SCC (%)
	Wk 1-6	Wk 7-24		Incidence (%)	Volume (mm^3)	
A	-	-	15	-	-	-
B	0.5% DMBA	-	26	84.6	89.2±76.2	76.9
C	0.5% DMBA	3% zileuton	24	58.3	39.9±48.0 *	45.8 *
D	0.5% DMBA	6% zileuton	28	46.4 *	22.9±31.4 *	32.1 *
E	0.5% DMBA	3% celecoxib	26	61.5	42.2±41.2 *	57.6
F	0.5% DMBA	6% celecoxib	24	54.2 *	33.3±32.5 *	50.0 *
G	0.5% DMBA	3% zileuton + 3% celecoxib	25	44.0 *	21.4±31.5 *	36.0 *

The left cheek pouch of all the hamsters (except group A) was topically treated with 0.5% DMBA in 100 µl mineral oil using a paintbrush 3 times per week for 6 weeks. Group B served as the positive control and received no further treatment. Groups C (3% zileuton), D (6% zileuton), E (3% celecoxib), F (6% celecoxib) and G (3% zileuton and 3% celecoxib) were treated with the respective chemopreventive agents topically in 100 µl mineral oil 3 times per week for 18 weeks. The tumor incidence was compared by the $?^2$ test. The tumor volume was analyzed using the Wilcoxon signed rank test. *$p<0.05$.

Table 4

Effect of topical GW2974 in long-term DMBA-induced oral carcinogenesis in hamster cheek pouch

Group	Treatment	No. of animals	Visible Tumors			Microscopic Observations			
			Incidence % (No.)	No.	Average size (mm^3)	No. of hyperplasia	No. of dysplasia	No. of SCC	Incidence of SCC % (No.)
A	Negative control	10	0	0	0	0	0	0	0
B	Positive control	30	80% (24)	1.00±0.88	256.84±442.53	8.07±4.39	7.57±3.37	1.83±1.91	70% (21)
C	GW2974 (4 mM)	30	43.3% (13)**	0.57±0.82*	62.08±58.99	5.73±3.09**	6.80±3.74	0.67±0.99*	40% (12)*
D	GW2974 (8 mM)	30	36.7% (11) **	0.43±0.63**	50.50±54.88	5.30±2.89**	5.97±2.62*	0.43±0.68**	33% (10)**

The left cheek pouch of all the hamsters (except group A) was topically treated with 0.5% DMBA in 100 µl mineral oil using a paintbrush 3 times per week for 6 weeks. Group B served as the positive control and received no further treatment. Groups C (4 mM GW2974) and D (8 mM GW2974) were treated with the respective chemopreventive agents topically in 100 µl mineral oil 3 times per week for 18 weeks. The number and size of visible tumors, and the numbers of oral lesions (hyperplasia, dysplasia, and SCC) were expressed as mean ± SD. χ^2 test was used for comparison of incidences of visible tumor and SCC, signed rank test for visible tumor number, size and number of SCC, and ANOVA for number of hyperplasia, dysplasia and SCC. Values of Groups C and D were compared with Group B. * $p<0.05$, ** $p<0.01$.

Table 5

Incidence of visible tumors in 4-NQO-induced oro-esophageal carcinogenesis in A/J mice

Group	Genotype	Treatment		No. of animals	Incidence of visible tumors			
		Wk 1-8	Wk 9-24		Tongue	Esophagus	Fore-stomach	Overall[a]
A	Wild-type	-	-	6	-	-	-	-
B	Wild-type	4-NQO	-	26	10 (38%)	21 (80%)	26 (100%)	26 (100%)
C	Wild-type	4-NQO	1,000 ppm zileuton in diet	16	5 (31%)	5 (31%) *	8 (50%) *	14 (87%)
D	*p53*^{A135V}	4-NQO	-	21	8 (38%)	13 (61%)	13 (61%)	19 (90%)
E	*p53*^{A135V}	4-NQO	0.6% Polyphenon E in drink	23	7 (30%)	12 (52%)	15 (65%)	19 (82%)

4-NQO was administered at a concentration of 100 μg/ml for 8 weeks. Zileuton and PPE were administered from week 9 to week 24 in diet and drink respectively. All animals were sacrificed at week 24 and the visible tumors were counted. *$p<0.05$. Chi square test was used for statistical comparison of incidence of visible tumors. Group C and D are compared with group B. Group E is compared with Group D. [a] Overall means that animals have visible tumors in at least one site (tongue, esophagus and fore-stomach).

Table 6

Incidence of SCC in 4-NQO induced oro-esophageal carcinogenesis in A/J mice

Group	Genotype	Treatment		No. of animals	Incidence of SCC			
		Wk 1-8	Wk 9-24		Tongue	Esophagus	Fore-stomach	Overall [a]
A	Wild-type	-	-	6	-	-	-	-
B	Wild-type	4-NQO	-	26	26.9% (7/26)	34.6% (9/26)	23.1% (6/26)	65.4% (17/26)
C	Wild-type	4-NQO	1,000 ppm zileuton in diet	16	6.3% (1/16)	18.8% (3/16)	6.3% (1/16)	31.3% (5/16) *
D	*p53*^{A135V}	4-NQO	-	21	70% (13/21) *	33.3% (7/21)	33.3% (7/21)	90.5% (19/21) *
E	*p53*^{A135V}	4-NQO	0.6% Polyphenon E in drink	23	30.4% (7/23) *	30.4% (7/23)	17.4% (4/23)	74% (17/23)

4-NQO was administered at a concentration of 100 μg/ml for 8 weeks. Zileuton and PPE were administered from week 9 to week 24 in diet and drink respectively. All animals were sacrificed at week 24. [a] Overall means that animals have cancer in at least one site (tongue, esophagus or fore-stomach). In Group B one case had SCC in 3 organ sites and three cases in 2 organ sites. In Group D seven cases had SCC in 2 organ sites and one case in 3 organ sites. In Group E three cases had SCC in 2 organ site. One animal had SCC in spleen and gingival respectively. Group C and D are compared with Group B. Group E is compared with Group D. Chi square test was used for statistical comparison of incidence of SCC. *$p<0.05$.

Table 7

5-Lox expression in 4-NQO induced oro-esophageal carcinogenesis in A/J mice

Pathology	Staining Intensity			Total cases
	Weak	Moderate	Strong	
Tongue				
Normal mucosa	20	4	2	26
Dysplasia *	0	14	7	21
SCC **	2	3	2	7
Esophagus				
Normal mucosa	12	9	5	26
Dysplasia §	2	12	11	25
SCC	0	4	6	10

5-Lox IHC was conducted in animals from Group B (4-NQO treated group only, positive control). The semi-quantification was performed by grading the intensity of 5-Lox expression as weak, moderate and strong in different stages of pathology with the help of light microscopy. The numbers in the table represent the total number of animals with respective intensity in specific pathology states. Chi-square test was used for statistical analysis. * Significant when compared to normal mucosa ($p<0.001$). ** Significant when compared to dysplasia ($p<0.05$).
§ Significant when compared to normal mucosa ($p<0.01$).

Table 8

Incidence of visible tumors in 4-NQO induced oro-esophageal carcinogenesis in C57 black mice

Group	Treatment		No. of animals	Incidence of visible tumors		
	Wk 1-12	Wk 13-28		Tongue	Esophagus	Overall[a]
A	-	-	10	-	-	-
B	4-NQO	-	29	21 (72.4%)	10 (34.4%)	23 (79.3%)
C	4-NQO	500 ppm zileuton	30	21 (70.0%)	13 (43.3%)	22 (73.3%)
D	4-NQO	1,000 ppm zileuton	30	15 (50.0%)	12 (40.0%)	21 (70.0%)
E	4-NQO	2,000 ppm zileuton	30	15 (50.0%)	11 (30.56%)	20 (66.6%)
F	4-NQO	0.16% EGCG	29	20 (68.9%)	6 (20.6%)	22 (75.8%)
G	4-NQO	0.32% EGCG	29	15 (51.7%)	10 (34.4%)	20 (68.9%)
H	4-NQO	500 ppm zileuton + 0.16% EGCG	29	18 (62.0%)	11 (37.9%)	20 (68.9%)

4-NQO was administered at a concentration of 100 µg/ml for 12 weeks. Zileuton and EGCG were administered from week 13^{th} to week 28^{th} in diet and drink respectively. All animals were sacrificed at week 28. The number of visible tumors was calculated with naked eye. Chi square test was used for statistical comparison of incidence of visible tumors. No statistical significance was observed between various groups. [a] Overall means that animals have visible tumors in at least one site (tongue or esophagus).

Table 9

Incidence of SCC in 4-NQO induced oro-esophageal carcinogenesis in C57BL/6J mice

Group	Treatment		No. of animals	Incidence of SCC (#)		
	Wk 1-12	Wk 13-28		Tongue	Esophagus	Overall [a]
A	-	-	10	-	-	-
B	4-NQO	-	29	96.5% (28)	55.1% (16)	100% (29)
C	4-NQO	500 ppm zileuton	30	53.3% (16) *	56.6% (17)	90.0% (27)
D	4-NQO	1,000 ppm zileuton	30	36.7% (11) *	46.6% (14)	66.6% (20) *
E	4-NQO	2,000 ppm zileuton	30	30.0% (9) *	43.3% (13)	56.6% (17) *
F	4-NQO	0.16% EGCG	30	70.0% (21) *	43.3% (13)	80.0% (24) *
G	4-NQO	0.32% EGCG	29	72.4% (21) *	48.2% (14)	86.2% (25) *
H	4-NQO	500 ppm zileuton + 0.16% EGCG	29	37.9% (11) *	37.9% (11)	55.0% (16) *

4-NQO was administered at a concentration of 100 μg/ml for 12 weeks. Zileuton and EGCG were administered from week 13th to week 28th in diet and drink respectively. All animals were sacrificed at week 28. [a] Overall means that animals have cancer in at least one site (tongue or esophagus). Chi square test was used for statistical comparison of incidence of SCC. * $p<0.05$. All the groups were compared with Group B (positive control). 15, 6, 5, 5, 10, 10 and 5 cases in Groups B, C, D, E, F, G and H respectively had cancers in both tongue and esophagus.

Table 10

5-Lox expression in 4-NQO induced oro-esophageal carcinogenesis in C57BL/6J mice

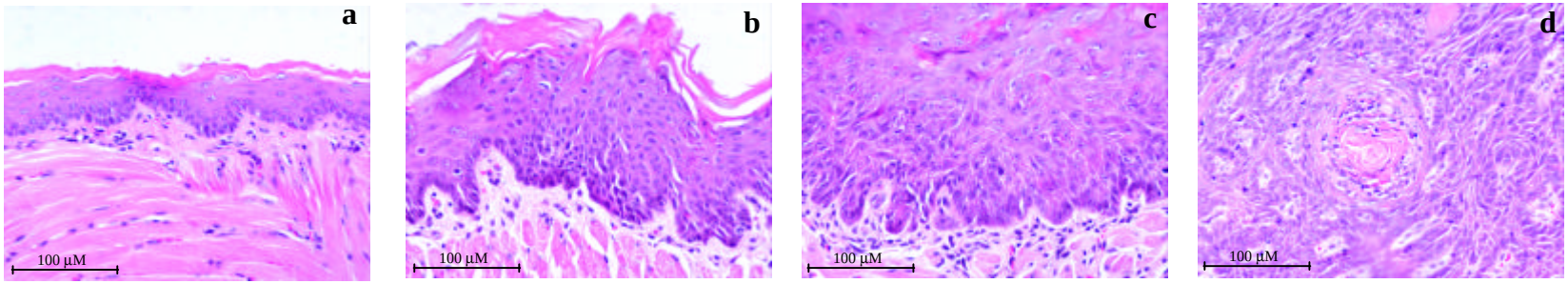
Pathology	Staining Intensity			Total cases
	Weak	Moderate	Strong	
Tongue				
Normal mucosa	24	2	2	28
Dysplasia *	4	8	7	19
SCC	7	10	3	20
Esophagus				
Normal mucosa	21	4	5	30
Dysplasia	6	4	6	16
SCC	8	6	1	15

5-Lox IHC was conducted in animals from Group B (4-NQO treated group only, positive control). The semi-quantification was performed by grading the intensity of 5-Lox expression as weak, moderate and strong in different stages of pathology with the help of light microscopy. The numbers in the table represent the total number of animals with respective intensity in specific pathology states. Chi-square test was used for statistical analysis. * Significant when compared to normal mucosa ($p<0.001$).

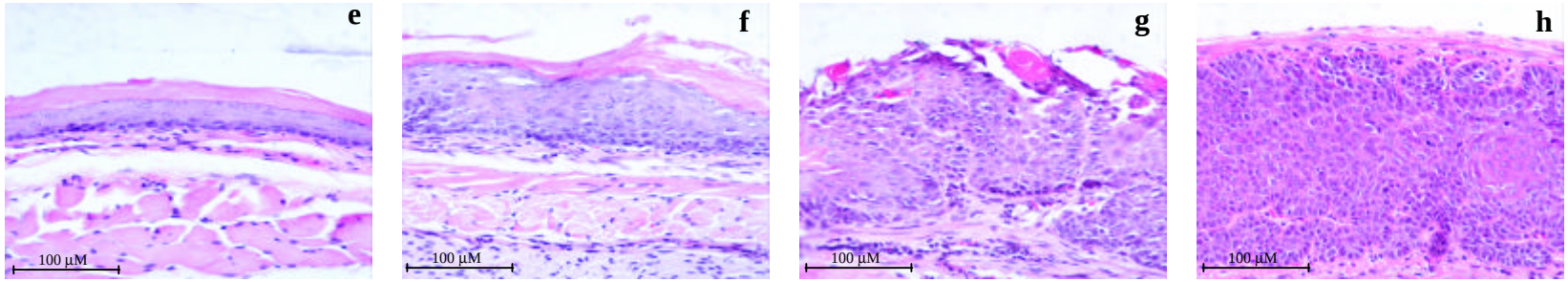
Figure 1

Histopathological progression of SCC in tongue and esophagus

Tongue



Esophagus



Normal mucosa Hyperplasia Dysplasia SCC

The above pictures represent the histopathological progression of normal mucosa to cancer in tongue (a-d) and esophagus (e-h). Hematoxylin and eosin staining was performed and the slides were viewed under the light microscope. Diagnosis was made according to the established criteria. Magnification is 400x.

Figure 2

Proposed mechanism for inflammation associated multi-stage carcinogenesis

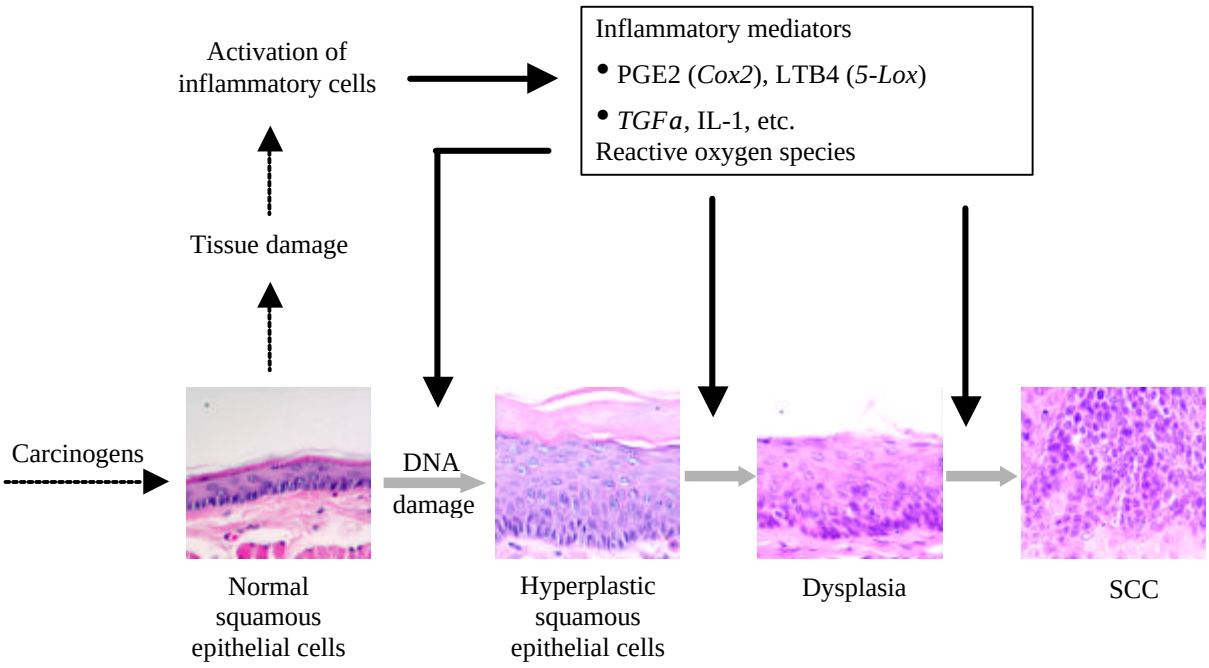


Figure 3

Arachidonic acid (5,8,11,14-eicosatetraenoic acid)

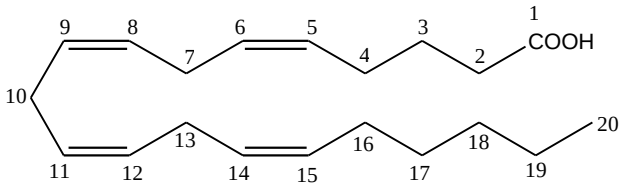


Figure 4

Cyclooxygenase pathway of AA metabolism

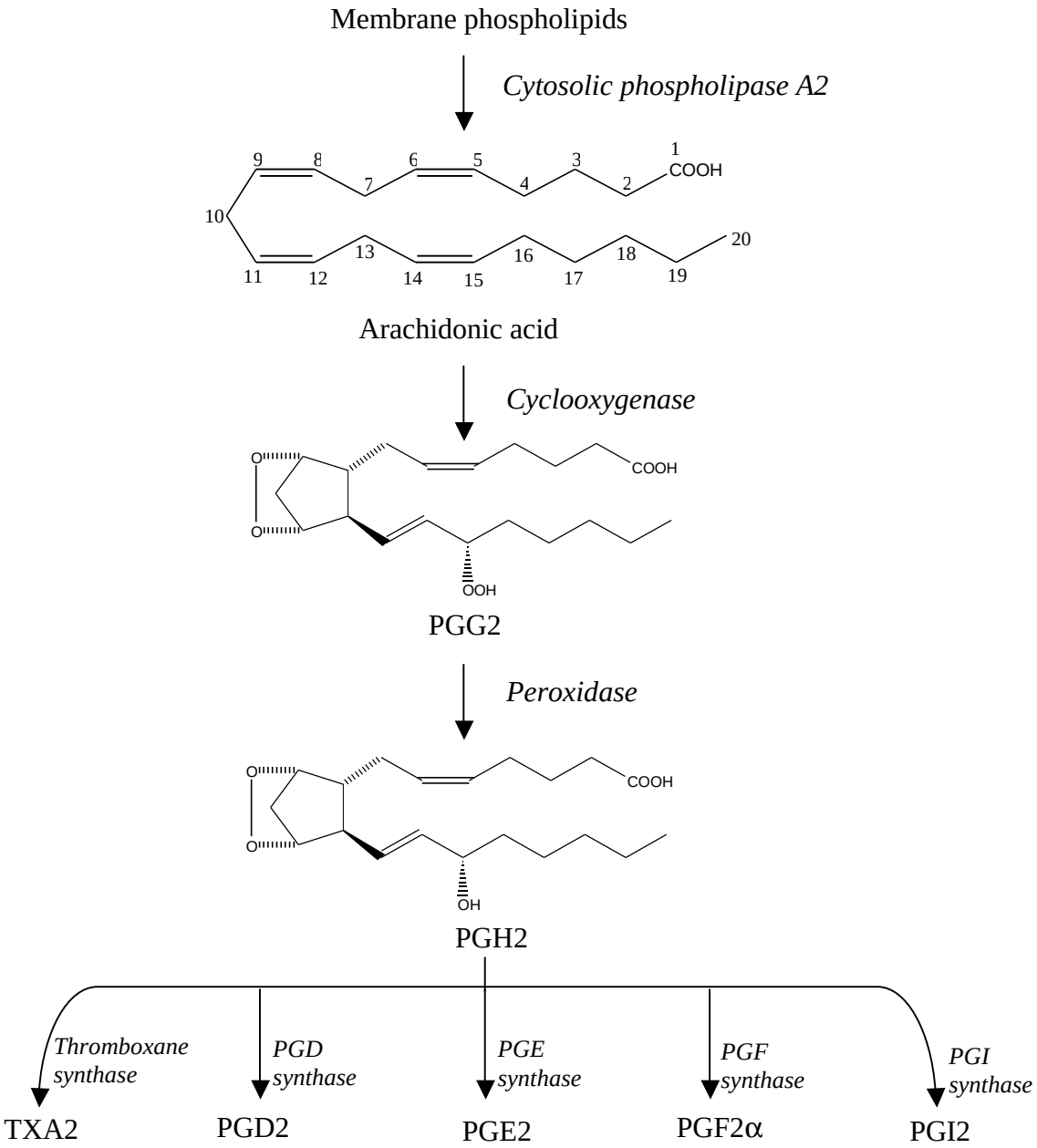


Figure 5

PGE2 (7-[3-hydroxy-2-(3-hydroxyoct-1-enyl)-5-oxo-cyclopentyl]hept-5-enoic acid)

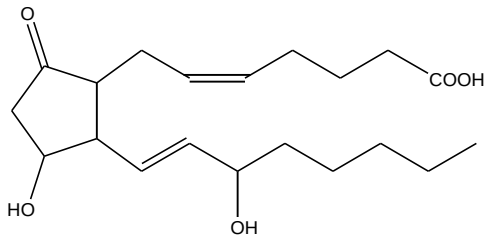


Figure 6

Celecoxib (4-[5-(4-methylphenyl)-3-trifluoromethyl]-1*H*- pyrazol-1-yl]-benzenesulfonamide)

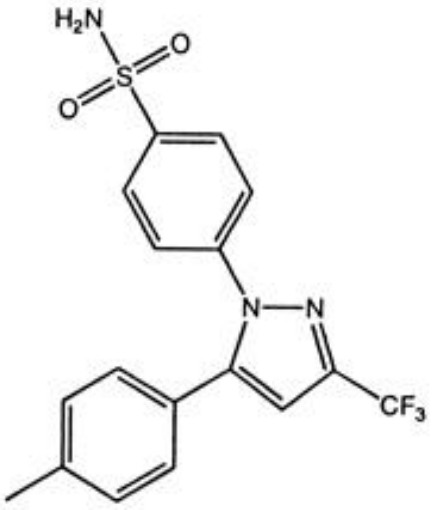


Figure 7

Lipoxygenase pathway of AA metabolism

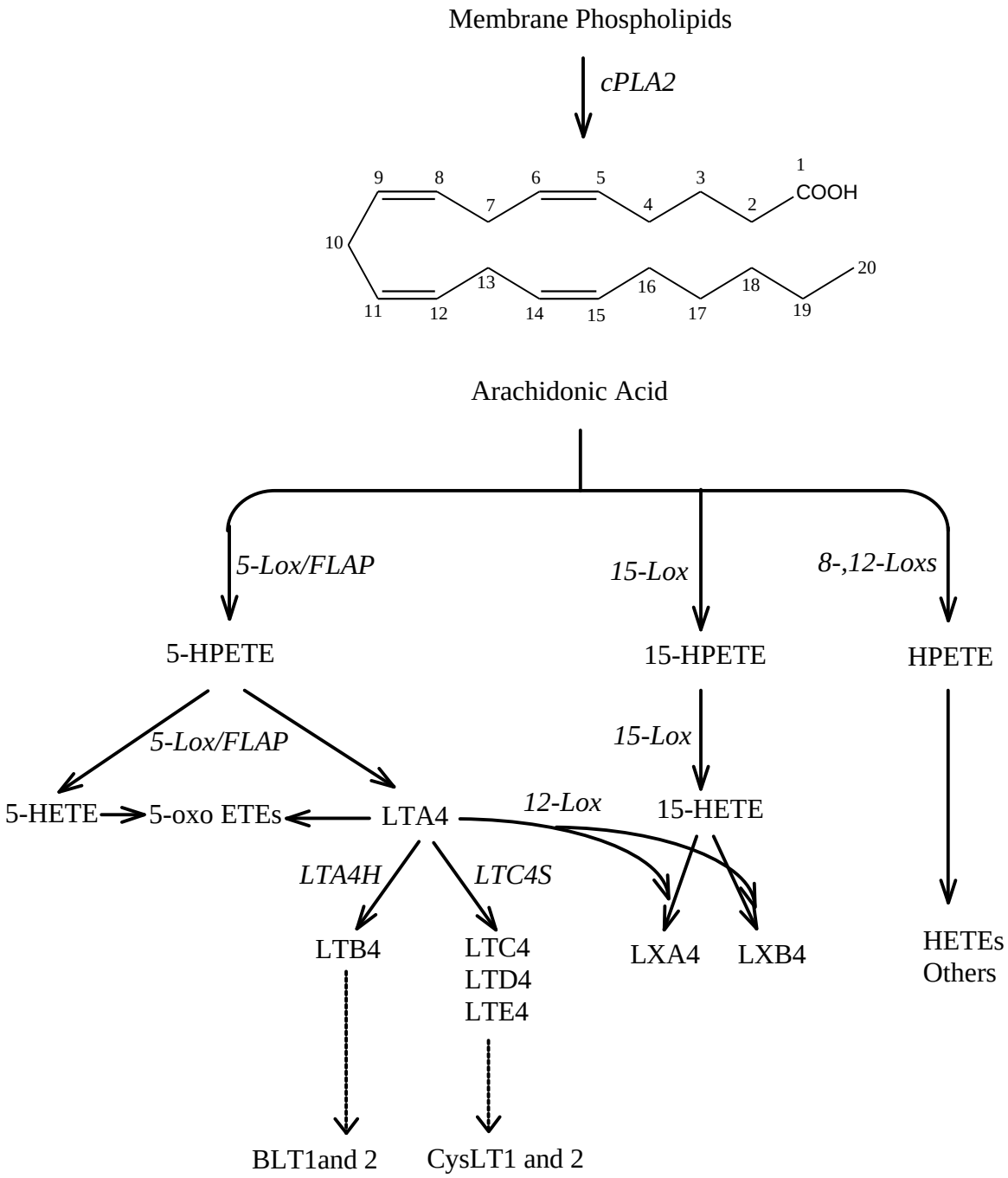


Figure 8

LTB4 (5,12-dihydroxyicosa-6,8,10,14-tetraenoic acid)

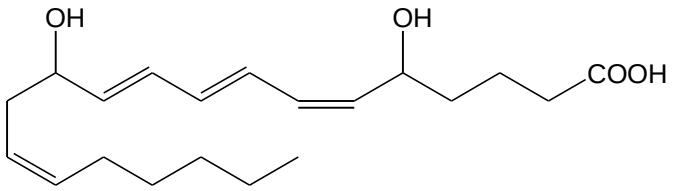


Figure 9

Zileuton (N-(1-benzo [b] thien-2-ylethyl)-N-hydroxyurea)

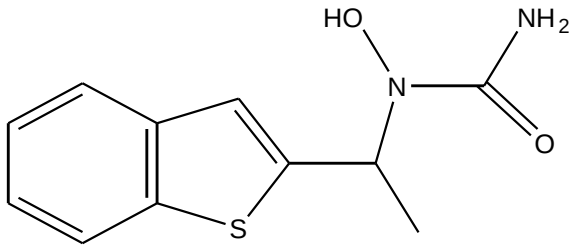
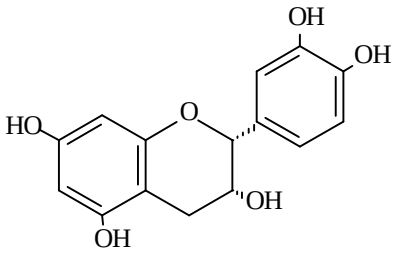
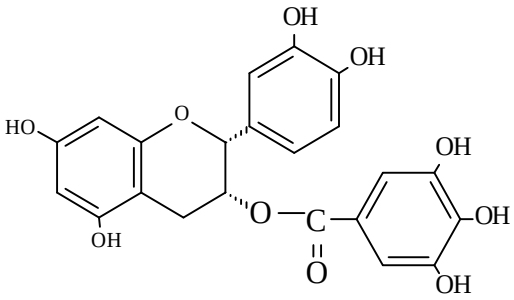


Figure 10

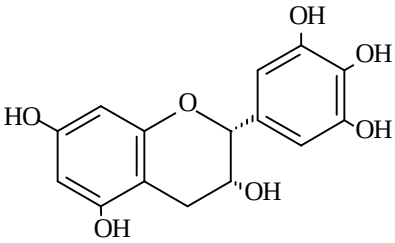
Structures of major green tea catechins



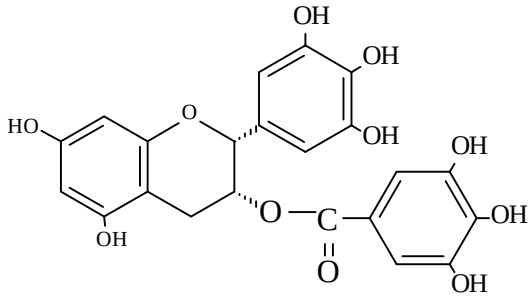
(-) Epicatechin (EC)



(-) Epicatechin-3-gallate (ECG)



(-) Epigallocatechin (EGC)



(-) Epigallocatechin-3-gallate (EGCG)

Figure 11

Activation of DMBA leading to the formation of DNA adducts

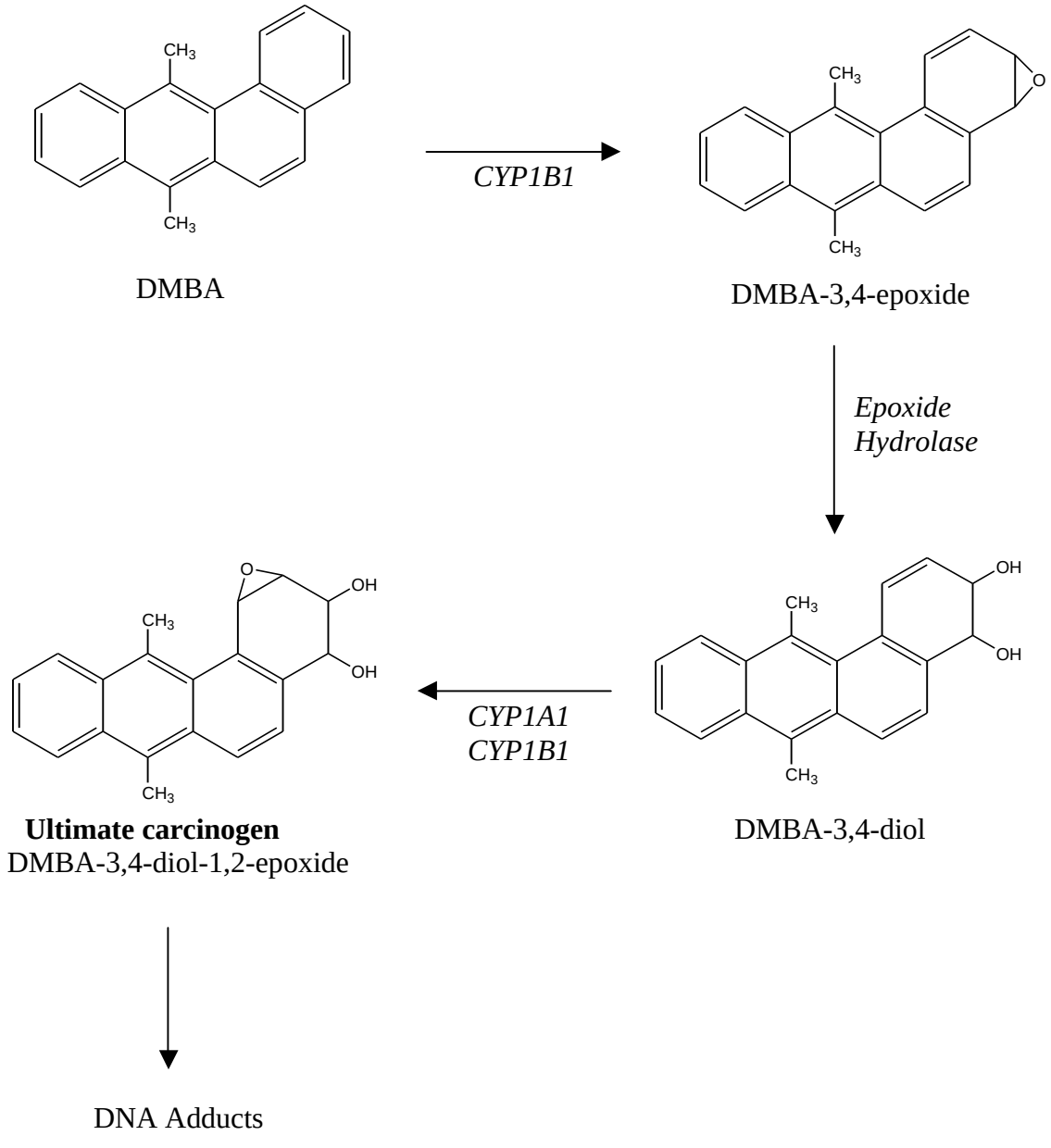
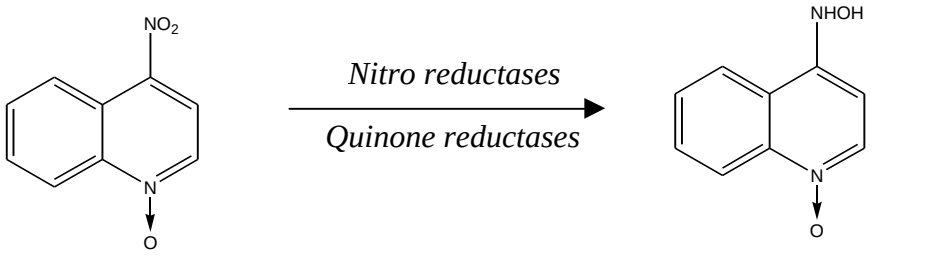


Figure 12

Activation of 4-NQO leading to formation of DNA adducts



4-NQO

Ultimate Carcinogen
4-hydroxyamino-quino-line-1-oxide (4HAQO)

DNA adducts

Figure 13

Short-term effects of topical zileuton, celecoxib and GW2974 on aberrant AA metabolism and cell proliferation in DMBA-treated hamster cheek pouch

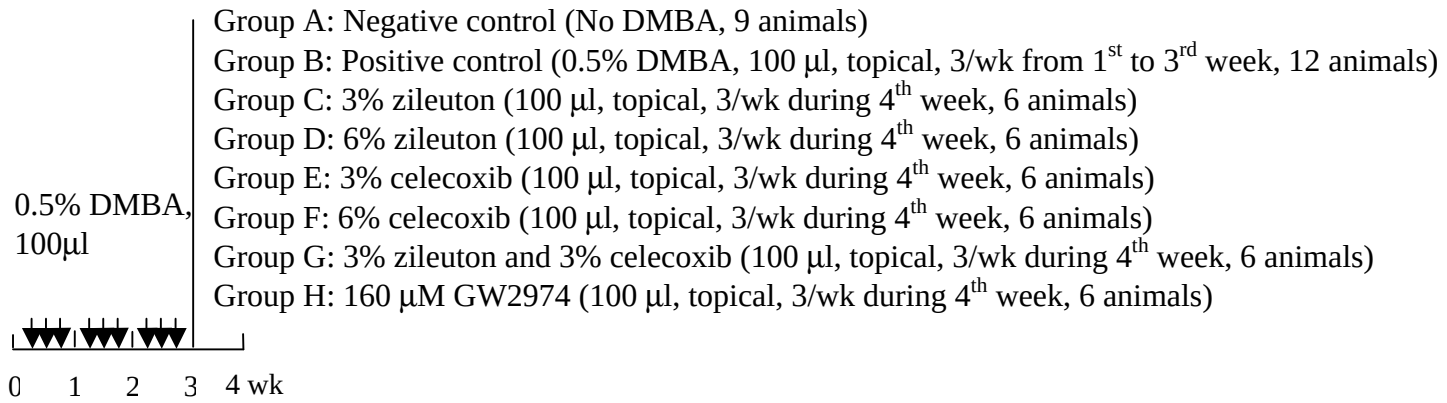


Figure 14

Long-term effects of topical zileuton and celecoxib on DMBA-induced hamster cheek pouch carcinogenesis

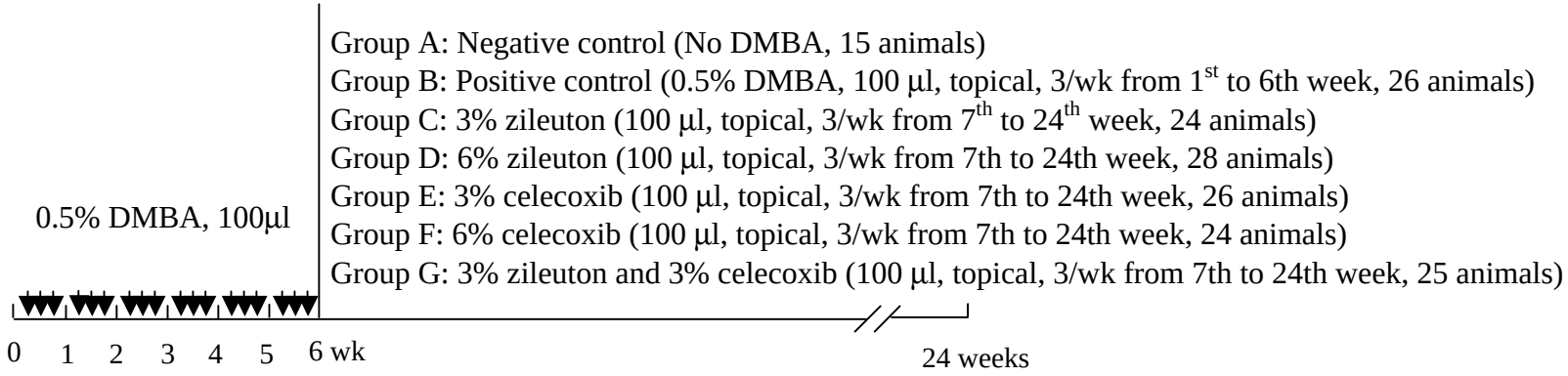


Figure 15

Long-term effects of topical GW2974 on DMBA-induced hamster cheek pouch carcinogenesis

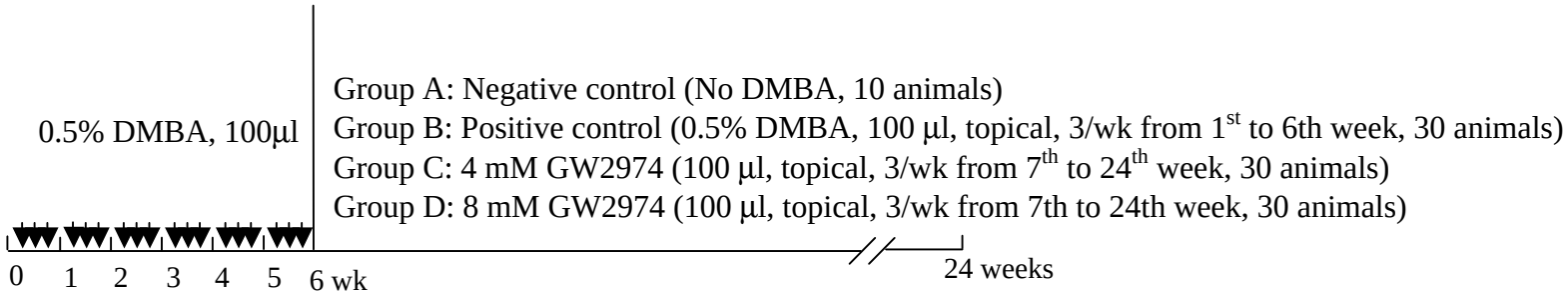


Figure 16

Long-term effects of zileuton and Polyphenon E on 4-NQO induced oro-esophageal carcinogenesis in A/J mice

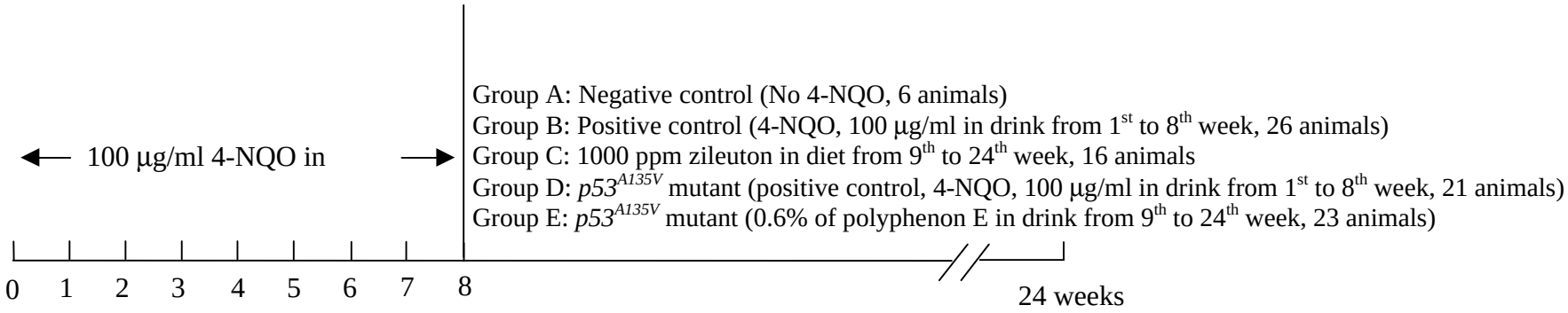


Figure 17

Long-term dose-dependent effects of zileuton and EGCG on 4-NQO induced oro-esophageal carcinogenesis in C57BL/6J mice

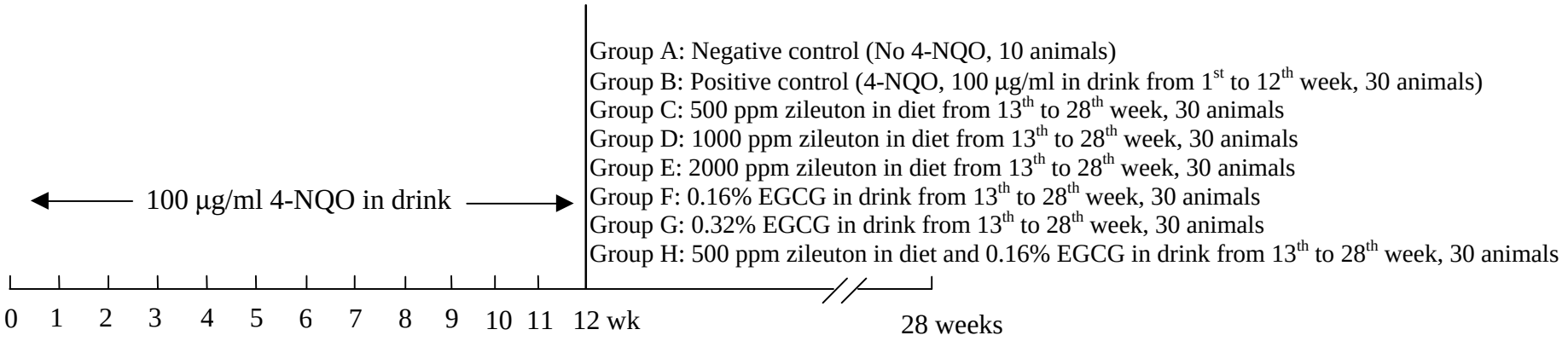
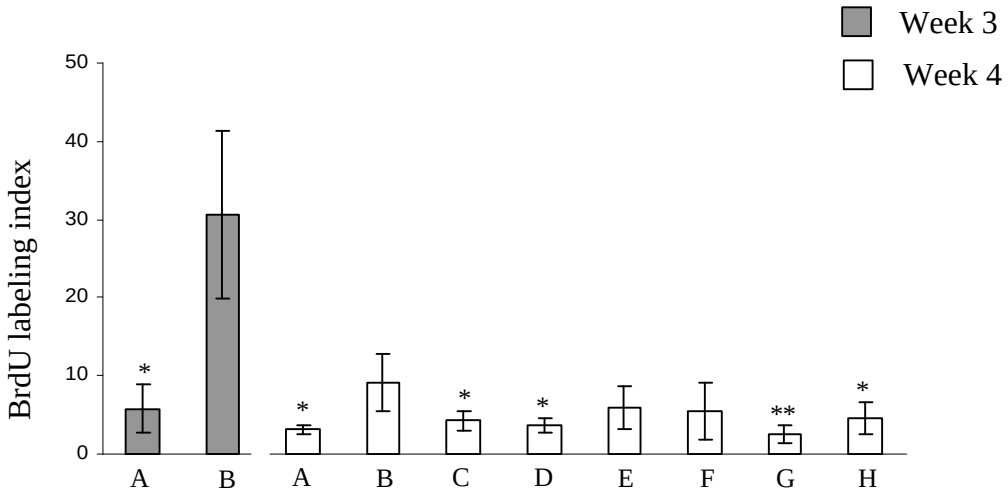


Figure 18

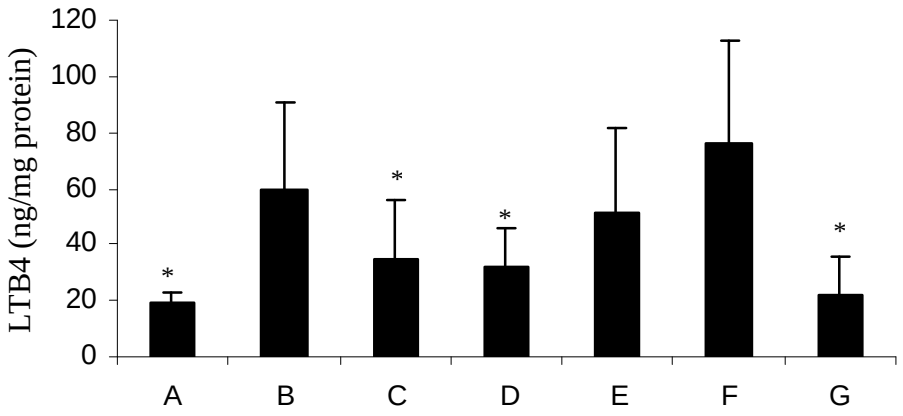
Cell proliferation analysis in short-term hamster study



Hamsters were topically treated with 0.5% DMBA in 100 µl mineral oil using a paintbrush 3 times per week for 3 weeks. They were then randomly divided into 7 groups with Group B serving as the positive control and receiving no further treatment. Groups C (3% zileuton), D (6% zileuton), E (3% celecoxib), F (6% celecoxib), G (3% zileuton and 3% celecoxib) and H (160 µM GW2974) were treated with respective chemopreventive agents in 100 µl mineral oil 3 times per week for 1 week. The avidin-biotin peroxidase method was used with a rat monoclonal antibody (Serotec, Raleigh, NC) at a dilution of 1:100. Three noncontiguous, randomly selected fields under 400x were photographed per sample. The sum of all positive cells was divided by total number of cells to calculate the percentage positive cells (BrdU labeling index). During entire analysis the slides were blinded so that identity of various groups was unknown. Image-Pro Plus 4.5 software (Media Cybernetics, Silver Spring, MD) was used for cell counting.

Figure 19

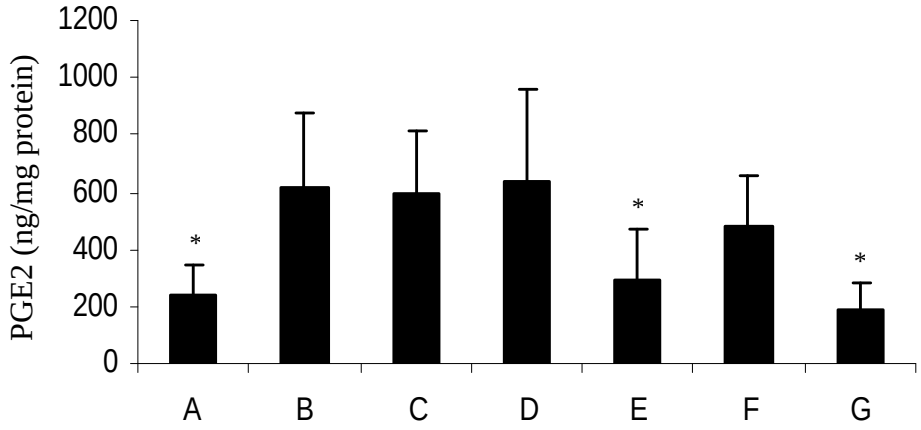
Concentration of LTB4 in long-term DMBA-induced hamster cheek pouch carcinogenesis



Hamsters were topically treated with 0.5% DMBA in 100 µl mineral oil 3 times per week for 6 weeks. They were then randomly divided into 6 groups with Group B serving as the positive control and receiving no further treatment. Groups C (3% zileuton), D (6% zileuton), E (3% celecoxib), F (6% celecoxib) and G (3% zileuton and 3% celecoxib) were treated with respective chemopreventive agents in 100 µl mineral oil 3 times per week for 18 weeks. Oral mucosa samples were snap frozen in liquid nitrogen. After pulverization and homogenization in a buffer containing 10 µM of zileuton and indomethacin, a part of the sample was used for analyzing the protein concentration while the other was extracted with an organic solvent. The organic extract was dried under nitrogen and reconstituted in the enzyme immunoassay buffer for analysis with a kit according to the manufacturer's instructions (Cayman Chemical Co., Ann Arbor, MI). The tissue levels were expressed as nanograms per milligram protein. Student's *t* test was used for statistical analysis. * $p<0.05$. All groups were compared to Group B.

Figure 20

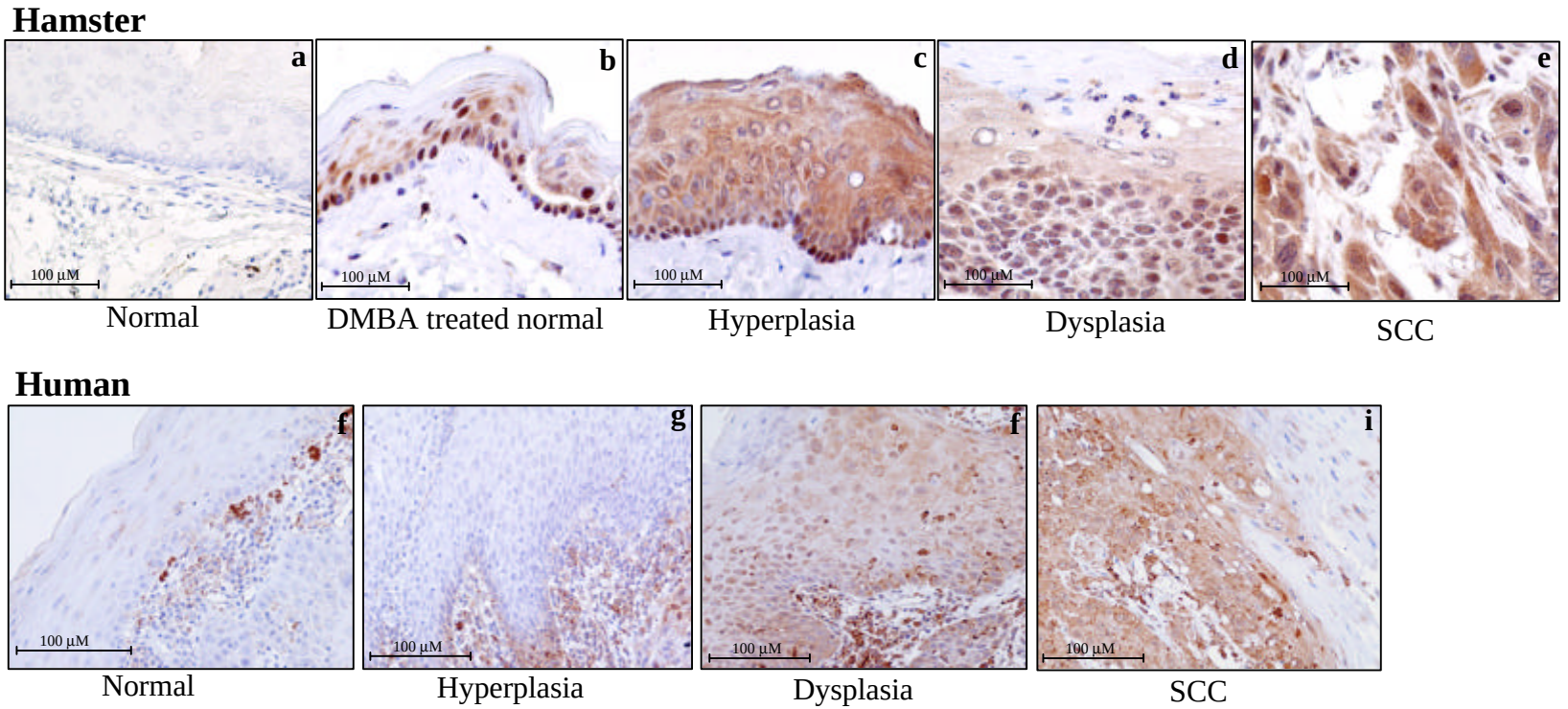
Concentration of PGE2 in long-term DMBA-induced hamster cheek pouch carcinogenesis



Hamsters were topically treated with 0.5% DMBA in 100 µl mineral oil 3 times per week for 6 weeks. They were then randomly divided into 6 groups with Group B serving as the positive control and receiving no further treatment. Groups C (3% zileuton), D (6% zileuton), E (3% celecoxib), F (6% celecoxib) and G (3% zileuton and 3% celecoxib) were treated with respective chemopreventive agents in 100 µl mineral oil 3 times per week for 18 weeks. Oral mucosa samples were snap frozen in liquid nitrogen. After pulverization and homogenization in a buffer containing 10 µM of zileuton and indomethacin, a part of the sample was used for analyzing the protein concentration while the other was extracted with an organic solvent. The organic extract was dried under nitrogen and reconstituted in the enzyme immunoassay buffer for analysis with a kit according to the manufacturer's instructions (Cayman Chemical Co., Ann Arbor, MI). The tissue levels were expressed as nanograms per milligram protein. Student's *t* test was used for statistical analysis. * $p<0.05$. All groups were compared to Group B.

Figure 21

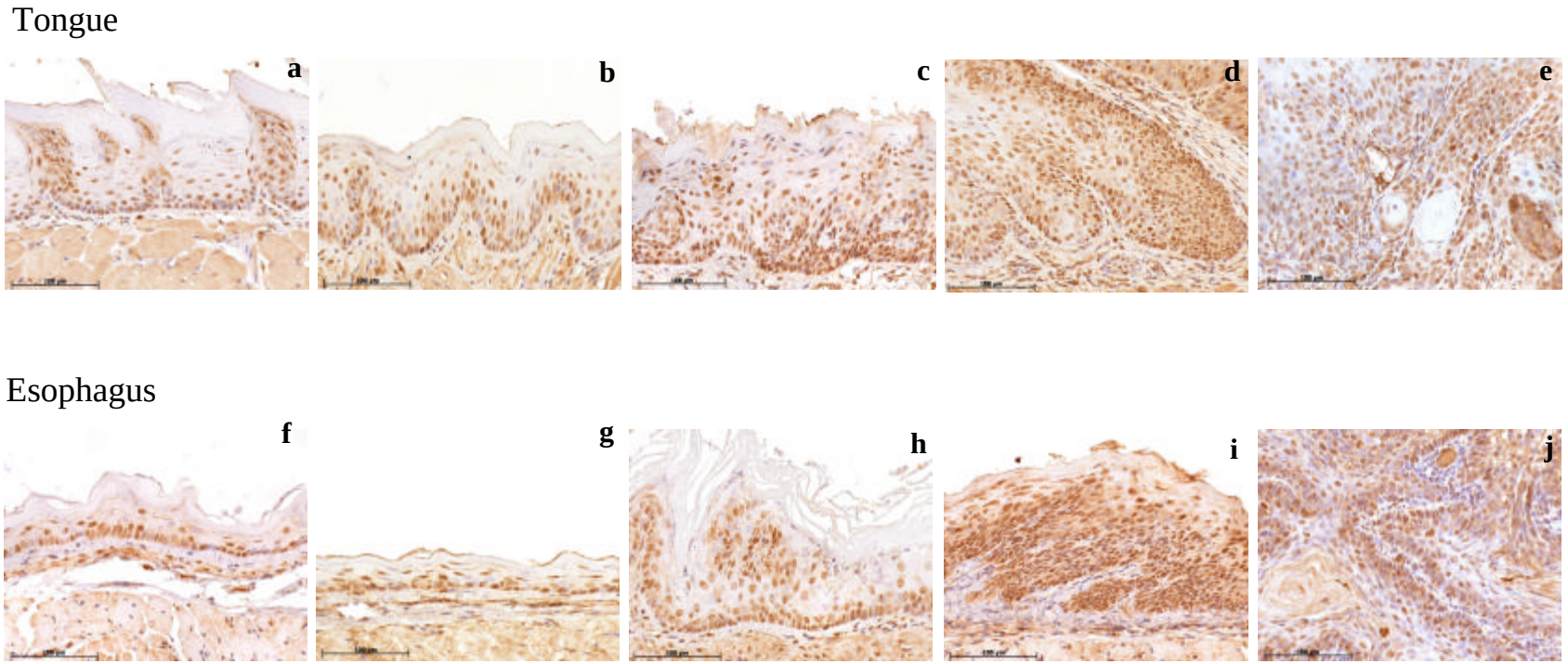
5-Lox expression in DMBA-induced hamster cheek pouch carcinogenesis and human SCC samples



5-Lox IHC was performed using avidin-biotin-peroxidase system. 5-Lox monoclonal antibody was used at a dilution of 1:50 on formalin fixed, paraffin embedded tissues. The paraffin sections were pretreated with antigen unmaking solution before being incubated with the primary antibody. 5-lox was expressed in different stages of pathology in both hamster and human samples.

Figure 22

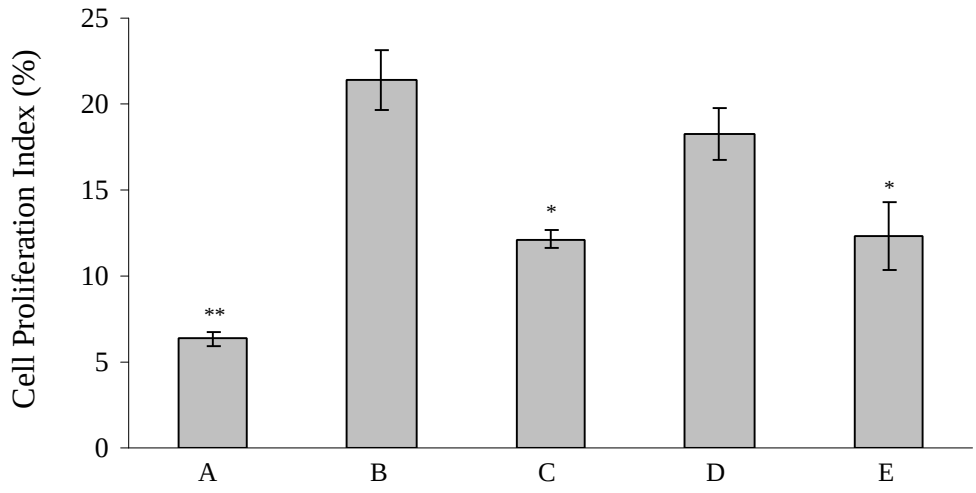
5-Lox expression in long-term 4-NQO induced oro-esophageal carcinogenesis in A/J mice



5-Lox IHC was performed using avidin-biotin-peroxidase system. 5-Lox monoclonal antibody was used at a dilution of 1:50 on formalin fixed, paraffin embedded tissues from the negative and the positive control. The paraffin sections were pretreated with antigen unmaking solution before being incubated with the primary antibody. 5-lox was expressed in different stages of pathology in both tongue and esophagus.

Figure 23

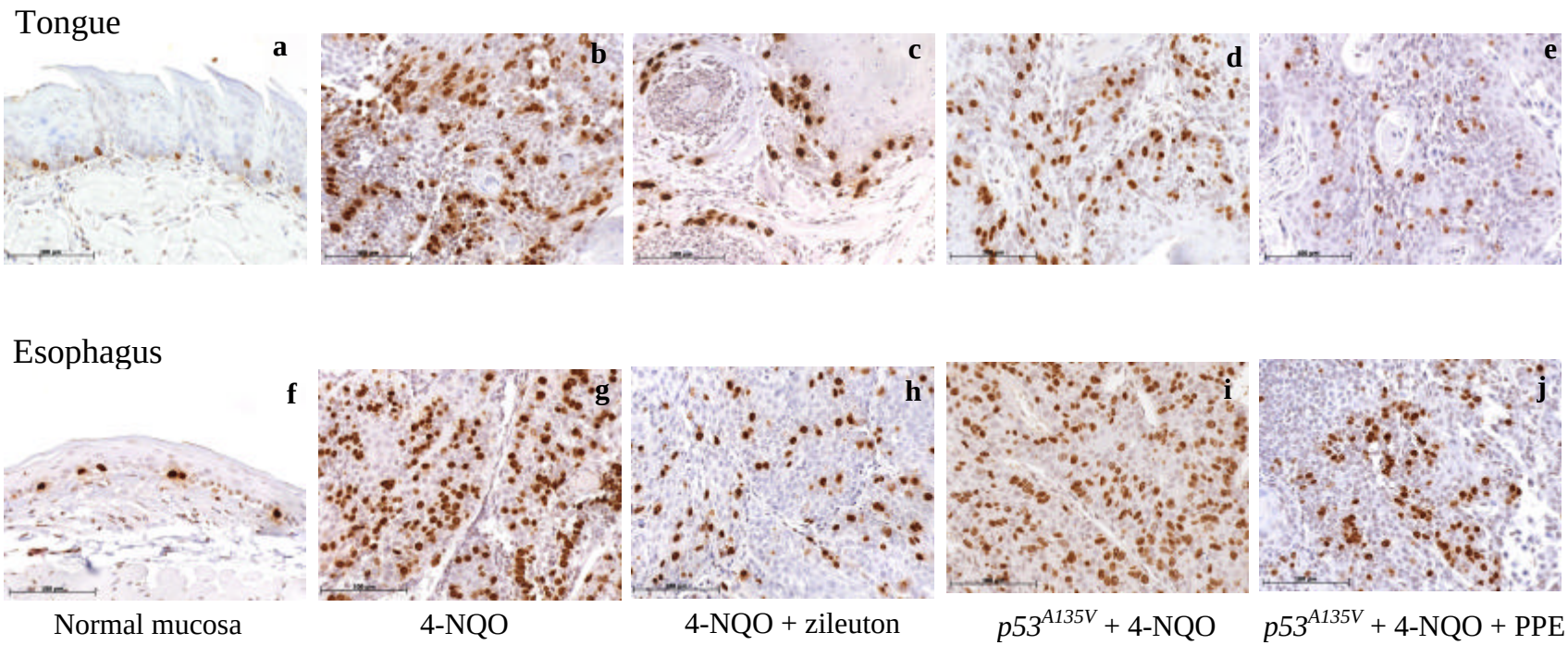
Cell proliferation analysis in cancer tissue from tongue and esophagus in 4-NQO induced oro-esophageal carcinogenesis in A/J mice.



4-NQO was administered at a concentration of 100 μg/ml for 8 weeks. Zileuton and PPE were administered from week 9 to week 24 in diet and drink respectively. For analysis of cell proliferation in tongue and esophagus, BrdU IHC was performed on formalin-fixed, paraffin-embedded tissue sections. The avidin-biotin peroxidase method was used with a rat monoclonal antibody at a dilution of 1:100. Groups A and C were compared with Group B while Group E was compared with Group D. Student's *t* test was used for the statistical analysis between various groups (* $p<0.05$; ** $p<0.01$). Groups A, B, C, D and E represents negative control, positive control, zileuton treated, $p53^{A135V}$ mutant, PPE treated respectively. The values are expressed as average mean±SE. One, two or three (depending on the cancer area available on the tissue) noncontiguous, randomly selected fields under 400x were photographed per sample. The sum of all positive cells was divided by total number of cells to calculate the percentage positive cells (BrdU or cell proliferation index).

Figure 24

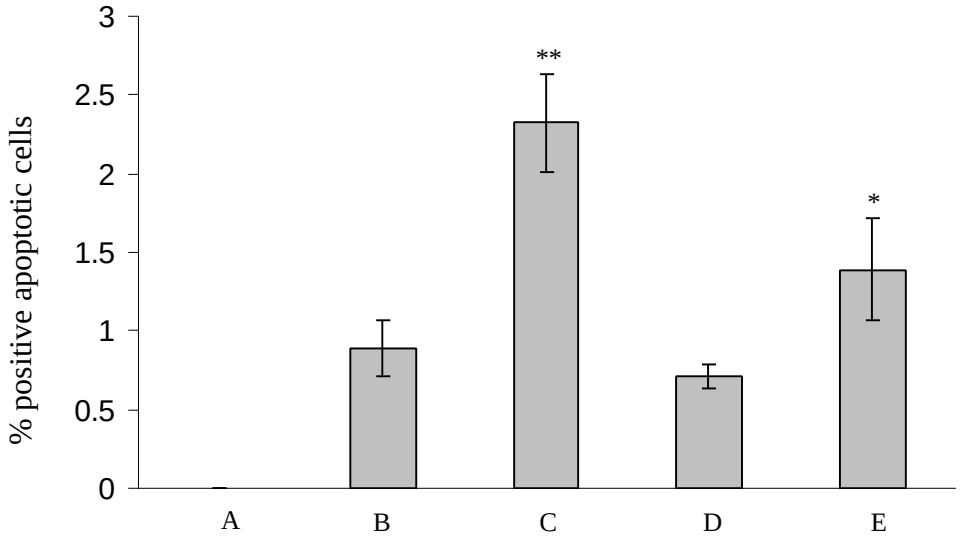
Representative pictures for cell proliferation analysis in cancer tissue from tongue and esophagus in 4-NQO induced oro-esophageal carcinogenesis in A/J mice



BrdU IHC was performed on formalin-fixed, paraffin-embedded tissue sections. The avidin-biotin peroxidase method was used with a rat monoclonal antibody at a dilution of 1:100. One, two or three (depending on the cancer area available on the tissue) noncontiguous, randomly selected fields under 400x were photographed per sample. The sum of all positive cells was divided by total number of cells to calculate the percentage positive cells (BrdU or cell proliferation index). During entire analysis the slides were blinded so that identity of various groups was unknown. Magnification is 400x.

Figure 25

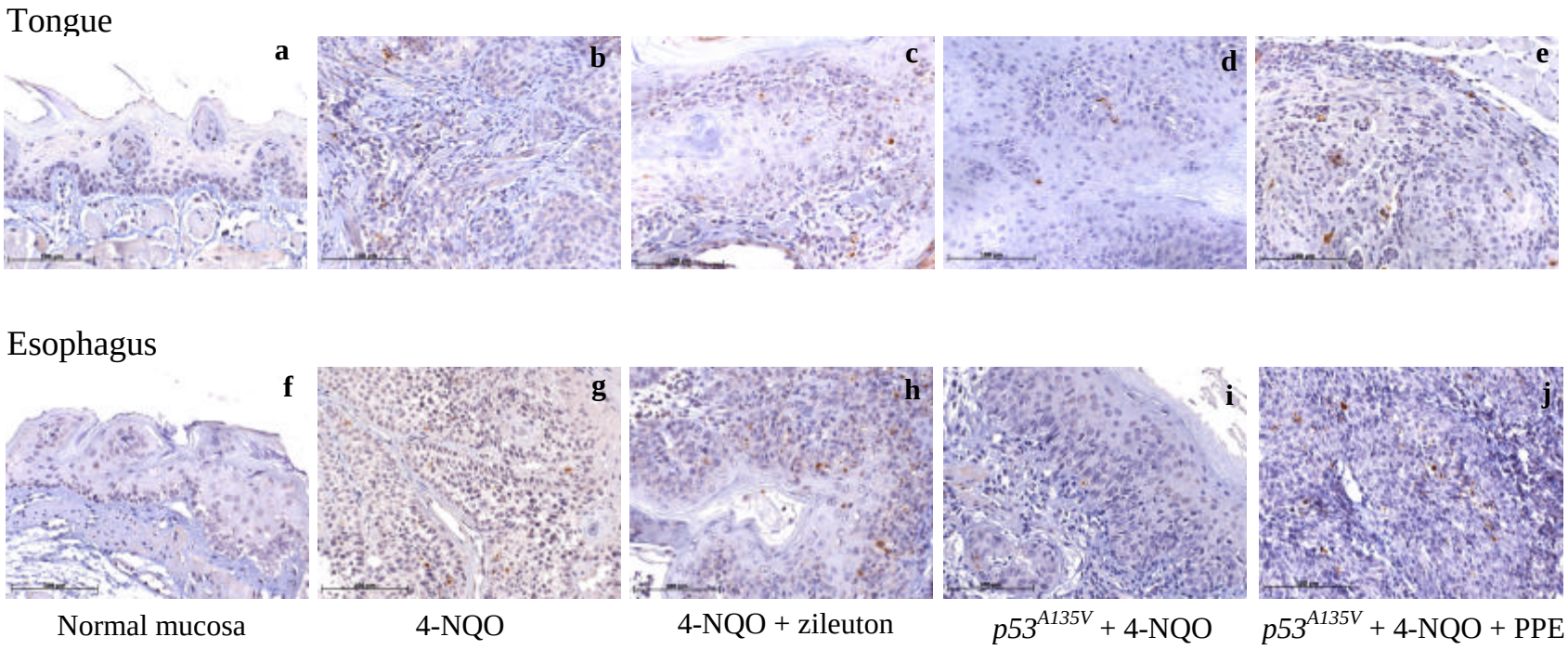
Apoptosis analysis in cancer tissue from tongue and esophagus in 4-NQO induced oro-esophageal carcinogenesis in A/J mice



4-NQO was administered at a concentration of 100 μg/ml for 8 weeks. Zileuton and PPE were administered from week 9 to week 24 in diet and drink respectively. For analysis of apoptosis in tongue and esophagus, cleaved caspase 3 IHC was performed on formalin-fixed, paraffin-embedded tissue sections. The avidin-biotin peroxidase method was used with a rabbit monoclonal antibody at a dilution of 1:100. No apoptosis was seen in the mucosa of the negative control. Group C was compared with Group B while Group E was compared with Group D. Student's *t* test was used for the statistical analysis between various groups (* $p<0.05$; ** $p<0.01$). Groups A, B, C, D and E represents negative control, positive control, zileuton treated, $p53^{A135V}$ mutant, PPE treated respectively. The values are expressed as average mean±SE. One, two or three (depending on the cancer area available on the tissue) noncontiguous, randomly selected fields under 400x were photographed per sample. The sum of all positive cells was divided by total number of cells to calculate the percentage positive cells (apoptotic index).

Figure 26

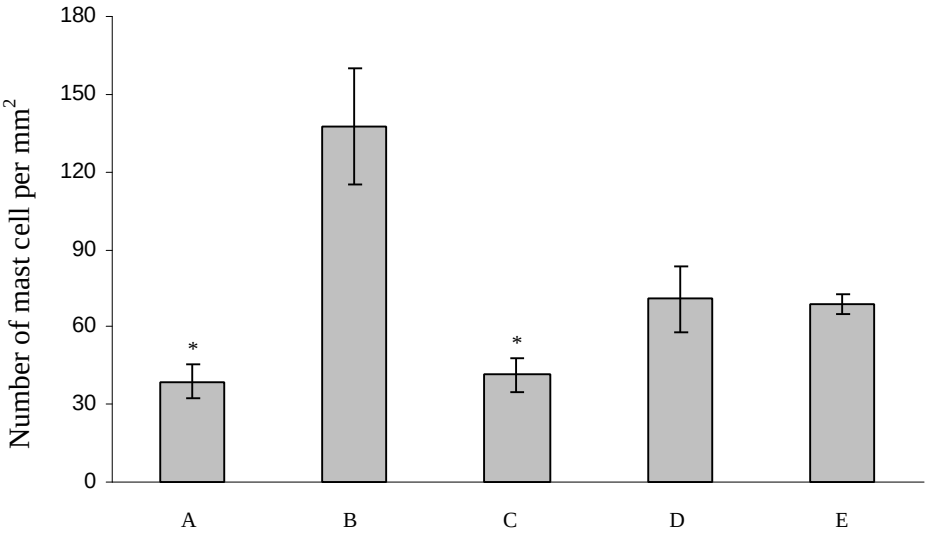
Representative pictures for apoptosis analysis in cancer tissue from tongue and esophagus in 4-NQO induced oro-esophageal carcinogenesis in A/J mice



Cleaved caspase 3 IHC was performed on formalin-fixed, paraffin-embedded tissue sections. The avidin-biotin peroxidase method was used with a rabbit monoclonal antibody at a dilution of 1:100. One, two or three (depending on the cancer area available on the tissue) noncontiguous, randomly selected fields under 400x were photographed per sample. The sum of all positive cells was divided by total number of cells to calculate the percentage positive cells (apoptotic index). During entire analysis the slides were blinded so that identity of various groups was unknown. Magnification is 400x.

Figure 27

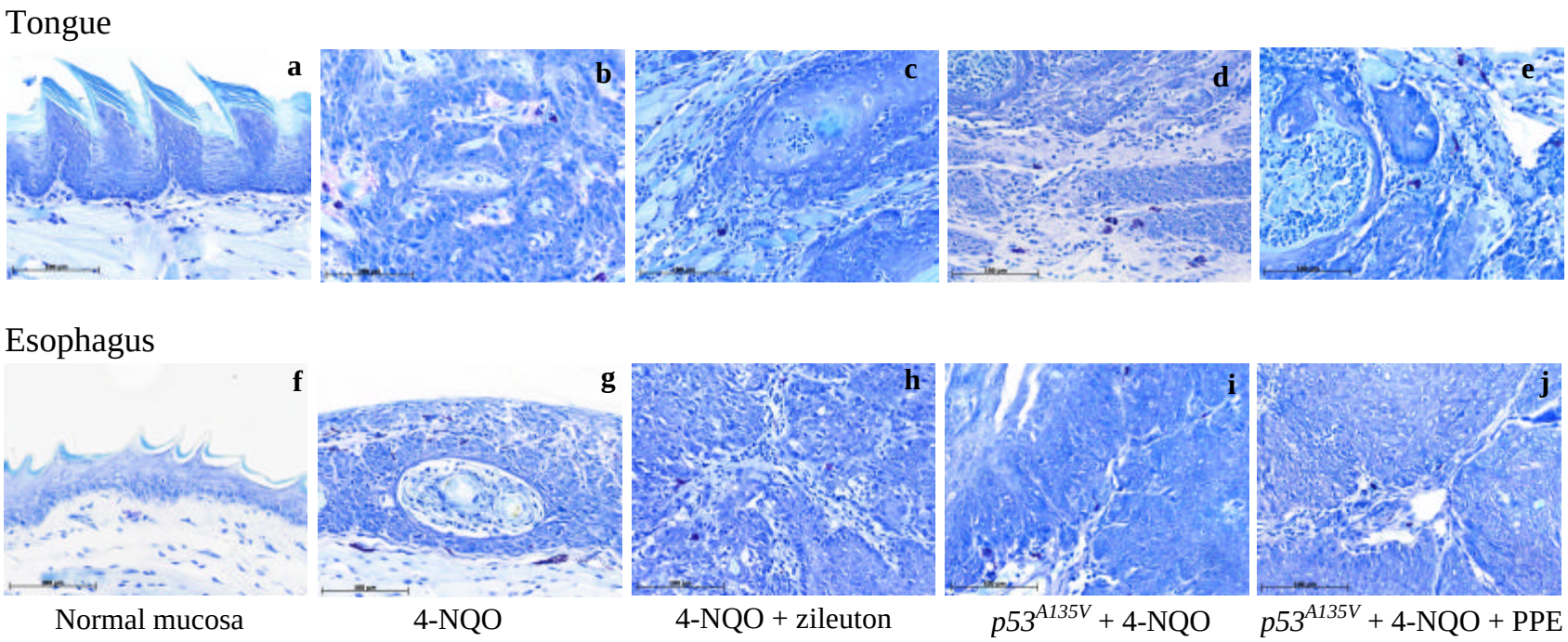
Mast cell analysis in cancer tissue from tongue and esophagus in 4-NQO induced oro-esophageal carcinogenesis in A/J mice



4-NQO was administered at a concentration of 100 μg/ml for 8 weeks. Zileuton and PPE were administered from week 9 to week 24 in diet and drink respectively. For analysis of mast cell density in tongue and esophagus, toluidine blue staining was performed on formalin-fixed, paraffin-embedded tissue sections. One, two or three (depending on the cancer area available on the tissue) noncontiguous, randomly selected fields under 200x were photographed per sample. During entire analysis the slides were blinded so that identity of various groups was unknown. Number of mast cell was counted per mm^2 for each sample using AxioVision imaging software version 4.1 (Thornwood, NY). The average number of mast cells was counted for each group to give the mast cell density per mm^2. Groups A and C were compared with Group B while Group E was compared with Group D. Student's *t* test was used for the statistical analysis between various groups (* $p<0.05$).

Figure 28

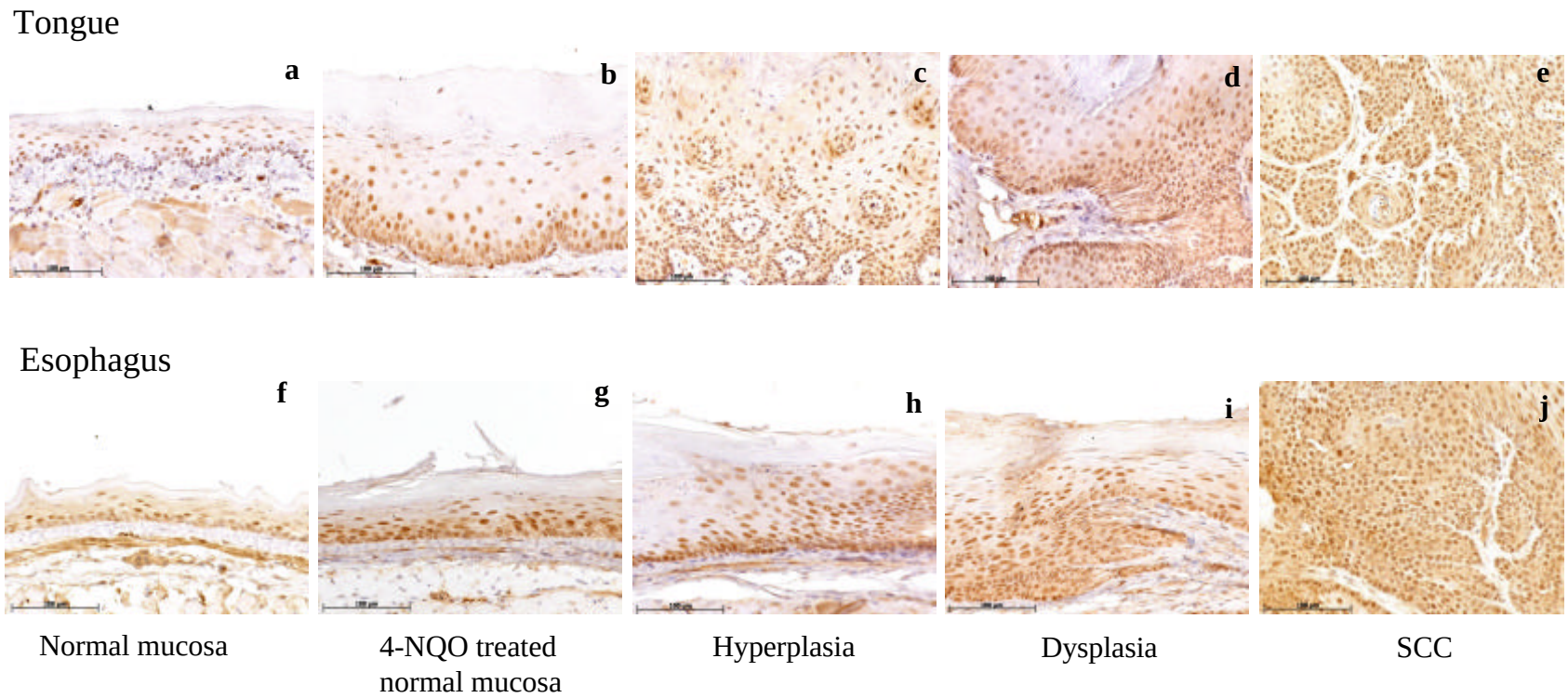
Representative pictures for mast cell density in cancer tissue from tongue and esophagus in 4-NQO induced oro-esophageal carcinogenesis in A/J mice



Toluidine blue staining was performed on formalin-fixed, paraffin-embedded tissue sections. One, two or three (depending on the cancer area available on the tissue) noncontiguous, randomly selected fields under 200x were photographed per sample. During entire analysis the slides were blinded so that identity of various groups was unknown. Number of mast cell was counted per mm^2 for each sample using AxioVision imaging software version 4.1 (Thornwood, NY). The average number of mast cells was counted for each group to give the mast cell density per mm^2. Magnification is 400x.

Figure 29

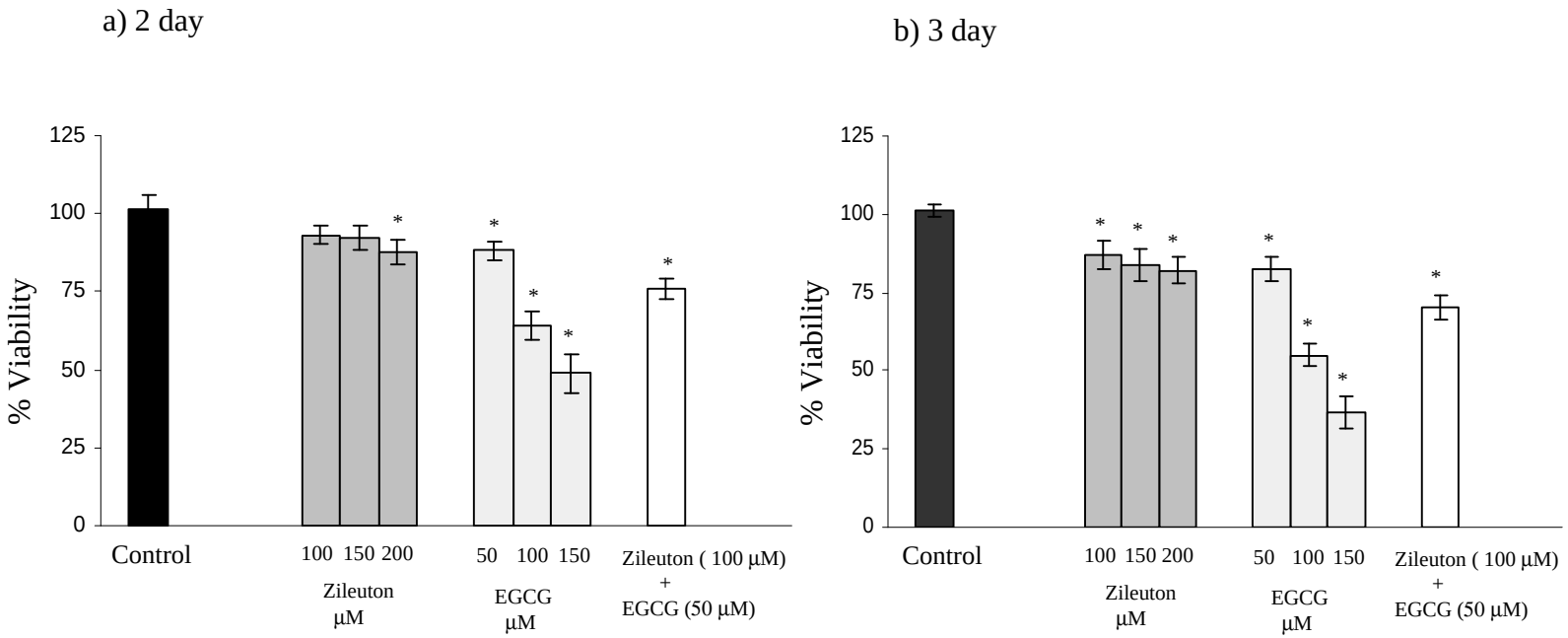
5-Lox expression in long-term 4-NQO induced oro-esophageal carcinogenesis in C57 black mice



5-Lox IHC was performed using avidin-biotin-peroxidase system. 5-Lox monoclonal antibody was used at a dilution of 1:50 on formalin fixed, paraffin embedded tissues from the negative and the positive control. The paraffin sections were pretreated with antigen unmaking solution before being incubated with the primary antibody. 5-lox was expressed in different stages of pathology in both tongue and esophagus.

Figure 30

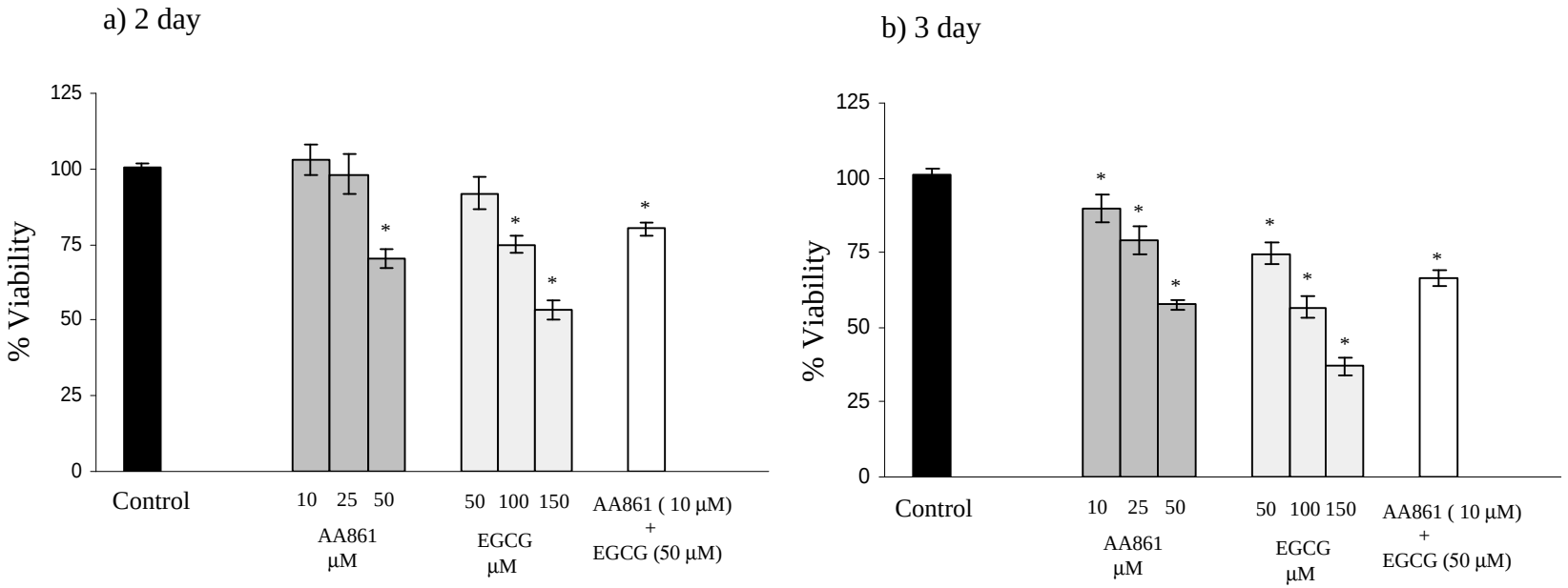
Effect of zileuton and EGCG on viability of CAL27 cells



CAL27 cells were plated at a density of 3 x 10^3 cells/well in a 96 well plate and incubated at 37 ºC in a 5% carbon dioxide atmosphere and were incubated for 24 hours. The cells were treated with various agents for another 48 and 72 hours and were then incubated 0.5 mg/ml MTT solution and for 2-3 hours. The resulting formazan product was solubilized in dimethlysulfoxide (DMSO) and absorbance was measured at 570 nm. Student's *t* test was used for the statistical comparison. All comparisons were made with the control group. $^{*}p<0.05$.

Figure 31

Effect of AA861 and EGCG on viability of CAL27 cells



CAL27 cells were plated at a density of 3 x 10^3 cells/well in a 96 well plate and incubated at 37 ºC in a 5% carbon dioxide atmosphere and were incubated for 24 hours. The cells were treated with various agents for another 48 and 72 hours and were then incubated 0.5 mg/ml MTT solution and for 2-3 hours. The resulting formazan product was solubilized in dimethlysulfoxide (DMSO) and absorbance was measured at 570 nm. Student's *t* test was used for the statistical comparisons. All comparisons were made with the control group. $^{*}p<0.05$.

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EDUCATION

2003-2007	Ph.D. Food Science, Graduate School-New Brunswick, Rutgers, The State University of New Jersey, New Brunswick, New Jersey, USA
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Aug1999-Aug 2001	Medical Representative, GlaxoSmithKline, India

PUBLICATIONS

Chen X, **Sood S**, Yang CS, Li N, and Sun Z (2006). Five-lipoxygenase pathway of arachidonic acid metabolism in carcinogenesis and cancer chemoprevention. *Current Cancer Drug Targets*, **6**, 519-32.

Sun Z[*], **Sood S**[*], Li N, Ramji D, Yang P, Newman RA, Yang CS, and Chen X (2006). Involvement of the 5-lipoxygenase/leukotriene A4 hydrolase pathway in 7,12-dimethylbenz[a]anthracene (DMBA)-induced oral carcinogenesis in hamster cheek pouch, and inhibition of carcinogenesis by its inhibitors. *Carcinogenesis*, **27**, 1902-8.
[*] Equal contribution to this work.

Sood S, Shiff SJ, Yang CS and Chen X (2005). Selection of topically applied non-steroidal anti-inflammatory drugs for oral cancer chemoprevention. *Oral Oncology*, **41**, 562-7.

Li N, **Sood S**, Wang S, Fang M, Wang P, Sun Z, Yang CS and Chen X (2005). Over-expression of 5-lipoxygenase and cyclooxygenase 2 in hamster and human oral cancer and chemopreventive effects of zileuton and celecoxib. *Clinical Cancer Research*, **11**, 2089-96.

Chen X, Wang S, Wu N, **Sood S**, Wang P, Jin Z, Beer DG, Giordano TJ, Lin Y, Shih WC, Lubet RA and Yang CS (2004). Overexpressi

on of 5-lipoxygenase in rat and human esophageal adenocarcinoma and inhibitory effects of zileuton and celecoxib on carcinogenesis. *Clinical Cancer Research*, **10**, 6703-9.