INHIBITION OF COLON CARCINOGENESIS BY STILBENOIDS AND MECHANISMS OF ACTION

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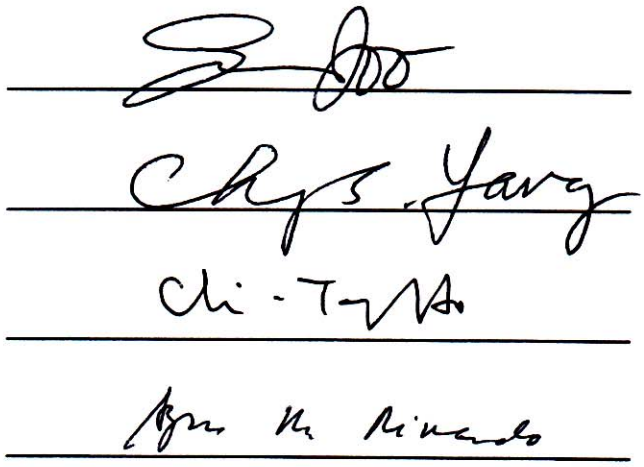

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ABSTRACT OF THE DISSERTATION

Inhibition of colon carcinogenesis by stilbenoids and mechanisms of action

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Stilbenes are a class of compounds present in small fruits such as grapes and berries and are known to present diverse pharmacological properties which include cholesterol lowering, serum glucose regulation, controlling lifespan and anti-cancer activity. Resveratrol is one of the extensively studied stilbene. Pterostilbene (*trans*-3, 5-dimethoxy-4'-hydroxystilbene), a structural analog of resveratrol, is present in heartwood of the tree, *Pterocarpus marsupium* as well as in many small fruits such as blueberries.

In an attempt to study the effects of pterostilbene on colon carcinogenesis, we identified that pterostilbene inhibited expression of certain inflammatory genes in the colon and suppressed aberrant crypt foci formation in an azoxymethane (AOM)-induced model of colon carcinogenesis in rats. We also investigated the mechanism of anti-inflammatory action of pterostilbene using cultured HT-29 colon cancer cells. Our studies identified that the p38-ATF2 pathway was significantly inhibited by

pterostilbene. More importantly, by silencing the expression of p38α isoform, there was significant reduction in iNOS and COX-2 induction. Interestingly, pterostilbene and the structurally similar compound, resveratrol, targeted different inflammatory pathways in HT-29 colon cells.

Pterostilbene given at 40 ppm of the diet of AOM-injected rats lowered the tumor multiplicity of non-invasive adenocarcinomas compared to the control diet. Pterostilbene lowered the β-catenin levels in HT-29 cells which can have implications in the action of the compound against cell proliferation. An evaluation of different structural analogs of pterostilbene revealed some compounds to have greater action than pterostilbene against colon cancer cells. Methoxylation, ester and amino modifications at the 4' position in the B ring conferred greater potency for the molecule against cell proliferation and inflammation *in vitro*. Amino substitutions and methoxy modifications with trans configuration reduced tumor burden significantly in the HT-29 xenograft tumor model in SCID mice. These compounds were present at higher levels in the serum of the animals compared to the levels of other compounds. In conclusion, stilbenoid class of compounds has promising effects against proliferation and inflammation in both *in vivo* and *in vitro* models of colon carcinogenesis.

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INTRODUCTION

1. Colorectal cancer

Colorectal cancers (CRC) are the cancers that arise either in the colon or the rectum and initiates as a polyp from the inner lining cells eventually growing into the center of the colon or the rectum. According to the statistics released by American Cancer Society for the year 2009, it is the third largest cause of cancer deaths in the United States. The estimated new cases of CRC for 2009 are 146,970 with cancer deaths of 49,920 [1]. CRC is most prevalent in North America, Argentina, Australia, New Zealand, parts of Europe, Japan and Israel, making it more correlated with western lifestyle and diet. However, CRC incidences are on a rise worldwide and is one of the frequent cancers of the planet.

1.1. Clinical overview of CRC.

CRC is categorized based on the stage of cancer development from stage I through stage IV, stage I being the initial stage during which cancer spreads from mucosa into sub mucosa and stage IV, the stage representing metastatic progression into distant organs and lymph nodes. Stages II and III present intermediate characteristics of cancer progression and its spread. Five-year survival rates in CRC based on the stage of cancer: patients with stage I cancers have a 5-year survival of approximately 93%; stage IIA, 84%; stage IIB, 72%; stage IIIA, 83%; stage IIIB, 64%; stage IIIC, 44%; and stage IV, 8% [2]. TNM staging is also one of the very commonly

used staging system and is based on the extent of the tumor **(T)**, the extent of spread to the lymph nodes **(N)**, and the presence of metastasis **(M)**.

Based on the releases of the National Cancer Institute, CRC incidence has fallen substantially by 26% from 1984 to 2004 [3]. This may be attributed to the improved availability and use of screening techniques, such as sigmoidoscopy, fecal occult blood testing and colonoscopy; improved surgical methods; advances in chemotherapy, such as targeted therapies against epidermal growth factor receptor (EGFR) by Cetuximab and development of recombinant antibodies (Bevacizumab) against vascular endothelial growth factor (VEGF) and development and clinical use of easily detectable tumor markers, such as serum carcinoembryonic antigen (CEA) and CA19-9 [3-6].

1.2. Prognostic markers for CRC

Tumor extent and staging, lymph node status, tumor grade and tumor invasion are the most important morphological prognostic markers in CRC [7]. In order to have an accurate prediction on prognosis and treatment, a large number of tissue based molecular, protein or carbohydrate prognostic markers have been investigated. This includes markers for loss of heterozygosity at various chromosomal sites (e.g. 1p, 8p, 17p or 18q), serum CEA, mutations in *KRAS,* markers for angiogenesis, such as vascular endothelial growth factor (VEGF), and markers for cell proliferation identified by immunohistochemistry of tumor tissues and tissue microarray, such as cyclin D1, c-MYC, PCNA, Ki-67, p21 and p27 [7,8]. The

reliability of the identified markers for exact prognosis of CRC in clinic is yet to be fully validated.

1.3. Surrogate end points for CRC detection based on colon anatomy changes

1.3.1. Basic function, anatomy and morphology of colon

Colon is the part of the intestinal tract that is involved in the absorption of water and salt from the feces and propelling the feces to the rectum for defecation. The length of the human colon is around 100-150 cm and is composed of four types of cells: columnar absorptive cells, mucus secreting goblet cells, peptide hormone-secreting endocrine cells and Paneth cells [9]. The mucus protects the colonic mucosa from the distress of progressively dehydrated feces. The cells are arranged in tightly packed glands or crypts which extend to the colonic mucosa, with the goblet cells occupying mostly the bottom of the crypts and absorptive cells lining the entire luminal surface. The space between the glands is filled by the lamina propria which contains collagen; blood and lymph vessels to absorb the water by passive diffusion; and lymphocytes and plasma cells to aid in defense mechanisms [10].

1.3.2. Aberrant crypt foci (ACF)

Stem cells reside near the base of the crypt and give rise to progenitor cells that move progressively to the top of the crypts and undergo cell cycle arrest and differentiation during the process. The process of cell proliferation and migration is compensated when the cells on the top of the crypts undergo apoptosis that

requires about 2-3 days [9,11-13]. However, the cells with damaged DNA or genetic mutations do not undergo apoptosis and continue proliferating at the uppermost part of the crypt leading to the precancerous stage called as aberrant crypt foci (ACF). One school of thought suggests that the genetic mutation may be first acquired by a stem cell in the niche which may be transferred to the progenitor cells and so on till the colonization of crypts with mutant cells by processes of clonal conversion and cell division; however this is not a completely verified theory [9].

ACF are widely used biomarkers in animal experiments for chemoprevention [14-16]. These lesions are thought to be the earliest identifiable precursor for CRC in rodents and humans, and not all of the ACF develop into adenoma/carcinoma. These are better than adenomatous polyps for use as a marker for sporadic cancers, since it takes around couple of decades for a normal cryptal cell to transform to adenoma. ACF may eventually evolve into polyps (adenoma) and further into carcinoma by the 'adenoma-carcinoma sequence' [17]. ACF are characterized by crypts with altered luminal opening, thickened epithelia and larger crypts compared to the adjacent crypts [18]. These lesions are divided into dysplastic and non-dysplastic ACF and dysplastic ACF can be readily identified in the mucosa of patients with inherited disorder called familial adenomatous polyposis and also in sporadic cancer patients [19,20]. ACF were recently identified as important biomarkers in dysplasia-carcinoma sequence in patients with ulcerative colitis [21].

1.3.3. Beta catenin accumulated crypts (BCAC)

These crypts, as the name suggests, carry a large amount of β-catenin in the nuclei and/or the cytoplasm as can be revealed by the highly basophilic cells in hematoxylin eosin stained sections and by β-catenin immunohistochemistry of horizontal cross sections. BCAC are not identifiable on the mucosal surface as is ACF and have greater dysplasia than ACF lesions [22]. Whether BCAC represents a subset of ACF is yet to be confirmed. However, frequent *β-catenin* gene mutations were observed in BCAC, which occurred early in rodent colonic carcinogenesis, with no appearance of aberrant crypt foci (ACF) [23]. Also, BCAC numbers were sequentially higher with the duration of carcinogen exposure, while ACF numbers were almost unchanged [22].

1.3.3. Mucin-depleted foci (MDF)

MDF represent dysplastic crypts that are characterized by the absence or scant production of mucus and have been identified as useful biomarkers in CRC of carcinogen-treated rodents [24] and, recently, also in humans at high risk of colon cancer [25]. MUC2 is the most abundant mucin in the colon and *Muc2*-deficient mice are prone to intestinal carcinogenesis [26]. Muc2 levels were drastically reduced in MDF [27], suggesting the relevance of MDF as a biomarker. Further, the number of MDF increased proportionally with the increase in dosage of the carcinogen [28] and the increase in MDF by carcinogen was reduced with colon cancer inhibiting drug, such as piroxicam [28]. MDF had mutations in genes affecting colon

carcinogenesis, such as *APC* and showed Wnt signaling activation like colonic tumors, features suggesting that these lesions are precancerous [28]. MDF is identified in nonsectioned colon as the crypts showing negative staining with high-iron diamine Alcian blue.

1.3.4. Adenomatous polyps (Adenoma)

Polyps denote a mass of cells of epithelial dysplasia with uncontrolled proliferation that project above the surface of the surrounding normal mucosa. Severe dysplasia in an adenoma is considered a selective marker for the increased risk for CRC, especially when the lesions exhibit villous morphology and are greater than 1.0 cm [29]. Colorectal adenomas show mutations of oncogenes, such as *KRAS*, inactivation of tumor suppressor genes such as *APC, p53* and *DCC* (deleted in colon cancer) and DNA repair genes (*MLH1, MSH2*) and aberrations in DNA methylation.[30]. Polyps are easily detectable by endoscopy since they project out from the mucosa unless they are 'flat' in nature. However, detection of these flat adenomas is critical owing to the considerably higher malignancy associated with these structures with greater villous compartmentalization compared to regular polyps [31].

1.4. Genetic alterations during CRC initiation and progression.

Sporadic CRCs develop through a multi-step process with acquired genetic alterations marking the entry into a higher grade of tumor. The chromosomal instability and the sequential genetic aberrations required for initiation and

progression from one stage to the next is reported in the initial studies by Vogelstein *et al.* [32] and are reviewed by Walther et al. [33]. The formation of premalignant lesions of the colon, also called adenomas is associated with the loss of the tumor suppressor gene, the *APC* (*Adenomatous Polyposis Coli*) gene. *APC* mutations occur in up to 80% of adenomas and adenocarcinomas and 4.3% in aberrant crypt foci (ACF) [34,35]. Large adenomas acquire mutations in the GTPase, *KRAS* to transform into intermediate adenomas. *KRAS* oncogene binds to cytoplasmic Raf-1and translocates it to the membrane and the active Raf-1 causes activation of mitogen activated protein kinase/extracellular receptor kinase (ERK) to result in increased cell proliferation. *Ras* gene mutations occur in 58% of adenomas larger than 1 cm and in 47% of carcinomas, while only 9% adenomas with size less than 1 cm show mutations in *KRAS [32]*. Controversies exist for the sequence that *APC* mutations precede *KRAS* mutations in case of sporadic CRC. Takayama et al. found no *APC* mutations in dysplastic ACF while *KRAS* mutations were present in 68% cases, although adenomas from same patients showed positivity for *APC* mutation [36]. These suggest the presence of an alternate genetic pathway for the development of ACF and adenomas; and *APC* mutations within the ACF may be central for its progression to adenoma [36]. Loss of 18q chromosome with Smad4, a known CRC predisposition gene that is also located on 18q, drives the adenoma to late adenoma stage. Loss of 18q is a marker of chromosomal instability (CIN) [37] while mutations in Smad4 are indicative of microsatellite instability (MSI) [38]. CIN occurs in around 85% of sporadic CRC with the remaining 15%

contributed by MSI. Further the mutations in the tumor suppressor gene *p53* transforms the adenoma to the carcinoma stage [32].

1.5. Risk factors for CRC

Risk factors for developing cancers of the colon and rectum include age, presence of polyps, family history for CRC, inherited syndromes such as, familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC), certain diets and lifestyle [39]. Also, a history of bowel disease that includes ulcerative colitis and Crohn's disease is considered a major risk factor for the development of colon cancer. In these conditions intestine remains inflamed over a long period of time and strong associations exist between the duration and extent of mucosal inflammation and cancer [40]. Although genetic mutations increase the risk for CRC, about 75% of CRC are sporadic. These are generally age related and are not known to be linked to hereditary factors [3]. 15-20% of CRC include moderate risk patients with a family history of CRC, while the high risk group of 5-15% include those with FAP or HNPCC or long standing IBD [41].

2. Inflammation and cancer

Chronic inflammation has long been associated with the process of tumorigenesis. Rudolf Virchow in 1863 noted lymphocytes in neoplasms and made the observation that "lymphoreticular infiltrate" reflected the origin of cancer at the site of chronic inflammation [42]. Over the past decades, our understanding of the tumor microenvironment and presence of inflammatory component in malignancies

has led to increasing appreciation of Virchow's suggestion. Many components of inflammation, such as cytokines, macrophages, reactive oxygen and nitrogen species and events such as, leukocyte migration and angiogenesis are also identified in a variety of tumors. Inflammation is a positive response of our body to any injury or infections and helps eliminate the invading pathogens when it appears in acute phase. However, when this inflammatory response becomes chronic, under inappropriate control it can flip into an imbalanced condition where the inflammatory mediators can act as enemies of tissue homeostasis and thereby act to promote tumors [43,44]. It has been estimated that chronic infections and inflammation is responsible for about one in four cancer cases worldwide [45]. For example, *Helicobacter pylori* infection is associated with gastrointestinal carcinogenesis; ulcerative colitis with colorectal cancer; Schistosomiasis with bladder carcinoma; human papilloma virus with cervical cancer [42,46].

2.1. Events in inflammation associated carcinogenesis

In response to proinflammatory signals, different inflammatory cells such as macrophages, neutrophils, mast cells, natural killer cells and dendritic cells are recruited at the sites of inflammation. These activated cells generate reactive oxygen and nitrogen species (ROS and RNS), which cause mutational damages to DNA and perturbation of DNA-protein cross-links to eventually effect in tumor initiation [46]. Aberrant DNA methylation [47], and histone modifications of cancer related genes [48] has been implicated in chronic inflammatory responses and related carcinogenesis. Importantly, initiated cells produce soluble mediators called

cytokines and chemokines that attract leukocytes, which in turn produce cytokines that stimulate further cell proliferation. Acceleration of cell proliferation, evasion from apoptotic cell death and neovascularization are the regulatory mechanisms for tumor promotion and progression and inflammation plays a key role in modulating these effects [49,50].

2.2. Cytokines and chemokines

Cytokines, chemokines, COX-2, iNOS, NF-κB and kinase pathways are recognized as the major molecular players in deregulated inflammation driven carcinogenesis. Cytokines can be broadly classified as inflammatory (interleukins, IL-1, IL-6, IL-17) and anti-inflammatory (IL-10) ones [44]. This classification is arbitrary since certain cytokines such as tumor necrosis factor (TNF-α) may act as tumor suppressors or promoters based on the tumor microenvironment [51]. Similarly, a low concentration of IL-1β is associated with protective immune response, while a high concentration is linked to inflammation-related tumor invasion [52]. Importantly, many of these cytokines are found to be present in human cancers including those of the colorectum, breast, prostate and bladder [42,53]. The action of cytokines to facilitate carcinogenesis is multi-fold: DNA damage by ROS, RNS; inhibition of DNA repair by ROS; functional inactivation of tumor suppressor genes; tissue remodeling via activation of matrix metalloproteinases (MMPs); stimulation of angiogenesis and control of cell adhesion molecules [42].

Chemokines are soluble chemotactic molecules usually produced by cytokines. Their major role is chemoattraction of leukocytes to sites of inflammation and directing the migration of tumor cells to the distal organs which is attributed to its ability to induce expression of MMPs [54].

2.3. Cyclooxygenase-2 and inducible nitric oxide synthase

Cyclooxygenase-2 (COX-2), is induced in inflammatory and epithelial cells by cytokines, bacterial lipopolysaccharide (LPS) and phorbol ester [55,56]. COX-2 knockout mice are found to have lower tumorigenesis of intestine [57], skin [58] and mammary gland [59]. The enzyme, COX-2 acts on arachidonic acid to produce a series of prostaglandins. PGE_2, $PGF_{2\alpha}$ and 15d-PGJ_2 represent prostaglandins that are generally regarded as proinflammatory and play crucial roles in carcinogenesis. Increased levels of PGE_2 have been seen in many human cancers [60,61]. PGE_2 promotes cell proliferation and tumor-associated neovascularization, suppresses apoptosis thereby favoring tumor growth [62].

Inducible nitric oxide synthase (iNOS) is another inducible enzyme similar to COX-2 that is an important mediator in inflammation related cancer. Inflammatory cytokines (mainly TNF-α, IL-1β and IFN-γ), LPS [63], UV [64] and phorbol esters [65] trigger the induction of iNOS, which acts on L-arginine to produce nitric oxide (NO). Regulation of NO production occurs during transcription and translation. Once active, iNOS is known to produce large amounts of NO [63]. Peroxynitrite ($ONOO^-$) radicals are produced by the reaction of nitric oxide with superoxide anions and

these radicals and NO cause oxidative and nitrosative stress which in turn is damaging to the DNA as previously described [66]. It also exerts angiogenic properties and induces expression of vascular endothelial growth factor (VEGF) [67]. Being an inflammatory enzyme, iNOS is overexpressed in many human tumors, especially in colorectal tumors [68,69]. Apart from the preneoplastic activity, NO is also associated with antineoplastic functions, the effects being dependent on NO concentration in tumors, cell type and interaction with other free radicals. NO is shown to be cytotoxic and iNOS is inversely correlated to metastatic activity in human colon cancer and in murine melanoma cells [63,70]. On the contrary, NO leads to hyperphosphorylation of retinoblastoma (Rb) protein and thus increased cell proliferation [71]. iNOS has also been shown to bind to COX-2 enzyme, nitrosylate it and cause the catalytic activation of COX-2 [72].

2.4. Major transcription factors in inflammation driven carcinogenesis: Nuclear factor-kappa B (NF-κB) and Signal transducers and activators of transcription (STATS)

Different transcription factors get to be activated during inflammation leading to aberrant induction of genes and synthesis of proteins which pave way to malignancies. Nuclear factor kappa B or NF-κB is the most extensively studied transcription factor in inflammatory responses and it is recognized as the potential molecular bridge between inflammation and cancer [73-75]. Mammals express five NFκB/REL genes, *NFKB1, NFKB2, RELA, c-REL* and *RELB* that give rise to seven

proteins- p105, p100, p50, p52, RELA/p65, c-REL and REL B. p50 and p52 corresponds to amino-terminal half of p105 and p100, respectively [76,77]. The canonical pathway for NF-κB activation is mediated through the activation of activation of inhibitory κB kinase (IKK) complex by signals such as those present during inflammation. This is followed by ubiquitin dependent degradation of IκBα/β and the nuclear translocation of NFκB dimers. The prototypical dimer is the p50-RELA complex [78]. The noncanonical NFκB pathway requires NFκB-inducing kinase (NIK) and its activation of IKKα leads to the processing of p100 and the nuclear translocation of p52:RELB complexes [79-81].

Synthesis of iNOS, COX-2 and inflammatory cytokines, such as TNF-α, is generally mediated through NF-κB, although other mechanisms exist [82]. They also transactivate genes involved in anti-apoptosis (e.g., Bcl-2, Bcl-XL), cell proliferation (e.g., cyclin D1) and angiogenesis (e.g., VEGF) and down regulate apoptotic genes (e.g., p53, Bax, Bad) [83]. Inhibition or genetic ablation of NF-κB or its upstream kinases are observed to reduce tumorigenesis in different cancer cell lines and animal models [84,85]. In a murine cancer metastasis model where colon adenocarcinoma generates lung metastasis, inhibition of NF-κB resulted in tumor regression [86]. The regression effects were mediated by a member of the TNF superfamily, TRAIL, whose receptor is induced in NF-κB-deficient cancer cells [86].

Signal transducers and activators of transcription (STATs), are a family of seven regulatory proteins (1, 2, 3, 4, 5A and 5B and STAT6) and get activated in response to ligand binding to non-receptor tyrosine kinase, janus kinase (JAK). This causes

the STATs to dimerize and bind to the the target genes, such as iNOS and COX-2 *via* interferon gamma activated sites (GAS) [87-91]. The association of STATs with interferon regulatory factor 9 (IRF-9) potentiates their binding to interferon-stimulated response element (ISRE) and IRF response element (IRE) on target genes [92]. STAT1 and 2 are important in inflammatory events mediated through interferons and regulate over 300 genes in response to interferons [89]. In many cancers including CRC, the STATs, namely STAT3 and STAT5, are tyrosine phosphorylated and activated constitutively. Uncontrolled STAT3 activation by IL-6 or interferon activation, promotes cell survival and proliferation by deregulating *cyclin D1, c-MYC, Bcl-X_L* and *survivin* genes [93,94] and enhances tumor angiogenesis by upregulation of vascular endothelial growth factor (VEGF) [95]. It is also identified as a crucial mediator of NF-κB and inflammation-associated cancer [91]. Mutant mice that lacked Stat3 specifically in all epithelial cell lineages of the intestine showed reduced tumor growth and multiplicity, while its hyperactivation promoted tumor incidence and growth [96]. STAT5 has active roles against apoptosis and also in aids in cell proliferation and invasion events eventually contributing to tumorigenesis [97].

2.5. Mitogen activated protein kinase (MAPK) signaling pathway in inflammation

Mitogen activated protein (MAP) kinases represent yet another pathway for implementing the inflammatory response in the cells. Intracellular signaling

following stimulation by cytokines and growth factors lead to rapid and reversible activation of the MAPK family, which essentially is composed of a cascade of kinases [98-101]. In mammalian cells, three subgroups of MAPK have been identified, i.e. extracellular-signal-regulated kinase (ERK), c-jun N terminal kinase (JNK) and p38 MAPK. These MAPKs activate a number of transcription factors that result in transcription of different genes involved in cell growth, proliferation, differentiation (mostly by members of ERK), and inflammation and apoptosis (JNK, p38 MAPK). p38 MAPK was shown to phosphorylate TATA box binding protein (TFIID) which was essential for its binding to TATA box and eventually for NF-κB dependent transcription [102]. MAPKs are also known to be the targets in suppressing the downstream inflammatory genes such as iNOS, COX-2 and proinflammatory cytokines, mainly TNF-α [100,103]. Different synthetic compounds that block the activation of MAPKs and the ensuing inflammation have entered clinical trials [104].

2.6. Inflammation in the context of colorectal carcinogenesis

Tumor microenvironment is set ablaze in the presence of inflammation, where it presents a wide range of tumor promoting effects. It aids in cell proliferation and leads to survival of malignant cells. Angiogenesis and metastasis are also promoted under these conditions in addition to alterations in responses to chemotherapeutics [105]. Colorectal cancer (CRC) development is a characteristic scenario in which inflammatory conditions predispose to carcinogenesis with about 20-fold increase in the risk [106]. Intestinal mucosal inflammation in mice administered with

dextran sulfate sodium was shown to be genotoxic to leukocytes and erythrocytes as determined by extent of oxidative DNA damage and single and double strand DNA breaks [107]. Reactive oxygen and nitrogen species caused accumulation of DNA damage in mice deficient in DNA base excision repair enzyme [108]. Suppressor of cytokine signaling 3 (SOC3) limited inflammation and hyper proliferation in mice colons by down regulating IL-6-STAT3 and NFκB signaling pathways, thereby suggesting the role of inflammation in proliferation events in the colon [109]. Overexpression of inflammatory enzymes, iNOS and COX-2 has been reported in colonic tumor models by the administration of colon specific carcinogen, azoxymethane (AOM) in rats [110,111] and in humans [68,69,112]. Administration of prostaglandin E2, product of COX-2, intraperitoneally in AOM injected rats increased colon carcinogenesis with increased cell proliferation and lower apoptosis [113]. More importantly, selective synthetic inhibitors of iNOS and COX-2 have proved to be potent in reducing the number of colorectal polyps, in suppressing hyperplastic aberrant crypt foci (ACF) formation and in preventing colon cancer [110,114-119].

3. Cell proliferation and its role in molecular pathogenesis of CRC

Colorectal cancer arises as a result of accumulation of genetic errors that lead to evasion from growth inhibitory effects and apoptosis and deregulation of the molecular pathways involved [120-122]. Chromosomal instabilities characterized by chromosomal translocation or alteration in numbers account for 85% of the CRC.

The remaining 15% display microsatellite instability wherein the failure to recognize and repair base pair mismatches lead to frame shift mutations in the microsatellite repeats [123,124]. The summary of molecular events and genetic mutations in CRC that lead a normal cell to transition into malignant stage has been described in the previous section [17]. It is known that as many as 11,000 genetic errors occur in adenomas which accumulate over time to develop into cancer [125,126]. Effective strategies in prevention and/or treatment of malignancies of the colon and rectum should resolve many of these abnormalities.

3.1. *Adenomatous polyposis coli (APC)*

Mutations in the tumor suppressor gene, *APC* gene, are observed in 80% of sporadic colorectal carcinomas. The gene encodes a cytoplasmic protein required for sequestering and down regulating catenin protein, the vital molecule in Wnt signaling [127]. The APC protein is an integral part of the degradation complex comprising of APC, β-catenin, glycogen synthase kinase 3 beta (GSK3β) and axin which recruits the β-catenin molecule for ubiquitination and degradation by the proteosome via its phosphorylation at serine and threonine residues by GSK3β. Another protein, Siah-1 interacts with the carboxyl terminus of APC and promotes the ubiquitination of β-catenin, leading to the degradation of β-catenin through a pathway independent of GSK-3β [128]. However, in the presence of *APC* mutations or activating Wnt signaling by Wnt ligands, APC protein cannot hold β-catenin, β-catenin fails to get degraded, gets accumulated in the cytosol and eventually gets translocated into the nucleus, a feature associated with progression along the

adenoma-carcinoma sequence [33,129,130]. In the nucleus, β-catenin functions as a cofactor for transcription factors of the T-cell factor/lymphoid enhancing factor (TCF/LEF) family that are involved in the transcription of a subset of genes involved in cell fate and regulating cell proliferation [131-133]. Of this family of transcription factors, TCF-4, LEF-1 and TCF-1 are the most relevant in CRC. Similar to the effects of APC on β-catenin, destabilization of actin also leads to the nuclear accumulation of β-catenin [134]. In parallel, Wnt signaling through the conventional transmembrane frizzled (Fz) receptors leads to hyperphosphorylation of dishevelled (Dsh), to inhibit GSK3β [135].

APC mutations are a hallmark for the pathology of familial adenomatous polyposis (FAP). FAP represents the inherited autosomal disease which leads to development of multiple adenomas in the small intestine and also in the colorectum. Malignancies may occur at other sites including the brain and the thyroid [136,137]. Mutations in the gene coding for β-catenin (*CTNNB1*) are present in 10% of CRC and mutation of either gene (*APC or CTNNB1*) has almost similar effects in terms of β-catenin stability and TCF transactivation. However, compared to *APC* mutations, the likelihood of small adenomas with β-catenin mutations to progress to larger adenomas is relatively low [138]. Most β-catenin mutations affect the phosphorylation sites of β-catenin by GSK3β, making it refractory to degradation [139]. Recently, it was shown that the Wnt signaling cooperates with the Notch pathway to regulate cell proliferation in humans and in other species [140]

Stem cells reside at the base of the crypts along with the cells of the mesenchyma, generally of the myofibroblast lineage and so are referred to as pericryptal myofibroblasts. These myofibroblasts are regarded as producers of Wnt signaling and that act on the Fz receptors on cryptal stem cells [141]. The effects of this include nuclear β-catenin accumulation and expression of β-catenin/TCF target genes. As the stem cells give rise to progenitor cells, they move up along the crypt and when these cells reach almost the mid-crypt region, β-catenin/TCF activity is down regulated leading to cell cycle arrest and differentiation [13,141].

The initial genetic change in most colorectal adenomas is thought to be somatic mutations in *APC* [17]. *APC* mutations cause expansion of the crypt base cell population, including crypt stem cells. These cells with *APC* mutations or even β-catenin gene mutations escape the physiological control signals on β-catenin/TCF activity and continue to proliferate giving rise to crypts occupied with mutated cells along with the chance of increased number of stem cells at risk for further mutations [13].

3.2. Cyclin D1

Cyclin D1 is the regulatory subunit of the holoenzymes whose catalytic subunit is cyclin dependent kinase (CDK4/6), and function during the G1 phase of cell cycle as “mitogen sensors”. They along with the CDKs phosphorylate and inactivate the cell cycle inhibiting function of the retinoblastoma protein (pRb). Hypophosphorylated Rb sequesters the transcription factor E2F in the cytosol, which blocks cell cycle progression in G1 phase or cells get accumulated in G1

phase. Hyperphosphorylation can release the E2F genes, which are responsible for transcription of genes required for cell cycle progression [142]. Also different cyclins such as cyclin E, A and B are important in different phases such as S, G2 and M phases in cell cycle progression. So depending on the cyclin that will be affected by pterostilbene, cells may accumulate in the respective phases.

Cyclin D1 is induced by a number of growth factors, such as insulin growth factor (IGF-I and II) and transforming growth factor β (TGFβ) and by hormones such as androgens, retinoic acid and peroxisome proliferator-activated receptor γ (PPAR γ) [143]. It is highly expressed in many tumors including that of the colon and rectum and is known to be overexpressed in patients with adenomatous polyps, primary colorectal adenocarcinoma [144] and familial adenomatous polyposis [145].

Cyclin D1 is a target gene of β-catenin/TCF activating signals [146,147], and in particular in colon cancer cells since it was identified that promoter that are related to consensus TCF/LEF-binding sites are necessary for activation [148]. In colon cancer cells, the oncoprotein $p21^{ras}$ further activates transcription of the *cyclin D1* gene, through sites within the promoter that bind the transcriptional regulators Ets or CREB [148]. Receptor tyrosine kinase signaling through the Ras/ MEK (MAPK kinase)/ ERK pathway is also implicated in the increase of cyclin D1 expression [149]. Prolonged ERK activity leads to G1 phase expression of cyclin D1 leading to cell cycle transition from G1 to S phase [150]. The Phosphatidylinositol-3-kinase (PI3K)/Akt pathway is another well known mechanism of regulating cyclin D1

expression. The effects on cyclin D1 translation are mediated through the mTOR (mammalian target of rapamycin)/S6 kinase 1 [151]. In another posttranscriptional mechanism, activation of PI3K/Akt leads to accumulation of cyclin D1 protein which involves GSK3β. Activation of GSK3β by Akt leads to the stabilization of cyclin D1 protein [152]. Also, Ras/MEK/ERK cooperates with PI3K/Akt pathways in regulating cyclin D1 [153].

3.3. c-MYC

The expression of *c-myc* gene is closely correlated with growth and deregulation of c-MYC proptein, which is associated with malignant potential of cells. *Myc* is a target gene of β-catenin/TCF signaling and as expected it is overexpressed in almost 70% of CRC [154]. Apart from the regulation via classical β-catenin/TCF signaling, the acetyltransferase of CREB binding protein (CBP) acetylates β-catenin at lysine 49 in the nucleus to improve the transactivation of c-MYC in particular [155]. Cooperative binding of β-catenin and another transcription factor, c-MYB was recently shown to upregulate of c-MYC promoter activity and lead to an increase in intestinal adenoma formation in APC^{Min+} mice [156].

3.4. RAS and RHO family of GTPases in transducing mitogenic signals and inflammation associated proliferation signals

The roles played by the RAS and Rho GTPases in regulating cell cycle are reviewed by Coleman et al. [157]. The three RAS proteins (H-RAS, K-RAS and N-RAS) are activated by the binding of GTP. Receptors including G-protein coupled

receptor and tyrosine kinase associated kinase activate RAS and activating mutations of RAS are present in approximately 40% of CRC [158,159]. RAF-MEK-ERK, RAL and PI3K/Akt signaling pathways are important in mediating the promotion of cell cycle progression. During the G1-S phase the primary role of RAS is to inactivate Rb protein and relieve the cell from the growth inhibitory action of Rb [160,161]. The expression of cyclin D1 and its complex formation with CDK4 or CDK6 require the RAS-RAF-MEK-ERK pathway [162,163]. The other signaling events regulating cyclin D1 expression such as, β-catenin/TCF, PI3K/Akt and GSK3β were mentioned in the section on cyclin D1. The RAS-RAF-MEK-ERK pathway further promotes cell cycle progression by reducing p27 CDK inhibitor by enhanced proteolysis and decreased protein synthesis [164]. The PI3K pathway represses p27 transcription [165] and also regulates the proteosome mediated degradation of p27 [166]. Contrary to the expectations, RAS signals mediate upregulation of p21 CDK inhibitor transcription and protein levels through the classical RAF-MEK-ERK pathway. Thus, moderate levels of RAS activation causes a balance between increased p21and decreased p27 levels, that actually promote cell proliferation [167].

RHO GTPases have been shown to promote G1-S transition in cell cycle [168]. The effects are partly mediated through its regulation of cyclin D1 and NF-κB at the transcriptional level [169]. Activating RHO GTPase is responsible for transducing Toll like receptor (TLR) stimulation effects in many cells, including the ones involved in innate immunity [170,171]. Src family kinases and RHOA were required

for NF-κB activation in human lung epithelial cells [172]. RHO-induced NF-κB and cyclin D1 transcription required STAT3 which was involved in NF-κB nuclear translocation. Also, loss of STAT3 inhibited RHO-promoted cell proliferation [173]. These results also signify the inflammation pathways lead to increased cell proliferation to mediate tumorigenesis.

3.5. *Survivin*

Survivin is one of the genes that are upregulated by the β-catenin/TCF pathway with important roles in cell proliferation and apoptosis. During the cell cycle it is specifically expressed in G2/M phase and binds to microtubules during mitosis and disruption of this binding leads to cell division defects [174,175]. Survivin is a member of inhibitor of apoptosis (IAP) family of proteins and is known to bind and inhibit caspases, mainly caspases 3 and 9 [176]. Localization of *survivin* is important, since its presence in the cytoplasm suppresses apoptosis and when in the nucleus, it affects G2/M phase of cell cycle [177].

4. Identification of effective chemopreventive agents against CRC

Cancer chemoprevention, by definition, is the use of natural, synthetic or biological agents to reverse, suppress or prevent either the initial phase of carcinogenesis or the progression of neoplastic cells to cancer [178,179]. In this regard, developing natural and synthetic cytotoxic compounds with anti-inflammatory property have received great attention since inflammation is implicated in all stages of carcinogenesis, and in particular during CRC. Detailed

understanding of molecular mechanisms involved in carcinogenesis lead us to identify target molecules critical for carcinogenesis of a particular organ/ organs. The clinical trials on chemoprevention have evaluated a few molecular targets, which includes COX-2 inhibitors against colon cancer [180-182], selective estrogen receptor modulators against breast cancer [183,184] and 5α-reductase inhibitors for prostate cancer prevention [185].

4.1. Colon cancer chemoprevention by synthetic anti-inflammatory drugs

The use of non-steroidal anti-inflammatory drugs (NSAIDS) to inhibit COX-2 and several synthetic compounds that target iNOS have been evaluated to prevent cancers of the colon, in studies ranging from those in cell culture to clinical trials [111,114,116-118]. Earlier studies with a non-selective COX inhibitor, sulindac, reduced the polyp numbers in patients with the genetic disorder, familial adenomatous polyposis (FAP) [186]. Steinbach *et al.* conducted studies and reported effectiveness with the selective COX-2 inhibitor, COX-2 in reducing the polyps in FAP patients which led to approval of the drug by FDA [118]. Further, in the adenoma prevention trial with celecoxib (400 mg twice daily and 200 mg twice daily for 3 years) conducted in 2035 patients, celecoxib significantly reduced the incidence of adenomas compared to the placebo. Three-year adenoma incidence was 37.5% in patients on 400 mg of celecoxib twice daily, 43% in 200 mg celecoxib group and 60% in the placebo [181]. iNOS inhibition as discussed in previous paragraphs is also a key to achieve the goal of chemoprevention against colon

cancer and compounds with potency against both COX-2 and iNOS are invaluable in this context. A good justification for this is based on the results of the preclinical studies conducted by Rao *et al.* [117]. The authors found that administration of COX-2 inhibitor along with the synthetic inhibitor against iNOS reduced AOM-induced colonic aberrant crypt foci (ACF) to a greater extent than did either of these agents alone.

4.2. Colon cancer chemoprevention by natural and dietary compounds

A number of dietary constituents and plant phytochemicals have been shown to possess chemopreventive potential in several *in vitro* and *in vivo* models [187]. Feeding experiments with western diet comprised of low calcium and vitamin D produced colon neoplasms in normal mice without carcinogen exposure [188]. This study highlights two messages; firstly the role of environment and nutrition in colon cancer pathogenesis and secondly, the importance of calcium and vitamin D in preventing the cancers of colon. Calcium increases the protein kinase C activation which drives the cell to undergo differentiation and vitamin D acts through its receptor to induce apoptosis by inhibiting anti-apoptotic molecules such as, BCl2 and IAP [189,190]. Polyunsaturated fatty acids (PUFAs) belonging to the omega-3 series are shown to reduce preneoplastic lesions and tumorigenesis in carcinogen induced animals and reduce polyps in Apc Min/+ mice and PPARγ is one of the molecular links between PUFA and cancer prevention etiology [191-193]. A few studies have reported higher intake of folate to be associated with a lowered risk for

CRC [194]. In the absence or lower amounts of folate in the diet, higher mutation rates and reduced stability of DNA methylation patterns lead to silencing of tumor suppressor genes and DNA repair genes leading to increased risk for CRC [195].

Tea polyphenols, such as epigallocatechin gallate (EGCG) showed growth inhibitory and proapoptotic effects against colon cancer cell lines and inhibited intestinal tumor formation in animal models [196-198]. The mechanisms included its inhibition of activation of insulin-like growth factor (IGF-1) receptor [199], epidermal growth factor receptor and the downstream targets, such as ERK1/2 and Akt [196-198,200], and down regulation of Wnt signaling pathway [196]. Some of the other promising nutrients identified as chemopreventive agents in colon cancer prevention include curcumin, a yellow pigment of turmeric (*Curcuma longa* L., Zingiberaceae), resveratrol from grapes (*Vitis vinifera*, Vitaceae), sulforaphane from broccoli, genistein from soybeans, quercetin from apple, onions and broccoli [201-203]. Almost all of these compounds are known to exert anti-inflammatory action and lower COX-2 protein level and/or its enzyme activity mainly by downregulating nuclear localization of p65, NFκB DNA binding and transcriptional activity and thereby contributing to anti-tumorigenicity [203] In addition to modulation of the NF-κB pathway, mechanisms relating to down regulation of other pathways, such as MAPK, PI3K/Akt, STAT-3 and STAT-1 have also been identified to contribute to anti-inflammation properties exhibited by many of the active dietary agents [202]. Topical application of [6]-gingerol, a pungent ingredient of ginger (*Zingiber officinale* Roscoe, Zingiberaceae), inhibited COX-2 expression in mouse skin

stimulated with tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) mainly by inhibiting NF-κB and p38 MAPK [204]. [6]-gingerol displayed anti-inflammatory action against HCT-116 colon cancer cells and suppressed tumor formation in nude mice by lowering the leukotriene A4 hydrolase protein [205].

Curcumin, tea polyphenols, resveratrol and many plant derived flavones such as genistein, apigenin from celery and parsley, and luteolin from leaves are identified as potent inhibitors of angiogenesis [206]. VEGF inhibition is an identified common mode of action for all these flavones. The effects of resveratrol in angiogenicity is ascribed to the scavenging of reactive oxygen species, while curcumin and green tea catechins mediate their effects through down regulation of NFκB and activator protein (AP-1) pathways [206].

Deregulation of cell cycle proteins and inhibition of apoptosis leads to tumor promoting effects. High calcium and vitamin D intake is associated with increased apoptosis of colonic epithelium [207]. Vitamin D target genes include p21, p27 causing cell cycle arrest and TGF-β causing growth inhibition [208]. Diets enriched with omega-3 PUFAs may protect against colorectal carcinogenesis by reducing DNA adducts, aiding DNA repair, and thus increasing apoptosis [209,210]. PUFAs are also known to reduce point mutations in KRAS gene [211]. Tea polyphenol, EGCG, induced apoptosis in HT29 colon cancer cells and inhibited the cell proliferation with an increase in cells in the G1 phase. The treatment decreased in the phosphorylated forms of EGFR and HER2 proteins, and subsequently caused a decrease in the phosphorylated forms of the extracellular signal-regulated kinase

and Akt proteins [200]. Curcumin targets the JNK and p38 MAPK pathway [212] and requires the proapoptotic Bax protein in inducing apoptosis in HCT-116 colon cancer cells [213]; causes S,G2/M cell cycle arrest in HCT-116 and Lovo cells while lowering the protein levels of cyclins D, E, and c-MYC [214]. Other mechanisms involved in mediating apoptotic effects include down regulation of antiapoptotic proteins, Wnt signaling pathway and upregulation of caspases [215].

4.3. Stilbenes against tumorigenesis

Stilbenes are a group of naturally occurring phytochemicals present in a wide variety of plants [216]. They can be present either constitutively or induced by environmental stresses to protect the host against excessive ultraviolet exposure, microbes/viral attacks and diseases [217]. The most studied and well characterized stilbenoid compound is resveratrol. Cancer chemopreventive potential has been established since resveratrol inhibits pre-neoplastic lesion formation in mouse mammary organ culture by inducing with 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced and reduces the incidence and multiplicity of DMBA/12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced papillomas in the two-stage mouse skin model [218]. Different biological effects of resveratrol include its role as an inducer of cell differentiation, mediator of anti-inflammatory effects, antioxidant and anti-aging agent [219-223]. Against colon cancer, resveratrol was shown to reduce formation of preneoplastic lesions called aberrant crypt foci (ACF) in rats [224], reduce the incidence and size of tumors in 1,2 dimethylhydrazine induced model of colon cancer in rats [225], and prevent the formation of colon and small

intestine tumors in *Apc*$^{Min/+}$ mice when administered along with drinking water [226]. The mechanisms responsible for this tumor suppression include regulation of genes involved in cell cycle progression, antioxidant action and inhibition of cyclooxygenase (COX) enzyme.

Cancer chemopreventive role of other stilbenes are also reported, although to a much lesser extent when compared to resveratrol. A recent review by Rimando and Suh reports the chemoprevention potency of a few stilbenes, such as astringin, rhapontigenin, pinosylvin, piceatannol and pinostilbene [216]. Piceatannol (*trans*-3,4, 3',5'-tetrahydroxystilbene) was found to exhibit similar and in many cases even better anti-oxidant capacity than resveratrol [227]. This may be attributed to the additional hydroxyl group of piceatannol making it more reactive radical scavenger. Piceatannol attenuated the proliferation of Caco-2 and HCT-116 at concentrations ranging from 0-200 μM and caused accumulation of cells in the S-phase of cell cycle [228]. Piceatannol and rhapontigenin caused inhibition of NF-κB and lowered induction of iNOS protein in lipopolysaccharide-activated macrophages with the major action of piceatannol was identified in inhibiting IkappaB kinase (IKK)-alpha and beta phosphorylation, and subsequently IkappaB-alpha phosphorylation [229,230]. Piceatannol was also shown to inhibit the NF-κB pathway by reducing phosphorylation of p65 and thus abrogate COX-2, matrix metalloproteinase 9 and cyclin D1 in human myeloid cells [231].

4.4. Biological role and chemopreventive action of pterostilbene, a stilbene present in blueberries

Pterostilbene, a natural dimethoxy analogue of resveratrol, has drawn significant attention lately. This compound was first isolated from red sandalwood and is present in the extracts of heartwood of the tree *Pterocarpus marsupium* and certain berries such as blueberries and deerberries [232,233]. Pterostilbene was found to be the potent inducer of peroxisome proliferator-activated receptor-α (PPAR-α) isoform, the nuclear receptor involved in lipid metabolism [234] and also to lower serum glucose levels in rats [235]. Recently, pterostilbene was identified as the most effective amongst a group of stilbenes in reversing cognitive behavioural deficits in F344 rats [236]. Against cancer, pterostilbene inhibited the formation of preneoplastic lesions in mammary organ culture [237], induced apoptosis and inhibited the growth of HL60 leukemia cells [238], inhibited metastatic cell growth in B16 melanoma cells [239], induced apoptosis in MDR and BCR-ABL-expressing leukemia cells unlike resveratrol [240] and in gastric carcinoma cells [241], inhibited the invasiveness and down regulated matrix metalloproteinase (MMP-9) in human hepatocellular carcinoma [242] and in heregulin-β1 (HRG-β1) transactivated human breast carcinoma cells, MCF-7 [243]. The major downside of resveratrol identified to give poor results in certain clinical trials is its low bioavailability [220,244,245]. A few studies highlight the bioavailability of pterostilbene outscores that of resveratrol with plasma half life of 1.73 ± 0.78 h in rats [246], 1.3 h in mice injected intravenously with 20 mg/kg pterostilbene [247].

On the other hand, half life for resveratrol was only 10.3 min in mice given i.v. at the dose of 20 mg/kg [247]. The promising observations made by different independent research groups on pterostilbene warrant the relevance for more studies on its role and mechanism of action in carcinogenesis.

MATERIALS AND METHODS

A. Reagents.

Pterostilbene (trans-3,5-dimethoxy-4'-hydroxystilbene; Fig.1) was synthesized at the National Products Utilization Research Unit, USDA (Mississippi) (purity > 99.9%). The compound was dissolved in dimethyl sulfoxide (DMSO) and the final concentration of DMSO used in the experimental set up was 0.1% or less. The controls were run with DMSO alone in all experiments. Recombinant human IFN-γ and TNF-α were purchased from R & D Systems, Inc. (Minneapolis, MN), and lipopolysaccharide (from Escherichia coli 0111:B4 γ-irradiated) was purchased from Sigma (St. Louis, MO). The kinase inhibitors, PD98059, SB203580, SP600125, U0126, LY294002, H-89 and Akt inhibitor, and Wnt agonist were obtained from Calbiochem (San Diego, CA).

B. Cell culture.

Human colon carcinoma cell lines HT-29 was obtained from the American Type Culture Collection (Manassas, VA). The cells were maintained in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% FBS and 1% penicillin /streptomycin at 37^0C and 5% CO_2. The cytokine mixture, consisting of 10 ng/ml of TNF-α, IFN-γ and LPS, was used to induce iNOS and COX-2, unless otherwise mentioned. The cells were treated with the test compound either alone or

in combination with cytokines for different time intervals and harvested for protein or RNA.

C. Preparation of total cell lysate

After treatment of cells with the test compounds, the medium was aspirated and the cells were washed in PBS, scraped and centrifuged at 2,500 rpm for 10 min. The cell pellet was incubated in 0.1 ml ice-cold lysis buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM ethylene diaminetetraacetic acid (EDTA), 1% (w/v) sodium deoxycholate, 50 mM Na_3VO_4, 0.1% (w/v) SDS, 1%(v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF) (pH 7.4), 10 μg/ml leupeptin, 0.5% aprotinin). The cells were then centrifuged at 12,000 g for 15 min at 4°C, and the supernatant (total cell lysate) was collected, aliquoted and stored at -80°C. The protein concentration was determined by using bicinchoninic acid: copper (II) sulfate solution (40:1) (Sigma, St. Louis, MO) and concentrations were read by spectrophotometer at 540 nm.

D. Preparation of cytosolic and nuclear lysate

Following treatment of cells with the test compounds, the medium was aspirated from 100 mm culture dish and the cells were washed in PBS, scraped and centrifuged at 2500 rpm for 10 min. The cell pellet was incubated in 0.1 ml ice-cold lysis buffer (HEPES (10 mM, pH 7.9), KCl (10 mM), EDTA (0.1 mM), ethyleneglycol-bis(aminoethylether)-tetraacetic acid (EGTA) (0.1 mM), DTT (1 mM), PMSF (1 mM)),

with freshly added protease inhibitor cocktail (P-8340, Sigma, St. Louis, MO), 1% Nonidet P-40 for 10 min, after which was added and the contents were mixed on a vortex and then centrifuged for 2 min (13 000 *g*) at 4°C. The supernatant was saved as cytosolic lysate and stored at -80°C. The nuclear pellet was resuspended in 20 µl of ice-cold nuclear extraction buffer (HEPES (20 mM, pH 7.9), NaCl (0.42 M), EDTA (1 mM), EGTA (0.1 mM), DTT (1 mM), PMSF (1 mM)) with freshly added protease inhibitor cocktail (P-8340, Sigma, St. Louis, MO) and shaking done on ice for 15 min. The tubes were centrifuged for 5 min (13,000 *g*) at 4°C, and the supernatant collected. To this supernatant 30 µl buffer consisting of HEPES (20 mM, pH 7.9), 20% (v/v) Glycerol, KCl (0.1 M), EDTA (1 mM), EGTA (0.1 mM), 1% Nonidet P-40, DTT (1 mM), PMSF (1 mM)) with freshly added protease inhibitor cocktail (P-8340, Sigma, St. Louis, MO) was added and the nuclear extract was stored at -80°C. The protein concentration was determined by the Bradford method (Bradford reagent from Sigma, St. Louis, MO) and concentrations read in a spectrophotometer at 590 nm.

E. Western blot analysis.

Whole cell and nuclear protein extracts from different experiments were collected and analyzed by Western blotting. In case of western blotting of tumor tissue, the tumor samples were homogenized in lysis buffer and the samples from each group were pooled together. The protein samples were separated on 4-15% SDS-PAGE gels (Bio-Rad, Hercules, CA) followed by transfer to a polyvinylidene

fluoride membrane. The membranes were blocked with 5% milk in Tris buffer for 1 h and then incubated with the appropriate primary antibody solutions overnight at 4^{o}C. The membranes were washed with Tris buffer, and incubated with horseradish peroxidase conjugated secondary antibody solutions for 1 h at room temperature. The protein bands were visualized using a chemiluminescence based kit from Amersham Biosciences (Buckinghamshire, UK). The primary antibodies against iNOS, COX-2, IκBα, p65, p-p65, cyclin D1, c-MYC, β-catenin, p-β-catenin, p21, p27 (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-STAT3, phospho-STAT1, phospho-Erk1/2, phospho-JNK1/2, phospho-p38, phospho-Akt, phospho-ATF2, phospho-Elk1, (Cell Signaling Technology Inc., Beverly, MA) and actin (Sigma, St. Louis, MO) and secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used for probing the abundance of the target protein in the samples. Western blot data shown are representative of three sets of independent experiments.

F. Immunofluorescence.

Cells were incubated with the vehicle, DMSO or cytokine with and without pterostilbene. Later, cells were fixed in 4% paraformaldehyde (pH 7.4), washed and blocked with 10% bovine serum albumin/ 0.5% Triton-X/ 1X PBS. Primary antibodies against phospho-p38 (1:100 dilution), phospho-ATF2 (1:25 dilution), and β-catenin (1:100 dilution) were added to the chamber, followed by the addition of fluorophore-conjugated secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG, Molecular Probes, OR). Florescence was observed with Zeiss510 microscope

when the cells were irradiated with green laser (488 nm). UV light (364 nm) was used to observe DAPI staining in the nucleus.

G. Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis.

To determine the changes of mRNA levels by pterostilbene and cytokines, we utilized quantitative RT-PCR analysis. The cells were incubated with compounds for indicated period and the cells were then lysed using Trizol to extract RNA. RNA was reverse transcribed into cDNA using a high capacity cDNA archive kit (Applied Biosystems, Foster City, CA). The cDNA was used for quantitative PCR which was run on the ABI Prism 700 sequence Detection System. The primers for the iNOS, COX-2, IFN-γ, TNF-α, IL-1β, IL-6 and GAPDH were obtained from Applied Biosystems (Foster City, CA).

H. Electrophoretic mobility shift assay (EMSA).

EMSAs were performed using the Lightshift Chemiluminescent EMSA kit (Pierce, Rockford, IL) for the NF-κB consensus oligonucleotide probe, 5'-AGTTGAGGGGACTTTCCCAGGC-3', 5’ end-labeled with biotin (IDT, Coralville, IA). Binding reactions were made in a total volume of 20 μl by adding 20 μg of nuclear extracts to 20 fmol of probe in binding buffer (20 mM HEPES (pH 8.0); 50 mM NaCl; 1 mM EDTA; 5% glycerol; 0.05 μg/μl poly [dI–dC] and 0.5 mM DTT). After incubation at room temperature for 20 min, the samples were loaded into 6% native PAGE. Competition assay with unlabeled oligonucleotide (cold DNA) was done in

parallel. This was followed by transfer of the DNA-protein complex to PVDF membrane, blocking and UV cross linking. The membrane was incubated in the substrate for the peroxidase enzyme and the shifted bands were observed by chemiluminiscence.

I. Measurement of cell proliferation by [^{3}H] thymidine incorporation.

HT-29 or Caco-2 cells were plated at a density of 20,000 cells/well in a 24 well plate and treated with varying concentrations of the structural analogs of pterostilbene for a period of 3 days at 37^{o}C. Before harvest, the cells were incubated with 1 μCi [^{3}H] thymidine for 4 h at 37^{o}C and were washed with phosphate buffered saline. The cells were precipitated with cold 10% trichloroacetic acid for 10 min and solubilized with 0.5 ml solubilization buffer (0.2 M NaOH, 40 μg/ml salmon sperm DNA) for 2 h at room temperature. The lysate was transferred to 5 ml Ecolume and the [^{3}H] thymidine incorporated into the DNA of HT-29 cells was determined using a scintillation spectrometer (Beckman Coulter, Fullerton, CA).

J. Experimental procedure for azoxymethane (AOM) induced colon carcinogenesis model in rats with aberrant crypt foci (ACF) as end point.

Weanling male F344 rats were obtained from Charles River Breeding Laboratories (Kingston, NY). All experimental diets were purchased from Research Diets (New Brunswick, NJ) and stored at 4^{o}C. All animals were randomly distributed by weight into control and experimental groups and housed in plastic cages with filter tops (three per cage) under controlled conditions of a 12-hr light and dark

cycle, 50% humidity and 21°C temperature. Animals had access to food and water at all times. Food cups were replenished with fresh diet two times weekly.

Beginning at 5 weeks of age, all rats were fed the modified American Institute of Nutrition-76A (AIN-76A) diet. At 7 weeks of age, the animals were given s.c. injections of azoxymethane (AOM) (CAS no. 25843-45-2, Ash Stevens, Detroit, MI) at a dose rate of 15 mg/kg body weight or saline as solvent control once weekly for 2 weeks. One day after the second AOM injection, groups of animals (n = 9 per group) were maintained on AIN-76A diet alone and AIN-76A diet containing 40 ppm pterostilbene (Fig 1). Dose selection of pterostilbene was based on our earlier study that 25 mg pterostilbene/kg diet lowered plasma cholesterol and lipoproteins in hypercholesterolemic hamsters [234]. On the average, the animal consumed about 0.6 mg pterostilbene per day. All rats were weighed once weekly until termination of the study at 8 weeks after the second AOM treatment. The animals were sacrificed by CO_2 asphyxiation. After laprotomy, the entire stomach, small intestine, and large intestine were resected. The organs were opened longitudinally, and the contents were flushed with normal saline.

K. Aberrant Crypt Foci (ACF) Analysis.

For the ACF analysis, the colons were fixed flat between two pieces of filter paper in 10% buffered formalin for a minimum of 24 hr. The colons were then cut into 2-cm segments, starting at the anus. They were stained with 0.2% methylene blue in Krebs-Ringer solution for 5-10 min, and were then placed mucosal side up on a microscope slide and observed through a light microscope. ACF were counted

and recorded according to standard procedures that are used routinely in our laboratory [116]. Aberrant crypts were distinguished from the surrounding normal crypts by their increased size, the significantly increased distance from lamina to basal surface of cells, and the easily discernible pericryptal zone. The parameters used to assess the aberrant crypts were their occurrence and multiplicity. Crypt multiplicity was determined as the number of crypts in each focus. Multicrypts were categorized as containing up to four or more aberrant crypts/focus.

L. Experimental procedure for azoxymethane (AOM) induced colon carcinogenesis model in rats with colon tumors as end point.

All the procedures used in this experiment were similar to that described in the previous section with certain modifications, especially since the experiment was a long term experiment compared to the ACF experiment and tumor was the endpoint in this experiment. One day after the second azoxymethane injection of rats, groups of animals (n = 31-34 per group) were maintained on AIN-76A diet alone and AIN-76A diet containing 40 ppm (0.004%) of pterostilbene. This was the dose that was shown to be effective in suppressing aberrant crypt foci formation in AOM injected rats in our previous study [248]. All rats were weighed once weekly until termination of the study after 45 weeks from the second azoxymethane treatment. The animals were sacrificed by CO_2 asphyxiation and the colon was removed, rinsed in PBS, opened longitudinally and flattened on a filter paper. The location and size of each tumor was noted. Mucosal scrapings were collected and stored at -80^{0}C for

further analysis. Tumors were removed, fixed in 10% buffered formalin for 24 h and transferred to 80% ethanol for histopathology analysis.

M. Histopathology.

The tumor tissues were dehydrated under vacuum in a series of alcohol and xylene solutions, embedded in paraffin and cut into 4 μm thick sections. These sections were hydrated and stained with hematoxylin and eosin according to the standard protocol. The stained sections were analyzed for the tumor grades by a pathologist, who was unaware of the treatments.

N. Immunohistochemistry.

Colon samples from each group were harvested at autopsy and fixed in 10% formalin for 24 hr. They were sectioned into 8 to 10 segments, paraffin embedded, and microtomed into 4 μm thick tissue sections. The slides were incubated overnight at room temperature with antibody to proliferating cell nuclear antigen (PCNA) (1:1000 diluted, BD Pharmingen, San Diego, CA), iNOS (1:100 diluted, Santa Cruz Biotechnology, Santa Cruz, CA), or mucin MUC2 (1:250 diluted, Santa Cruz Biotechnology, Santa Cruz, CA), cyclin D1 (1:500 diluted for colon tumor tissue analysis) and p27 (1:50 diluted) (Santa Cruz Biotechnology, Santa Cruz, CA). The slides were incubated with biotinylated secondary antibody, and then with avidin/biotinylated peroxidase complex for 30 min at room temperature (Vector Labs, Burlingame, CA), and were then incubated with 3'-diaminobenzamine (DAB) substrate. The sections were then counterstained with Modified Harris

Hematoxylin. The images were taken randomly at 400X using Zeiss AxioCam HRc camera fitted to a Zeiss Axioskope 2 Plus microscope. A positive reaction is noted by a reddish brown precipitate in the nucleus for PCNA, in the cytoplasm for iNOS or in the colon crypts for mucin MUC2.

O. Recording of ACF in colon and quantification and recording of protein markers analyzed by immunohistochemistry

The total number of ACF/colon and multiple aberrant crypts/focus were counted and the data were analyzed by the Student's t-test. The PCNA labeling index (PI) was calculated as the [(number of positive cells)/(total number of cells)] X 100 for each field which is averaged to get the PI for each section. Image-pro plus 6.2 software (Media Cybernetics, Inc., Bethesda, MD) was used for quantifying the images obtained after immunohistochemistry of the tumor samples. Positive red staining or negative blue counterstaining was assigned a unique color using the hue, saturation, intensity (HSI) model under the segmentation feature of the software. Threshold values enable to filter the dark positive staining from the background light staining. Five random fields per slide were selected at 400X magnification. PCNA staining was analyzed based on the cell counts, and reported as the PCNA labeling index (PI) which is [(number of positive cells) / (total number of cells)] x 100 for each field, which was averaged to get the PI for each tumor sample. Integrated optical density (IOD) calculated by the software for each field which was averaged to obtain the IOD for each tumor sample and finally for each treatment

group, was used to report the staining intensity for cyclin D1, p27. The significance of treatment between the groups was analyzed by the Student's t-test**.**

P. In vivo assay using HT-29 xenograft model in immunodeficient mice.

HT-29 colon cancer cells were trypsinized and suspended in DMEM at a density of 10^6 cells/ml in a 1:1 volume/volume mix of DMEM Matrigel (BD Biosciences, Bedford, MA). Seven to eight week old female severe combined immunodeficiency (SCID) mice were inoculated subcutaneously in the hind flank with 0.1 ml of cell suspension. Beginning one day after injection of the cells, the mice were treated with vehicle control (0.1 ml cremophor: PBS [1:8] mixture) or stilbene analogues (10 mg/kg body weight in 0.1 ml vehicle) intraperitoneally once daily for 3 weeks. The size of palpable lesions was measured twice a week with calipers. Tumors at autopsy were measured and weighed. All animal studies were performed in accordance with an institutionally approved protocol.

Q. Analysis of stilbenes in the serum and the colon tissues

Blood was collected into microvette (Sarstedt, Germany) at autopsy. The tubes were allowed to sit for 1 h at room temperature, and then centrifuged for 10 min at 2000 rcf (4000 rpm) in Eppendorf 5417 Microfuge to obtain serum. The serum samples were analyzed by Dr. Agnes M. Rimando's research group at USDA, Mississippi. Mice sera were stored at –20^{o}C until assayed. Sera were thawed in ice prior to analysis. To 50 µL of thawed serum was added 50 µL of sodium acetate trihydrate (1M, pH 5), then heated for 60 min at 37℃ in water bath. Serum mixture

was cooled to room temperature then partitioned with ethyl acetate (3x, 100 µL each). The combined ethyl acetate extracts was dried under a stream of nitrogen, and redissolved in 50 µL MeOH : $CHCl_3$ (1:1) for analysis by gas chromatography-mass spectrometry (GC-MS).

Colon tissue samples were frozen and kept at -80°C until extraction. For colon tissue extraction, tissues were homogenized with sodium phosphate buffer pH 7.4 (0.2 M NaH_2PO_4: 0.2 M Na_2HPO_4) (80:20) and centrifuged. The supernatant was collected and the tissue pellet was re-extracted with sodium phosphate buffer. The supernatants were extracted with ethyl acetate, combined, dried under a stream of nitrogen, then derivatized with *N*,*O*-bis[trimethylsilyl]-trifluoroacetamide: dimethylformamide (BSTFA: DMF, 1:1; Pierce Biotechnology, Inc., Rockford, IL), and heated. The derivatized samples were used for GS-MS. For serum extraction, serum was mixed with potassium phosphate buffer. Ice cold HPLC grade acetonitrile was then added, vortexed, and centrifuged. The supernatant was dried under a stream of nitrogen, and derivatized following procedures used for the colon tissues.

GC-MS analysis was performed using a JEOL GCMate II System (JEOL USA Inc., Peabody, MA) with a J&W DB-5 capillary column (0.25 mm internal diameter, 0.25 µm film thickness, 30 m length; Agilent Technologies, Foster City, CA). The GC temperature program was as follows: initial 190°C, raised to 240°C at a rate of 20°C/min, then raised to 280°C at the rate of 2°C/min, then finally raised to 300°C at the rate of 10°C/min. The carrier gas was ultra high purity helium at a flow rate of 1.0 mL/min. The inlet (splitless), GC interface, and ion chamber temperatures were

250, 300, and 300°C, respectively. The sample injection volume used was 2.0 μL. The retention times for the stilbenes are: **3**, 10.0 min; **4**, 6.1 min; **5**, 11.2 min; **6**, 6.7 min; **9**, 8.2 min; and **10**, 5.1 min. Quantitative analyses (Table 4.3) were performed from calibration curves of the individual stilbene, as external standards, monitored at the *m/z* 255 for **3** and **4**, *m/z* 298 for **5** and **6**, and *m/z* 270 for **9** and **10**.

R. Measurement of cytokine production by ELISA.

Mucosa scrapings from the colon were collected at autopsy and stored in -80^{o}C until analysis. Colonic mucosa samples to be used for cytokine analysis were first homogenized in a PBS based buffer solution (PBS, 0.4 M NaCl, 10 mM EDTA, 0.1 mM PMSF, 0.1 M Benzethonium ion, 0.5% BSA, 3.0% Aprotinin, 0.05% Tween 20) on ice using a Tekmar Tissuemiser (Fisher Scientific International, Inc., Pittsburgh, PA). The homogenized solution was centrifuged at 10,000 rpm, 4^{o}C for 10 minutes. The supernatant was collected for determination of protein concentration, samples pooled (n=6 per group) and stored at -20^{o}C. Invitrogen Immunoassay Kits (BioSource International Inc., Carmillo, Ca) were used to determine the levels of IL-1β (cat.# KRC0012), IL-4 (cat.# KRC0042) and TNF-α (cat.# KRC3012). For determination of IL-1β, IL-4 and TNF- α levels, tissue homogenates were normalized down to a concentration of 1.0 mg/ml of total protein. The normalized tissue homogenates were diluted 10-fold in diluent buffer and used for analysis. Procedures for each assay was carried out as per the manufacturer's protocols.

S. Statistical analysis.

Quantitative data are reported as mean±standard deviation for the individual experiments as specified in the figure legends. Statistical significance analysis was performed using the Student's t-test. The number of observations for each treatment, represented as n, and the measure of significance of treatments, the p-value, is given in the figure legend.

PART I

Pterostilbene suppresses aberrant crypt foci formation in the azoxymethane-induced colon carcinogenesis model in rats

1.1. Introduction

Administration of the well characterized stilbene, resveratrol, is shown to have chemopreventive activity against colon cancer. Resveratrol suppressed colon tumorigenesis in Min mice [226] and reduced aberrant crypt foci (ACF) formation in azoxymethane (AOM)- or 1,2-dimethylhydrazine-induced colon cancer in rats [224,249]. Experiments to determine the chemopreventive efficacy of pterostilbene on colon carcinogenesis were planned using ACF induced by azoxymethane (AOM), a colon-specific carcinogen, in a relevant laboratory animal model. ACF are recognized as early preneoplastic lesions consistently observed in experimentally induced colon carcinogenesis in laboratory animals [250]. Aberrant crypts are precursor lesions from which adenoma and carcinoma develop in the colon, and these lesions have been demonstrated to occur in the colonic mucosa of patients with colon cancer [251]. There is also evidence that several inhibitors of ACF development reduce colon tumorigenesis in laboratory animals [250,252].

The study was designed to examine the chemopreventive efficacy of pterostilbene against colon carcinogenesis using colonic ACF as an endpoint. Whether pterostilbene may suppress colon carcinogenesis by exerting anti-inflammatory activity, such as an inhibitory effect on iNOS, was also investigated.

Mucins are secreted gastrointestinal proteins that protect underlying intestinal epithelium, and mucin MUC2 is critical for colonic protection [253]. Expression of mucin MUC2 is lowered in inflammatory bowel disease and mucin MUC2 has been implicated in the suppression of colorectal cancer [26,253]. Recently, tumorigenesis in MUC2 mutant mice was suggested to develop from inflammation related pathways [254].

Our results indicate that pterostilbene 1) significantly lowered incidence and multiplicity of ACF in the colon of the rats, 2) reduced cell proliferation as observed by the proliferation marker, proliferating cell nuclear antigen (PCNA), 3) reduced iNOS expression in the colonic crypts and ACF and 4) increased the levels of the protective glycoprotein, mucin 2 (MUC2) in the crypts.

1.2. Results

1.2.1. General Observations.

Body weights of animals fed the experimental diet containing pterostilbene were comparable to those fed the control diet throughout the study, indicating that the dose of pterostilbene (Fig 1.1) used did not cause any overt toxicity.

1.2.2. Efficacy of Pterostilbene on ACF Formation

ACF were predominantly observed in the distal colon. Endpoints used in this study were the occurrence of total ACF as well as multicrypt clusters (more than 4 crypts/focus) of aberrant crypts (Table 1.1). Rats treated with AOM and fed with the

pterostilbene diet showed a significantly lower number of total ACF/colon compared to AOM-treated rats fed the control diet (57% inhibition, $p<0.001$). The incidence of multiple aberrant crypts/focus was also significantly inhibited in rats fed the pterostilbene diet as compared to those fed the control diet (29% inhibition, $p<0.01$).

1.2.3. PCNA Staining of Colons and Cell Counting.

The proliferating cell nuclear antigen (PCNA) was evaluated as a marker for cell proliferation in the colon specimens. Sections of colon samples from the control group or pterostilbene fed group are shown (Fig. 1.2.A, B). The PCNA staining of the normal-appearing mucosa of the colon tissue was much stronger in AOM-treated rats fed the control diet (A) than in the pterostilbene-fed group (B). The PCNA labeling index is also shown in Fig. 2. The colon sections from the AOM + control group showed a higher number of positive cells than those from the AOM + pterostilbene diet group. The PCNA positive cells (%) of the colon tissue in the control group were 56.6 ± 2.8%, whereas PCNA positive cells (%) from pterostilbene fed group were 44.2 ± 2.9% (Fig. 1.2.C). The two groups were significantly different ($p<0.01$).

1.2.4. Inducible Nitric Oxide Synthase (iNOS) Staining of Colons.

Since the inhibition of inflammatory genes such as iNOS may contribute to the suppression of ACF formation in colon carcinogenesis, it was important to determine whether pterostilbene might inhibit AOM-induced iNOS in the colon. The

iNOS expression was evaluated as a marker for inflammatory response in the colon specimens. Two independent sections of colon samples from the control group or pterostilbene fed group are shown (Fig. 1.3.). The iNOS staining of the colon tissue was stronger in control group than in pterostilbene-fed group. The colon sections from the control group showed higher staining of iNOS in the cytoplasm than those from pterostilbene-treated rat colons. We found that ACF with moderate dysplasia from the control group displayed strong cytoplasmic staining of iNOS, whereas ACF with moderate dysplasia from the pterostilbene-fed group showed weaker cytoplasmic staining of iNOS.

1.2.5. Increased Staining of Mucin MUC2 in the Colons by Pterostilbene.

We determined changes in the secretion of mucin MUC2 in the colonic crypts. MUC2 is the structural component of the colonic mucus layer which is critical for colonic protection. The colon mucosa from the AOM-treated control diet group showed little expression of mucin MUC2. However, there was abundant secretion of mucin MUC2 from goblet cells lining the colonic crypts in the AOM + pterostilbene-fed group. The staining of cross sections is also shown in the figure 1.4.

1.3. Discussion

The study was able to identify the efficacy of pterostilbene against colon carcinogenesis which included evaluation of preneoplastic marker, ACF, and other markers for cell proliferation and inflammation. Overexpression of inflammatory enzyme, iNOS, in colorectal tumors has been reported previously and its inhibition

has been linked to reduced tumorigenicity in the colon [111,255,256]. In addition, iNOS can be detected in most adenomas and dysplastic ACF, suggesting that iNOS plays an important role in colon carcinogenesis [35,110]. The similar animal model of azoxymethane-induced tumors in F344 rats was used to show that the selective iNOS inhibitor L-N6-(1-iminoethyl)lysine tetrazole-amide significantly suppress azoxymethane-induced colonic ACF at 100 ppm [117]. Another selective iNOS-specific inhibitor, S,S'-1,4-phenylene-bis(1,2-ethanediyl)bis-isothiourea, inhibited the formation of AOM-induced ACF formation and reduced crypt multiplicity containing 4 or more crypts in the colon at 50 ppm [111]. Pterostilbene in this study was able to significantly lower the ACF counts by 57% compared to the AOM treated control rats. Since we also observed statistically significant reduction in the PCNA counts and iNOS expression levels in the colonic crypts, the effects of pterostilbene in lowering ACF may be mediated through a reduction in cell proliferation and inflammation.

Mucins are a class of secreted glycoproteins from goblet cells that offer protection to the intestinal epithelium [257]. MUC2 is an important mucin and it is implicated in the reduction of intestinal tumorigenesis [26,253,258]. In our study, pterostilbene fed group showed increased expression of MUC2 compared to the control treatment. These results clearly indicate the anti-tumorigenic potential of pterostilbene against colon cancer development in the AOM-injected rat model.

1.4. Summary

Pterostilbene, the stilbene present in blueberries and heartwood of tree, *Pterocarpus marsupium,* was able to significantly lower the incidence and multiplicity of preneoplastic lesions, ACF, in AOM-injected rats. Cellular proliferation was inhibited by the stilbene. Furthermore, the expression levels of inflammatory marker; iNOS was also down regulated in the colonic crypts and ACF. This is mainly relevant in the perspective of inflammation being a major culprit in colon carcinogenesis. The role of pterostilbene to protect the colonic mucosa and thus to forestall any deregulatory mechanisms in colon was exemplified by its ability to increase the protective glycoprotein, MUC2. The promising results obtained from the study signify the chemopreventive role of pterostilbene against colon carcinogenesis.

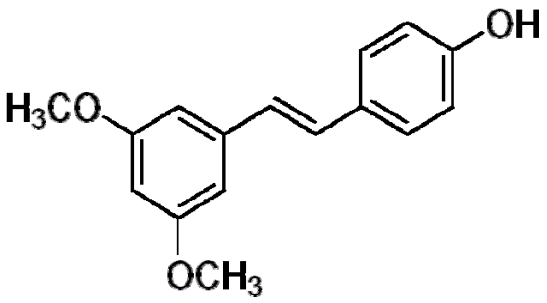


Fig. 1.1. Structure of pterostilbene.

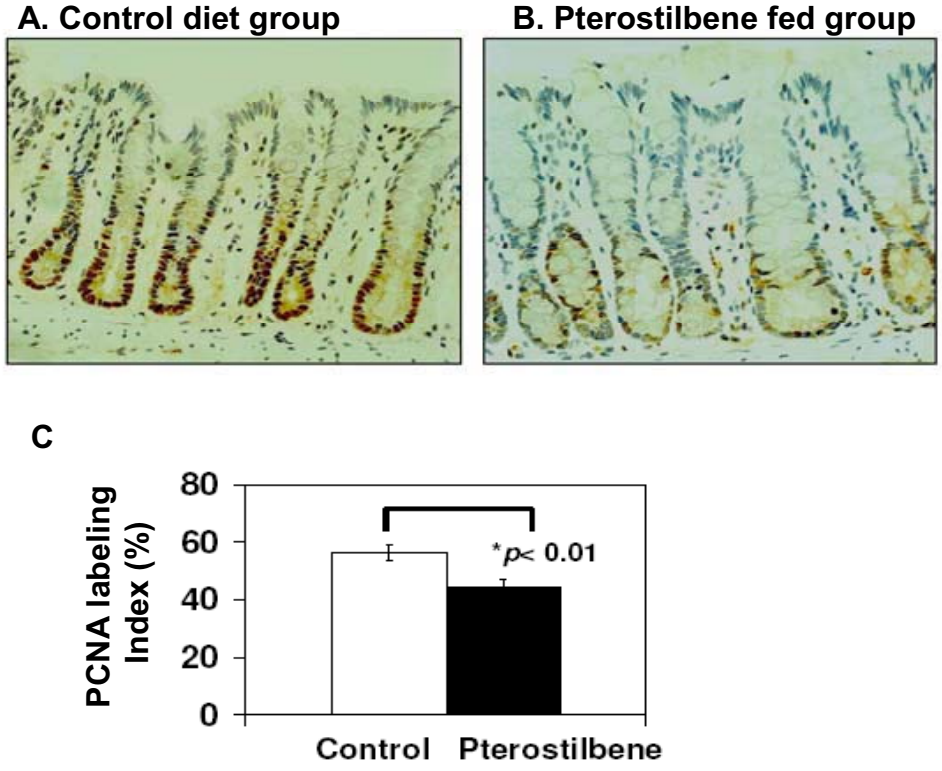


Fig. 1.2. PCNA staining of colon mucosa and cell counting. A representative section of colon samples from the control group (*A*) or pterostilbene-fed group (*B*). PCNA-positive cells in the nucleus (*brown*) and PCNA-negative cells (*blue*), were stained with hematoxylin. Four independent sections of the colon per animal were stained, and approximately 1,500 cells were counted from each section in total. C.The PCNA labeling index (PI) was calculated as the [(number of positive cells) / (total number of epithelial cells)] x 100 for each field. These PI values for all the different colon sections from the animals belonging to same group were then averaged. Statistical significance of treatment between the groups was analyzed by Student's *t* test (*, $P < 0.01$).

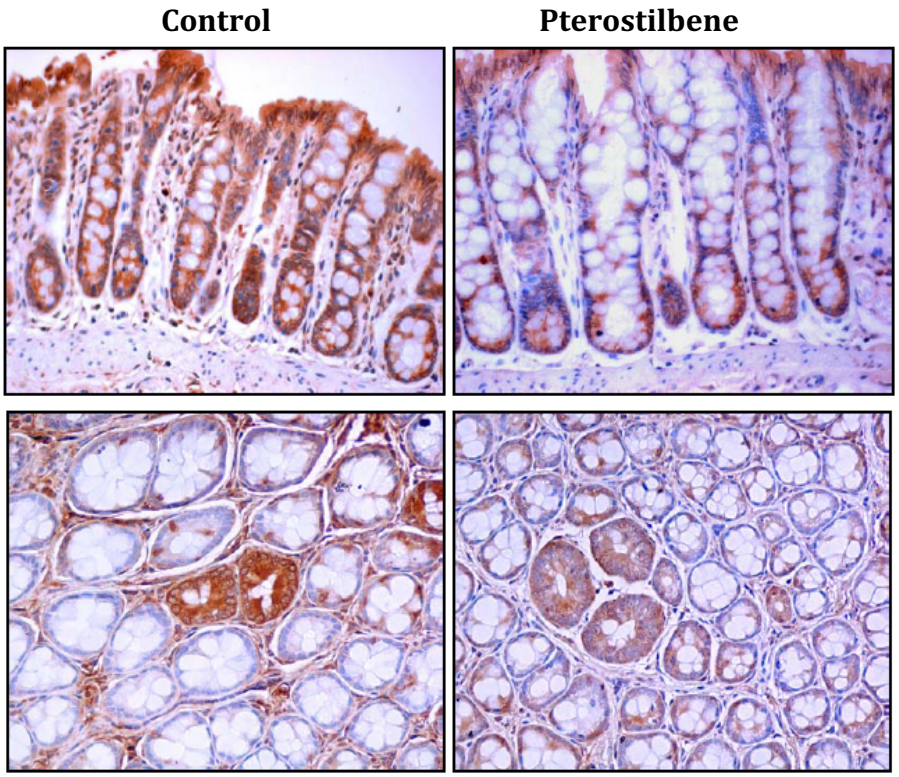


Fig. 1.3. Inhibition of iNOS expression in the colons by pterostilbene. Eight different sections of the colon per animal were stained, and a representative section for each group is shown. The positive cells for iNOS show cytoplasmic staining. The effect of pterostilbene on histologically normal colon sections (*top*), and the effect of pterostilbene on ACF (*bottom*).

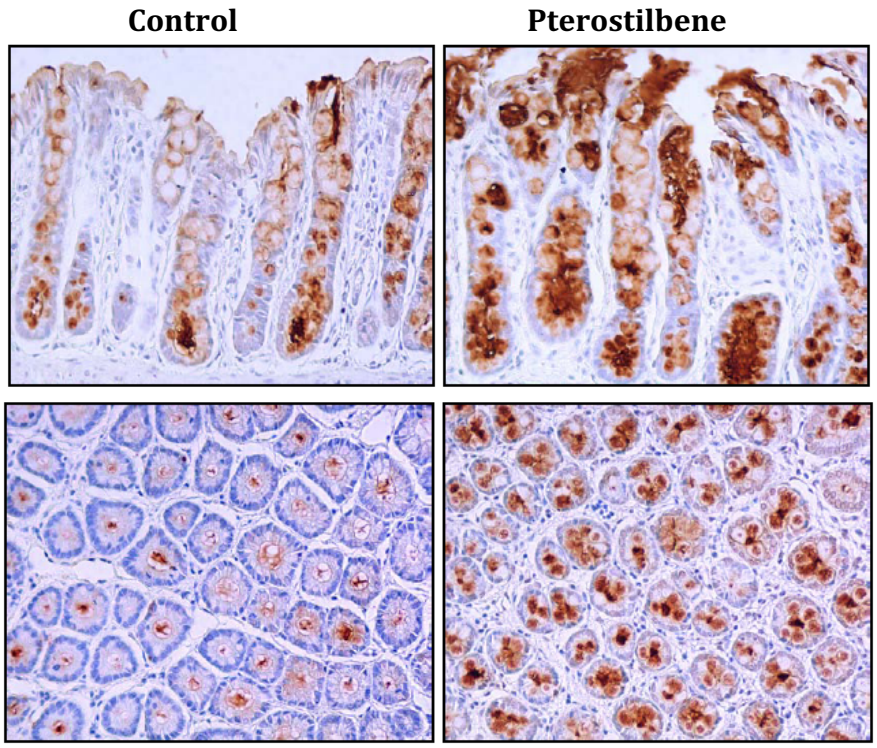


Fig. 1.4. Increased mucin MUC2 expression in the colons by pterostilbene. Eight different sections of the colon per animal were stained, and a representative section is shown for each group. Positive staining for mucin MUC2 (*brown-stained crypts*). Colonic crypt sections (*top*), and cross-sections of the colon (*bottom*).

Table 1.1. Inhibitory effects of dietary pterostilbene on the formation of azoxymethane-induced ACF in male F344 rats

Experimental diets	ACF/colon*	Multicrypt foci*
Control diet (AIN-76A)	273 ± 17	35.6 ± 8.3
40 ppm pterostilbene	117 ± 12†	25.1 ± 5.6‡

* Mean ± SD (n = 9).

†Significantly different from the control diet group, $P < 0.001$ by Student's t test.

‡ Significantly different from the control diet group, $P < 0.01$ by Student's t test.

PART II

Pterostilbene exhibits anti-inflammatory action in colon cancer cells and the effects are mediated through the p38 MAPK pathway

2.1. Introduction

Epithelial cells express iNOS and COX-2 in response to inflammatory cytokines and the bacterial endotoxin, lipopolysaccharide (LPS), although expression levels vary with cell type. The transcriptional regulation of iNOS and COX-2 is complex [259,260]. This process involves a number of transcription factors, including nuclear factor κB (NF-κB), activator protein-1 (AP-1), CCAAT-enhancer binding protein (C/EBP), activating transcription factor/cyclic-AMP response element binding protein (ATF/CREB), and Janus Kinase Signal Transducers and Activators of Transcription (JAK-STAT) family [259,261]. Depending on cell types, various downstream signaling pathways are also involved in the transcriptional regulation of iNOS and COX-2 [51,259-261].

There are several upstream kinase pathways responsible for transcriptional regulation of COX-2 and iNOS. Mitogen activated protein kinases (MAPKs) are composed of extracellular receptor kinase (ERK), p38 kinase and c-jun NH2 terminal kinase (JNK) [262]. These MAPKs are activated by MAPK kinase (MAPKK) and once activated, these MAPKs in turn activate a number of transcription factors such as Elk1, ATF2 and c-jun, which are the major activators of iNOS and COX-2 genes [100,263,264]. It was reported that COX-2 expression induced by IL-1β in HT-

29 cells was upregulated by all three MAPKs [265] Furthermore, p38 MAP kinase has been shown as the major signaling pathway, other than NF-κB, involved in the regulation of inflammatory cytokine synthesis [103,265].

Resveratrol, which is structurally similar to pterostilbene, reduced iNOS and COX-2 induction in rat glioma cells and inhibited iNOS induction by LPS in macrophages by reducing NF-κB [266,267]. Down-regulation of c-jun and c-fos (components of AP-1), NF-κB, and decreased phosphorylation of ERK and p38 have been implicated in the effect of resveratrol to lower COX-2 in mouse skin [222]. Although there have been some detailed studies on the chemopreventive effect of resveratrol, very little is known about the mechanism of action of pterostilbene. Pterostilbene was identified to suppress inflammatory components AP-1, NF-κB, iNOS and COX-2 with efficacy similar to resveratrol in mouse epidermis [268]. A recent study by Pan *et al.* identified pterostilbene to suppress the activation of ERK, p38 MAPK, PI-3-kinase and NF-κB in LPS-induced murine macrophages [269]. These pathways were suggested to play crucial roles in the action of pterostilbene to inhibit iNOS and COX-2.

Based on our studies on reduction of ACF by pterostilbene, the anti-inflammatory property of pterostilbene may be regarded as a key attribute for its role against colon tumorigenesis. The present study aims to understand the inhibitory effects of pterostilbene on the induction of inflammatory markers in the HT-29 colon cell line. The effects of pterostilbene on the activation of upstream signaling pathways and transcription factors involved in NF-κB, JAK-STAT, and

MAPK pathways were investigated. p38α MAP kinase was identified as a key mediator for the inhibitory effect of pterostilbene on the induction of iNOS and COX-2 in HT-29 cells. Interestingly, we observed a lowering in DNA binding ability of NF-κB which may be contributed by the lowering of p-p65 levels within the nuclei of the cells.

2.2. Results

2.2.1. Cytokines act synergistically to induce iNOS and COX-2 in HT-29 cells.

Induction of iNOS and COX-2 has been reported to be maximal with a combination of cytokines for a number of cell lines [270,271]. HT-29 cells were treated with TNF-α, IFN-γ, LPS and IL-1β either alone or in combination for 15 h (Fig. 2.1.). Addition of the cytokines individually to HT-29 cells did not cause a noticeable induction of iNOS. Although the addition of IFN-γ plus LPS caused a strong induction, even stronger induction of iNOS amongst the combinations tested was exhibited by a triple combination of TNF-α, IFN-γ and LPS. COX-2 was induced by TNF-α, LPS or IL-1β individually, and TNF-α was the most potent inducer. The combination of TNF-α with either IL-1β or LPS yielded the strongest induction. The triple combination of TNF-α, IFN-γ and LPS caused a moderate induction of COX-2. Since the triple combination induced both iNOS and COX-2, this combination was selected for additional studies. Apart from HT-29 cells, other colon carcinoma cells were evaluated for induction of iNOS and COX-2 by TNF-α, IFN-γ and LPS at 10 ng/ml each (data not shown). These cell lines include HCT-116, DLD-1 (with no

observable induction of iNOS and COX-2), Caco-2 (with a slight induction of iNOS and COX-2) and LoVo (with good induction of iNOS, but it had relatively high basal level of COX-2 and the cytokines failed to cause a further increase).

2.2.2. Cytokine induction of iNOS and COX-2 is time-dependent, and pterostilbene dose-dependently blocks the induction.

To determine the kinetics of induction of iNOS and COX-2 in HT-29 colon cancer cells, the cells were treated with the cytokine mixture of TNF-α, IFN-γ and LPS for periods of 9, 12 and 15 h. The induction of iNOS was highest at 15 h while the COX-2 level was high at 9-12 h and low at 15 h (Fig. 2.2.A). Similar experiments conducted for 6 and 24 h showed weaker induction of iNOS and COX-2 (data not shown). These data show that maximal induction of COX-2 occurs earlier than that of iNOS. As also shown in Fig. 2.2.A, pterostilbene at 50 μM markedly blocked the induction of iNOS and COX-2 by the cytokine mixture at each time point. In addition, we determined the effect of treatment of the cells with different concentrations of pterostilbene on the induction of iNOS and COX-2 by the cytokine mixture. Pterostilbene inhibited the induction of iNOS and COX-2 in a dose-dependent manner (Fig. 2.2.B).

2.2.3. Pterostilbene down-regulates mRNA levels of inflammatory genes iNOS and COX-2 and pro-inflammatory cytokines IL-1β, IL-6 and TNF-α.

The gene-mediated expression of iNOS and COX-2 are regulated both at the transcriptional and translational levels [104,259,272]. In order to understand the effect of pterostilbene on cytokine-induced expression of proinflammatory enzymes

and cytokines, RNA samples after treatment of HT-29 cells with cytokines and/or pterostilbene were analyzed by quantitative RT-PCR for the induction of mRNA levels of iNOS, COX-2, IL-1β, IL-6, IFN-γ and TNF-α genes. Pterostilbene at 10 and 30 μM strongly inhibited iNOS, COX-2, IL-6 and IL-1β mRNA induction by the cytokine mixture (Fig. 2.3 and 2.4). Induction of TNF-α mRNA by cytokines was observed, but pterostilbene showed a weak inhibitory effect. We also measured the mRNA level of IFN-γ induced by the cytokine mixtures, but it was too low to be detected in HT-29 cells (data not shown).

2.2.4. Multiple signaling pathways are involved in regulating iNOS and COX-2 formation in HT-29 cells.

Cytokines induce iNOS and COX-2 through various signaling pathways [261,272]. In an attempt to evaluate the role of relevant kinases in the suppression or activation of iNOS and COX-2, pharmacological inhibitors for these kinases were used. HT-29 cells were treated with PD98059 (inhibitor of MEK1/2, upstream kinase of ERK1/2), SB203580 (p38 kinase inhibitor), SP600125 (JNK inhibitor), U0126 (inhibitor of MEK1/2), LY294002 (PI-3-kinase inhibitor) and an Akt inhibitor for 15 h (Fig. 2.5.A). Interestingly, all the kinase inhibitors tested, except the AKT inhibitor, reduced the induction of iNOS and COX-2 proteins. The results obtained from pharmacological inhibitors indicate that the ERK1/2, p38 MAPK and the PI-3-kinase pathways may be important for the induction of iNOS and COX-2 in HT-29 cells by the cytokine mixture of TNF-α, IFN-γ and LPS.

2.2.5. Pterostilbene down-regulates iNOS and COX-2 by blocking p38 activation

To elucidate the mechanism responsible for the anti-inflammatory action of pterostilbene, we examined the upstream pathways for iNOS and COX-2 formation, which are activated rapidly after cytokine treatment. As shown in Fig. 2.5.B, cytokine treatment for a short time (15 min) decreased IκBα levels. IκBα is phosphorylated and degraded to activate the NF-κB pathway. Pterostilbene, however, did not block the effects on IκBα induced by the cytokines. The non-involvement of NF-κB was confirmed by examining the nuclear protein levels of the p65 subunit of NF-κB. Cytokine treatment led to the nuclear import of p65 from cytosol. Addition of pterostilbene, however, did not reduce this elevated nuclear levels of p65 brought about by the cytokines (data not shown). The significance of the JAK-STAT pathway in HT-29 cells was evaluated by the level of phospho-STAT3 protein. Cytokines activated the STAT pathway, as shown by a strong induction of phospho-STAT3. However, pterostilbene did not alter the level of induced phospho-STAT3 (Fig. 2.5.B).

When we determined the activation of ERK1/2 and p38 kinases by cytokines by measuring the levels of phosphorylated ERK1/2 and p38, we found that pterostilbene did not block ERK1/2 activation but strongly inhibited activation of p38 (Fig. 2.5.C). JNK activation was noticeable, and there was a weak inhibitory effect of pterostilbene on p-JNK1/2 protein levels. Interestingly, cytokine treatment

or pterostilbene did not change the level of p-Akt, which is the downstream effector of the PI3-kinase pathway (Fig. 2.5.C).

2.2.6. The p38 MAP kinase pathway is a key signal transduction pathway for eliciting the anti-inflammatory action of pterostilbene in colon cancer cells.

Since pterostilbene is effective in down-regulating the cytokine-induced activation of p38, we further examined the involvement of pterostilbene on some of the known upstream effectors and downstream targets of p38 kinase (Fig. 2.6.A, 2.6.B). Phospho-MKK3/6 is known as the major molecule responsible for activating p38 MAPK which in turn gets activated by the upstream kinase in the MAPK cascade. Our results show that pterostilbene was effective in inhibiting cytokine-induced phosphorylation of MKK3/6 at 15 minute time point, suggesting that MKK3/6 activation occurs in close proximity in timeline to p38 activation (Fig. 2.6.A). Further, the activation of well-known downstream targets of p38, ATF2 and Elk-1, was also blocked by pterostilbene at 30 min, which was determined by their phosphorylation (Fig. 2.6.B).

Changes in the intracellular expression pattern of p-p38 and p-ATF2 were detected by immunofluorescence. With regard to p-p38, cytokine treatment induced p-p38 and its localization mainly in the nucleus. Recently, Siddiqui *et al.* have reported similar localization pattern for activated p38 in endothelial cells [273]. Pterostilbene treatment attenuated this increase (Fig. 2.7.A), parallel to the observations from western blot analysis (Fig. 2.6.B). Activated transcription factor, p-ATF2, was prominent in the nucleus on cytokine treatment and pterostilbene

virtually nullified these elevated levels (Fig. 2.7.B). Thus, significantly lower levels of the activated p38 and ATF-2 were observed in the nucleus of HT-29 cells on treatment with the stilbene.

2.2.7. p38α isoform is crucial for the induction of iNOS and COX-2 in HT-29 colon cancer cells by the cytokine mixture.

Four mammalian p38 isoforms, p38α, p38β, p38γ and p38δ, have been identified. p38α and p38β forms are ubiquitously expressed, while p38γ is present mostly in skeletal muscle, heart, lung and thymus and p38δ in lungs, pancreas, testis, kidney and small intestine [274]. SB203580, the pharmacological inhibitor that specifically targets the alpha and beta isoforms [275], was shown to lower iNOS and COX-2 induction in our study. Thus to confirm the role of p38 MAPK for the induction of iNOS and COX-2, we used siRNA against p38α and p38β. Results show that absence of p38α expression almost completely blocked the induction of iNOS (Fig. 2.8.). Since deletion of p38α expression by itself resulted in almost no induction of iNOS, there was hardly any induction on cotreatment of pterostilbene with the cytokine mixture in the p38α siRNA treated group. Also, siRNA against p38α significantly reduced COX-2 induction.

Moreover, p38α is the most abundant isoform of p38 (Fig. 2.8.; total p38 blot). The treatment with cytokine mixture or pterostilbene does not affect p-p38 levels at a longer time-point (15 h) compared to a shorter time (15 min; Fig. 2.6.B). Notably, the expression of phospho-p38 was almost absent by silencing p38α (Fig. 2.8.). This

observation is particularly relevant since pterostilbene was shown to lower phospho-p38 in shorter time point experiments (Fig. 2.6.B). This implies that pterostilbene may be mainly lowering the activation of p38α. These results suggest that p38α is the key molecule in inducing iNOS and COX-2 with the cytokine mixture and pterostilbene may be acting on p38α isoform to block the inflammatory enzyme expression in HT-29 cells.

2.2.8. Pterostilbene blocks the NF-κB binding activity.

The NF-κB pathway was not affected by pterostilbene from the evaluation of cytoplasmic IκBα degradation or nuclear translocation of p65 subunit of NFκB (Fig. 2.5.B). However, DNA binding experiments using EMSA (procedure described under Materials and Methods section) showed that pterostilbene blocked the NF-κB binding activity and thus may downregulate transcription of target genes which include iNOS and COX-2 (Fig. 2.9.). In the figure, upper bands represent the DNA-protein complex while the lower band is the biotin labeled probe. Cytokine treatment increased NF-κB binding activity compared to control, which is observed as a shift in the band. Pterostilbene lowered this binding and the effects were pronounced at 1 h time point as shown in the figure.

2.2.9. Phosphorylation of p65 is affected by pterostilbene

In order to identify the events that may cause the observed lowering in NF-κB binding activity by pterostilbene, we evaluated the changes in IκBα in the cytoplasmic and p65 in the nuclear fractions from 0 minutes to 3 h of treatment

time with cytokines and pterostilbene. Fig. 2.10.A represents the results obtained by Western blotting of cytosolic and nuclear protein fractions. In the cytosolic fraction, IκBα was degraded by pterostilbene at an early time point as 30 minutes, although there was some restitution at later time points as 1 h and 3 h (Fig.2.10A.). Pterostilbene, however, did not have any further effects beyond that of cytokine on IκBα. This is consistent with our observations in the previous section (Fig. 2.5.B.).

Nuclear fractions, on the other hand, contained p65 only when cells were treated with cytokines (Fig. 2.10.A.). But pterostilbene had little effects on this translocalization of p65. Interestingly, we observed that phosphorylation of p65 by cytokines was significantly lowered by pterostilbene. The effects were pronounced at 1 h time point, which coincided with the time when the lowering of DNA binding by pterostilbene was also evident (Fig. 2.9. and 2.10.A).

2.2.10. Phosphorylation of p65 may be regulated through the p38MAPK-MSK1 pathway in HT29 colon cancer cells

In order to identify the molecular mechanism responsible for the down regulation of p-p65 by pterostilbene, we treated the cells with pterostilbene, pharmacological inhibitors, SB203580 (against p38 MAPK) and H-89 (against mitogen stress kinase-1 (MSK-1)) in the presence of cytokine mixture. The assumption for this experiment was based on the previous report suggesting that p-p65 regulation in the nucleus is mainly regulated through the TNF-α-p38MAPK-MSK1-p-p65 pathway [276]. This pathway seemed to be relevant in our colon

cancer studies with pterostilbene; especially since pterostilbene down regulated the p38MAPK activation as described in the previous sections. As shown in Fig. 2.10B, the inhibitors against p38MAPK and MSK-1 reduced the phosphorylation of p65 induced by the cytokine mixture. Also, we observed down regulation of MSK-1, the downstream kinase of p38 MAPK, by pterostilbene. So based on these results, it appears that pterostilbene lowers activation of p38MAPK, leading to down regulation of p-MSK1 which further affects its downstream target p-p65 in the nuclear compartment.

Discussion

This part of the study indicates that pterostilbene exerts an anti-inflammatory action in HT-29 colon cancer cells. TNF-α, IFN-γ, IL-1β and LPS are effective inducers of the expression of inflammatory genes in macrophages and epithelial cells, although expression levels vary with cell type [277]. The upregulation of iNOS and COX-2 is mediated by multiple pathways, which vary with cell type and cytokines used. The involvement of NF-κB, AP-1, MAPKs and JAK-STAT in the expression of these genes has been evaluated for a variety of compounds with anti-inflammatory potential. Resveratrol, which is structurally similar to pterostilbene, reduced iNOS and COX-2 induction in rat glioma cells and inhibited iNOS induction by LPS in macrophages by reducing NF-κB [266,267]. Recently, pterostilbene was found to suppress the activation of ERK, p38, PI-3-kinase and NF-κB in LPS-induced murine macrophages, suggesting that these pathways play crucial roles in the action

of pterostilbene to inhibit iNOS and COX-2 in macrophages [269]. However, our results demonstrate the p38 MAP kinase cascade as a major signaling pathway inhibited by pterostilbene in HT-29 colon cancer cells, suggesting that the cell type specificity may contribute to this difference (Fig. 2.5.).

The p38 MAPK cascade is activated by its upstream kinase MKK3/6, which is the MAPK kinase for p38, and we found that pterostilbene strongly inhibited the activation of both MKK3/6 and p38 (Fig. 2.6.). This suggests that pterostilbene may inhibit p38 MAPK signaling through the conventional kinase cascade which has small GTP proteins, such as Rac, Rho, cdc42 acting on MAPKKK or MAP3K followed by its substrate, MAPKK or MAP2K (MKK3/6) and finally p38 MAPK [278]. In the MAPK cascade, we also observed that pterostilbene acts on downstream targets of p38, namely ATF2 and Elk-1 (Fig. 2.7.B). There are a myriad of transcription factors and kinases that are affected by p38 such as MEF2, MSK, CHOP and MAPKAP [100], but we examined two key mediators, ATF2 and Elk-1, which are known to play important roles in inflammatory gene responses [279]. ATF2 is a subunit of the AP-1 complex and binds to the CRE promoter sequence on iNOS and COX-2, and Elk-1 belongs to the ETS transcription factor and binds to the ETS DNA-binding domain on the promoter sequence of inflammatory genes [259]. In the present study, we found that pterostilbene blocked the phosphorylation and nuclear translocation of p38 and ATF-2 induced by cytokine mixture (Fig. 2.7.A, B), providing evidence for the involvement of the p38 MAPK-ATF-2 pathway for the anti-inflammatory action of pterostilbene in HT-29 colon cancer cells.

Among the different isoforms of p38 MAPKs, p38α is known to play a key role in inflammatory processes [280]. This MAPK was originally identified as a molecular target of the pyridinyl imidazole class of compounds, such as SB203580, that were known to inhibit pro-inflammatory cytokine synthesis, and many of these inhibitors have entered clinical trials for inflammatory diseases [104]. Since the p38 inhibitor, SB203580, suppresses activation of both p38α and p38β, it is difficult to distinguish the effects produced by each isoform independently. In our study, we employed RNA interference to show that p38α is the most abundant isoform in HT-29 colon cancer cells, and p38α is the key molecule involved in iNOS and COX-2 expression (Fig. 2.8.). This observation along with coordinated results from short time point experiments (Fig. 2.5. and 2.6.) indicate that pterostilbene may inhibit iNOS and COX-2 expression primarily through its action on p38α MAPK.

The NF-κB pathway is generally deregulated under chronic inflammatory conditions and in some cancers. Anti-inflammatory compounds including resveratrol target this pathway to bring about lowering of production of inflammatory cytokines, genes and their products [266,267]. The typical NF-κB complex is a heterodimer of p50 and p65 (REL A) subunits [281]. p65 subunit contains DNA binding domains and also transactivation domains while p50 has only the DNA binding domains. The biological events involved in the activation of NF-κB can be visualized as two actions. The first part comprises the IκBα activation and its degradation and the ensuing nuclear import of the NF-κB complexes. The second part is the post-translational modification by phosphorylation and acetylation of the

NF-κB subunits within the nucleus [282]. These events are particularly found in the p65 subunit and are known to alter dimerization properties and enhance the DNA binding property of p65 [283,284]. Thus, in our study, the action of pterostilbene on reducing p65 phosphorylation may contribute to the observed lowering of DNA binding by pterostilbene (Fig. 2.9.).

Both REL homology domain (RHD) and transactivation domains of p65 are phosphorylated by kinases both in the cytoplasm and nucleus. Major kinase involved is the protein kinase A, which phosphorylates serine 276 within the RHD of p65. Differential effects by cytokines are also known. LPS treatment triggers PKA [285,286] while TNFα stimulates MSK1 to phosphorylate p65 [276]. While PKA phosphorylates p65 in the cytoplasm, MSK1 action is in the nucleus. Our results show that p65 is mainly phosphorylated in the nucleus (Fig. 2.10.A), suggesting the role of MSK1 mediated phosphorylation of p65. Also, in the previous section we identified p38 MAPK as a major target of pterostilbene under inflammatory conditions. p38 is responsible for activation of MSK-1 [287] and activated MSK-1 phosphorylates p65 at serine 276 [276,288] revealing the plausible link between p38 MAPK, MSK-1 and p-p65. In our experiments, pharmacological inhibitors, SB203580 and H-89 against p38MAPK and MSK-1, respectively lowered p-p65 levels induced by the cytokine mixture in the colon cancer cells (Fig. 2.10.B.). Moreover, activation of MSK-1 was reduced by pterostilbene and by the inhibitors (Fig. 2.10.B.). However, further detailed experiments are required to confirm the exact role of pterostilbene on this phosphorylation pathway.

Summary

In this section of the project, we investigated the anti-inflammatory effects of pterostilbene. The compound was able to inhibit the inducible enzymes known to be the key molecules in inflammation, namely iNOS and COX-2, in HT-29 colon carcinoma cells. Synthesis of inflammatory cytokines such as interleukin-1β and interleukin-6 was also lowered by pterostilbene, as observed from quantitative RT-PCR analysis. Further, we examined the intracellular signaling pathways that may be involved in inflammation, to identify the probable mechanism or mechanisms of action of pterostilbene. However, amongst a number of possible pathways, only p38 MAPK activation was affected by pterostilbene. The anti-inflammatory action by the stilbene may be transduced through MKK3/6- P38 MAPK- ATF2/Elk1/other downstream transcription factors to finally control the *de novo* protein synthesis of inflammatory enzymes. More importantly, siRNA data suggest p38α to be important in regulating iNOS and COX-2 expression in HT-29 cells. Pterostilbene lowered the DNA binding of NF-κB, although there were no evident changes in the IκBα or p65 levels in the cytoplasm and nucleus respectively. However, phosphorylation of p65 was affected by pterostilbene and this may contribute to the observed lowering of DNA binding and finally affect transcription of NF-κB target inflammatory genes, iNOS and COX-2.

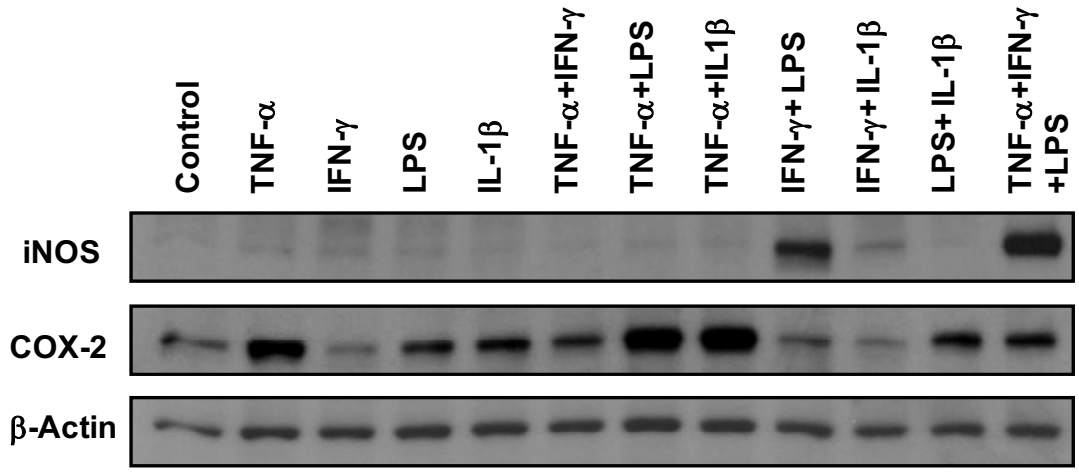


Fig. 2.1. A combination of cytokines yield strong induction of iNOS and COX-2 in HT-29 cells HT-29 cells (1.5×10^6 cells/ 100 mm dish) were treated with 10 ng/ml of TNF-α, IFN-γ, LPS, IL-1β either alone or in different combinations. The cells were harvested for protein measurements after 15 h, and the samples were immunoblotted to determine induction of iNOS and COX-2. β-Actin levels were used as the gel loading control.

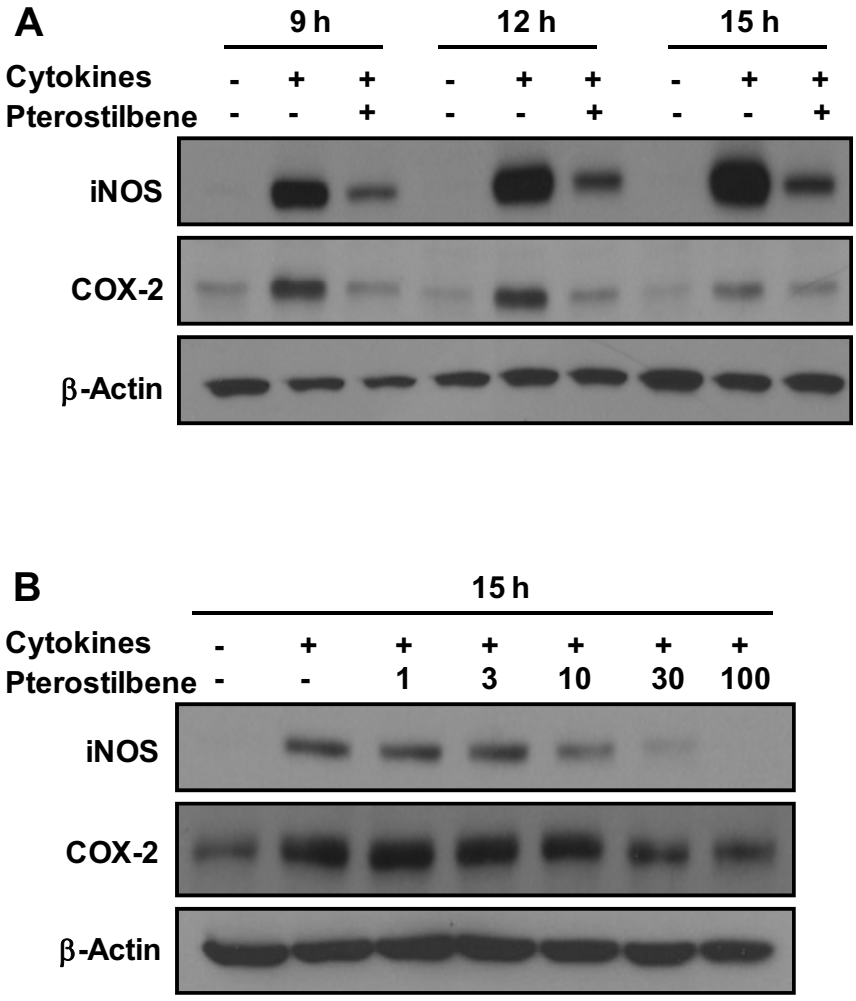


Fig. 2.2. iNOS and COX-2 induction by cytokines is time dependent and pterostilbene dose dependently inhibits this induction A. iNOS and COX-2 induction by cytokines is time dependent HT-29 cells (1.5×10^6 cells/ 100 mm dish) were treated with 10 ng/ml of TNF-α, IFN-γ, LPS, for 9, 12 or 15 h in the presence or absence of pterostilbene (50 μM). The cells were harvested and protein samples immunoblotted for iNOS and COX-2 induction by the cytokine mixture and their suppression by pterostilbene. B. Pterostilbene blocks the induction of iNOS and COX-2 in a dose-dependent manner. HT-29 cells ($1.5X10^6$ cells/ 100 mm dish) were treated with with 10 ng/ml of TNF-α, IFN-γ, LPS in the presence or absence of different concentrations of pterostilbene for 15 h. The protein levels of iNOS and COX-2 were determined by Western Blot analysis.

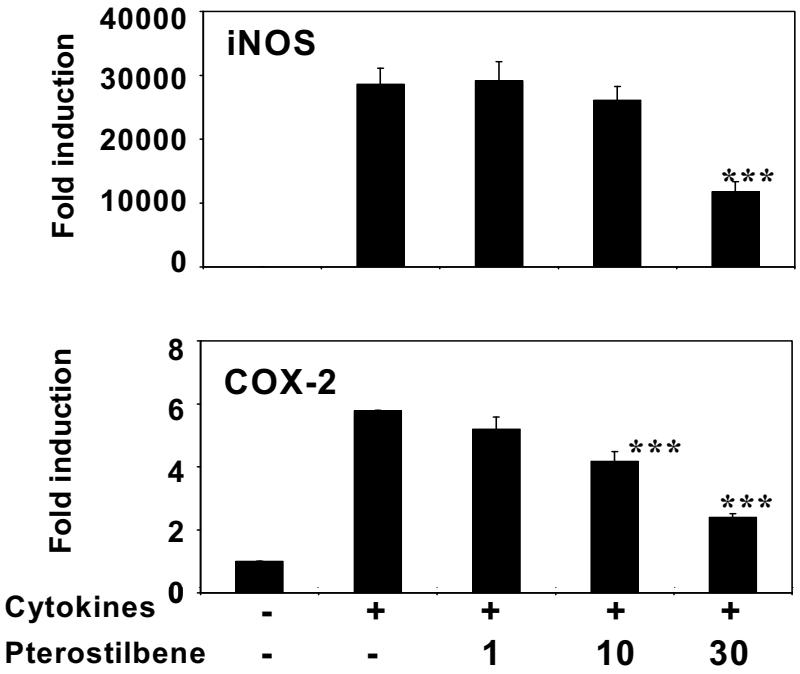


Fig. 2.3. Pterostilbene lowers the cytokine induced mRNA of iNOS and COX-2. HT-29 cells (1.5 X10^6 cells/ 100 mm dish) were treated with a mixture of TNF-α, IFN-γ and LPS (each at 10 ng/ml) alone or together with different concentrations of pterostilbene for 9 h. Total RNA was isolated and mRNA levels for iNOS and COX-2 were measured using quantitative RT-PCR analysis as described in Materials and Methods. The data are represented as mean ± standard deviation. Statistical significance was calculated by the Students t-test, n=4 and *** corresponds to p value <0.001.

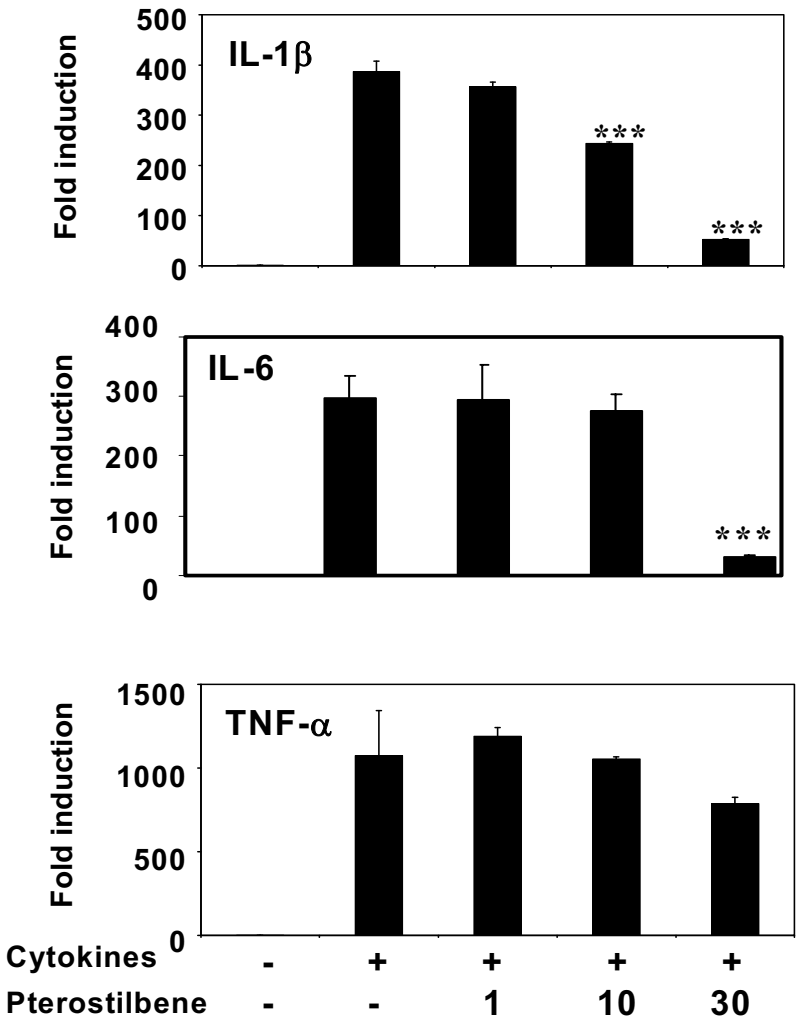


Fig. 2.4. Pterostilbene downregulates the mRNA synthesis of pro-inflammmatory cytokines. HT-29 cells (1.5 X10^6 cells/ 100 mm dish) were treated with a mixture of TNF-α, IFN-γ and LPS (each at 10 ng/ml) alone or together with different concentrations (μM) of pterostilbene for 9 h. Total RNA was isolated and mRNA levels for IL-1β, IL-6 and TNF-α were measured using quantitative RT-PCR analysis as described in Materials and Methods. The data are represented as mean ± standard deviation. Statistical significance was calculated by the Students t-test, n=4 and *** corresponds to p value <0.001.

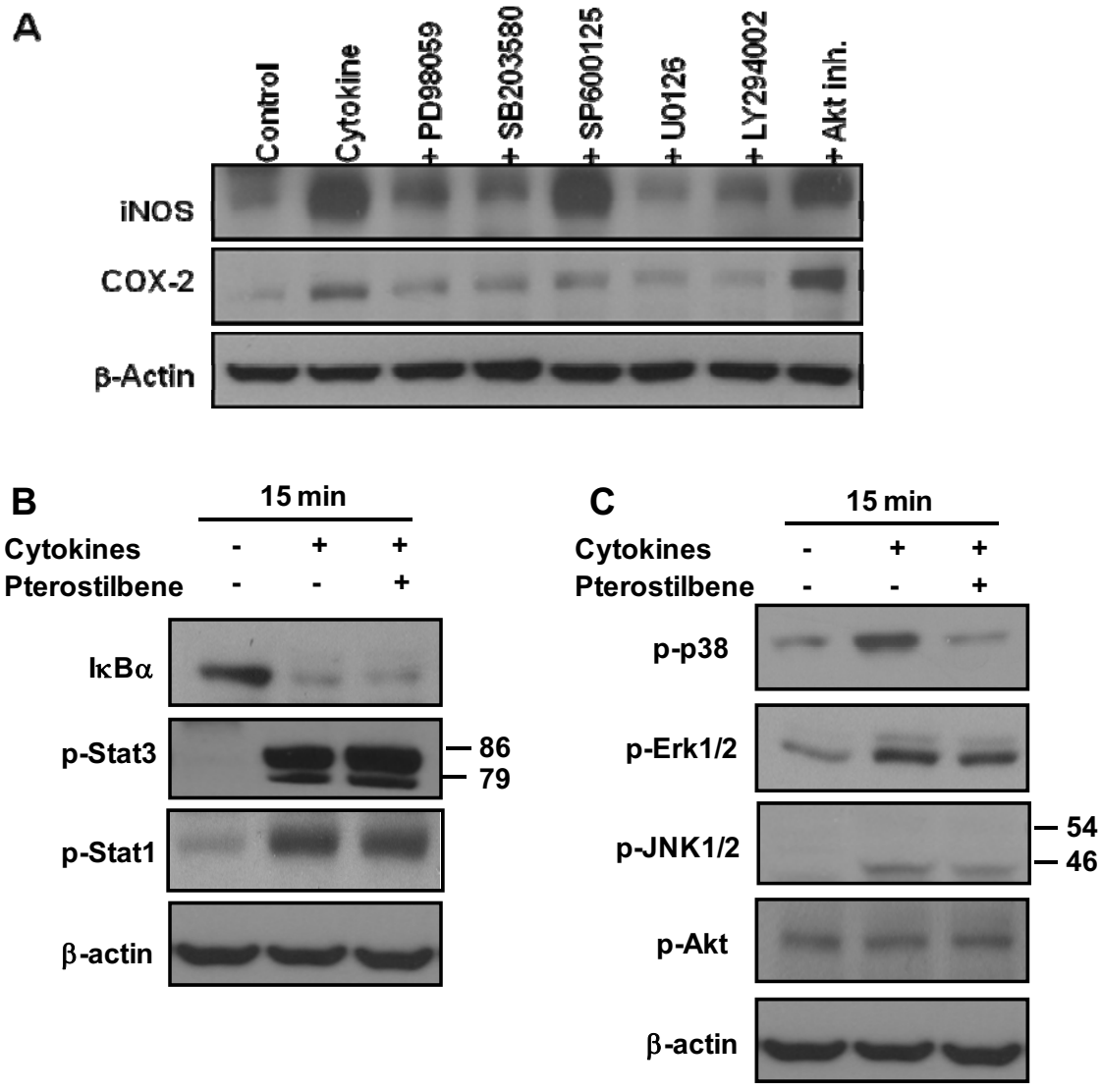


Fig. 2.5. p38 MAPK is a major target for the action of pterostilbene. A. Effect of different kinase inhibitors on the induction of iNOS and COX-2 in HT-29 cells. HT-29 cells (1.5×10^6 cells/ 100 mm dish) were incubated with a cytokine mixture of TNF-α, IFN-γ and LPS (each at 10 ng/ml) for 15 h to induce iNOS and COX-2. The following kinase inhibitors were added to the cytokine mixture: PD98059 (MEK1/2 inhibitor, 10 μM), SB203580 (p38 MAPK inhibitor, 10 μM), SP600125 (JNK inhibitor, 10 μM), U0126 (MEK1/2 inhibitor, 10 μM), LY294002 (PI3K inhibitor, 10 μM), and Akt inhibitor (10 μM). B. Effect of pterostilbene on the IκBα and p-STAT3. HT-29 cells (1.5×10^6 cells/ 100 mm dish) were incubated with cytokine mixture and pterostilbene (30 μM) for 15 min. The cells were harvested and

protein samples were immunoblotted for IκBα, p-STAT3 and β-actin. C. Effect of pterostilbene on the phosphorylation of p38 MAPK. C.HT-29 cells (1.5×10^6 cells/ 100 mm dish) were incubated with cytokine mixture and pterostilbene (30 μM) for 15 min. The cells were harvested and protein samples were immunoblotted for p-ERK1/2, p-p38, p-JNK1/2, p-AKT and β-actin.

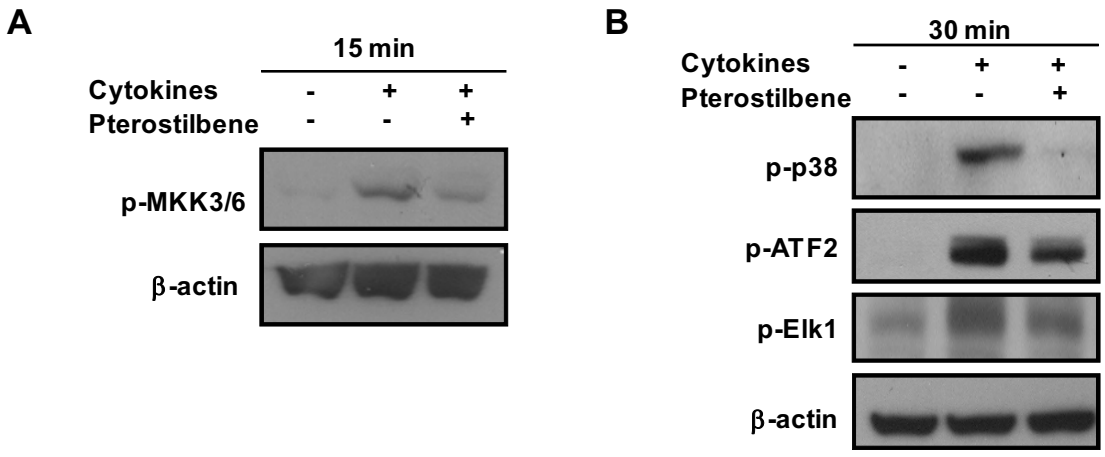


Fig. 2.6. Pterostilbene lowers the activation of upstream and downstream kinases for p38 signaling in HT-29 colon cancer cells. A. Pterostilbene down-regulates the phosphorylation of upstream kinase of p38. HT-29 cells (1.5X 10^6 cells/ 100 mm dish) were incubated with a mixture of TNF-α, IFN-γ, LPS (each at 10 ng/ml) in the presence or absence of pterostilbene (30 μM) for 15 min, and protein samples were immunoblotted for p-MKK3/6 and β-actin. B. Pterostilbene down-regulates the phosphorylation of downstream targets of p38. HT-29 cells (1.5X 10^6 cells/ 100 mm dish) were incubated with a mixture of TNF-α, IFN-γ, LPS (each at 10 ng/ml) in the presence or absence of pterostilbene (30 μM) for 30 min, and protein samples were immunoblotted for p-p38, p-Elk1 and p-ATF2 and β-actin.

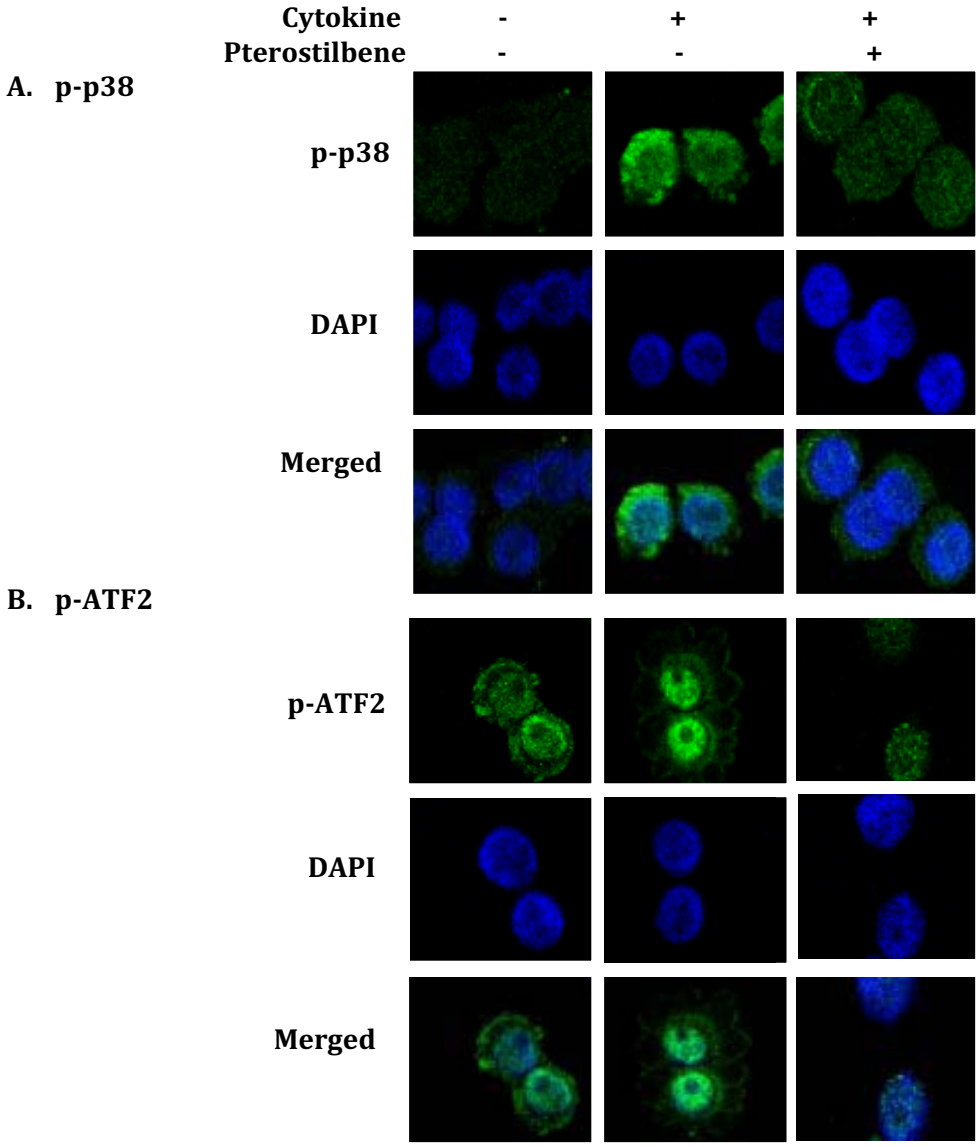


Fig. 2.7 Pterostilbene reduces the cellular levels of activated p38 and ATF2. A. Pterostilbene abolishes cellular abundance of p-p38 induced by cytokine mixture of TNF-α, IFN-γ, LPS (each at 10 ng/ml). HT-29 cells (30,000 per chamber in a 4-well chamber slide) were incubated with the cytokine mixture and pterostilbene (30 μM) for 15 min. Staining procedure is mentioned under Materials and Methods section. Green, staining for p-p38; blue, nuclear staining by DAPI; magnification, 63X. B. Pterostilbene abolishes nuclear abundance of p-ATF2 induced by cytokine mixture. HT-29 cells (30,000 per chamber in a 4-well chamber slide) were incubated with the cytokine mixture and pterostilbene (30 μM) for 30 min. Green, staining for p-ATF2; blue, nuclear staining by DAPI; magnification, 63X.

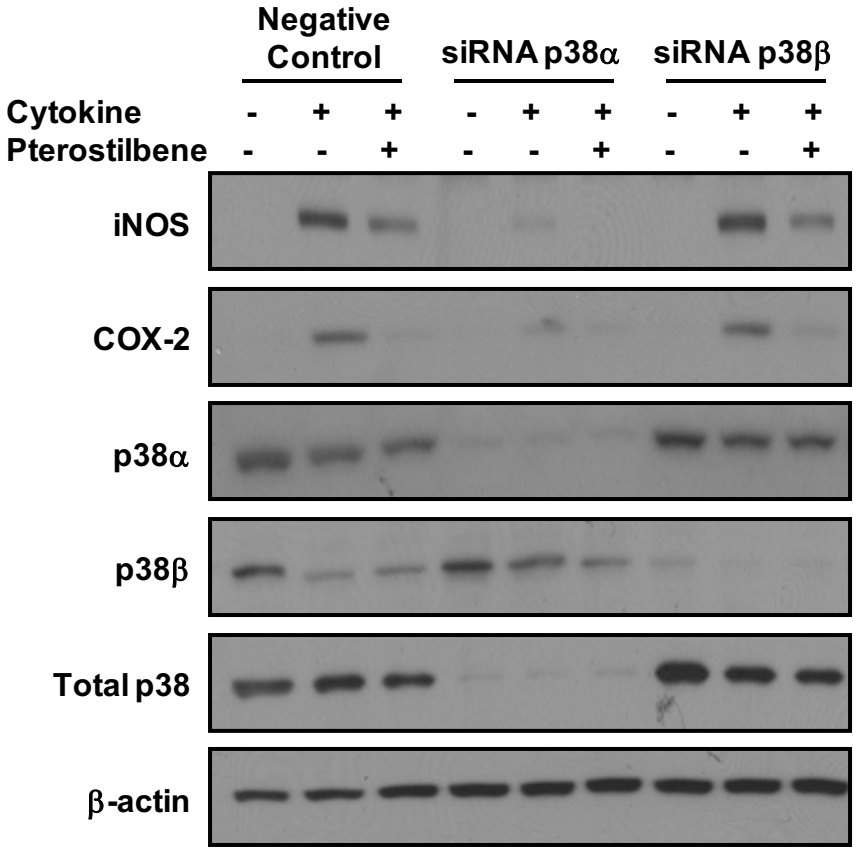


Fig. 2.8. p38α is required for the induction of iNOS and COX-2 in HT-29 cells. HT-29 cells (150,000 cells per well in 6-well plate) were transfected with siRNA against p38α and p38β in serum free AccelC delivery medium for 72 h. Medium was changed to regular medium with serum and cells were incubated with a mixture of TNF-α, IFN-γ and LPS (each at 10 ng/ml) in the presence or absence of pterostilbene (30 μM) for additional 15 h. Cells were harvested for protein and the samples analyzed by western blotting.

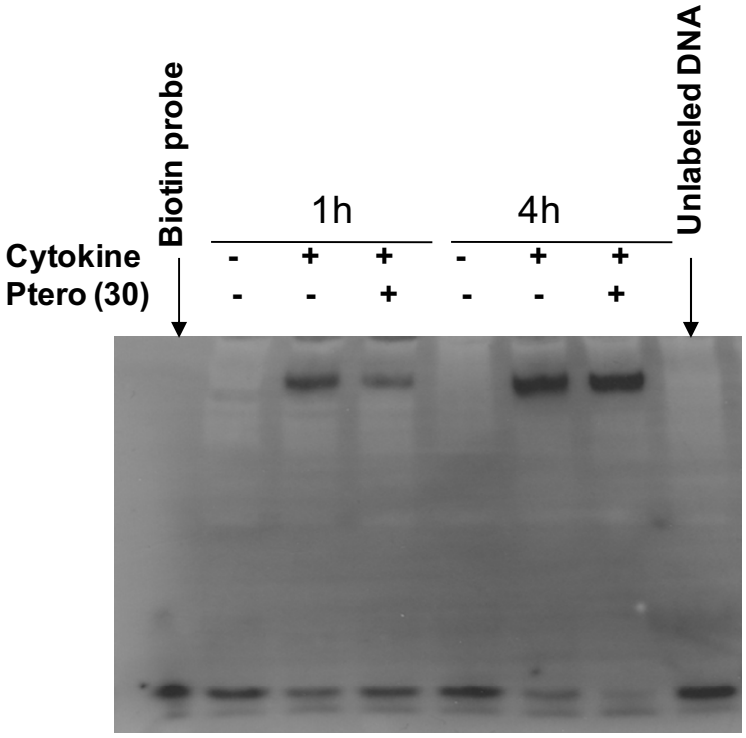


Fig. 2.9. NF-κB binding activity is inhibited by the treatment with pterostilbene. HT-29 cells (1.5X 10^6 cells/ 100 mm dish) were incubated with a mixture of TNF-α, IFN-γ and LPS (each at 10 ng/ml) in the presence or absence of pterostilbene (30 μM) for 1 h or 4 h. Nuclear fractions were collected from the cells and 20 μg protein was used for each DNA binding experiment. The nuclear fractions, biotinylated probe (labeled or unlabeled) and loading buffer were combined and loaded into a 6% non-denaturing polyacrylamide gel. Detailed procedure for the EMSA is provided under the materials and methods section.

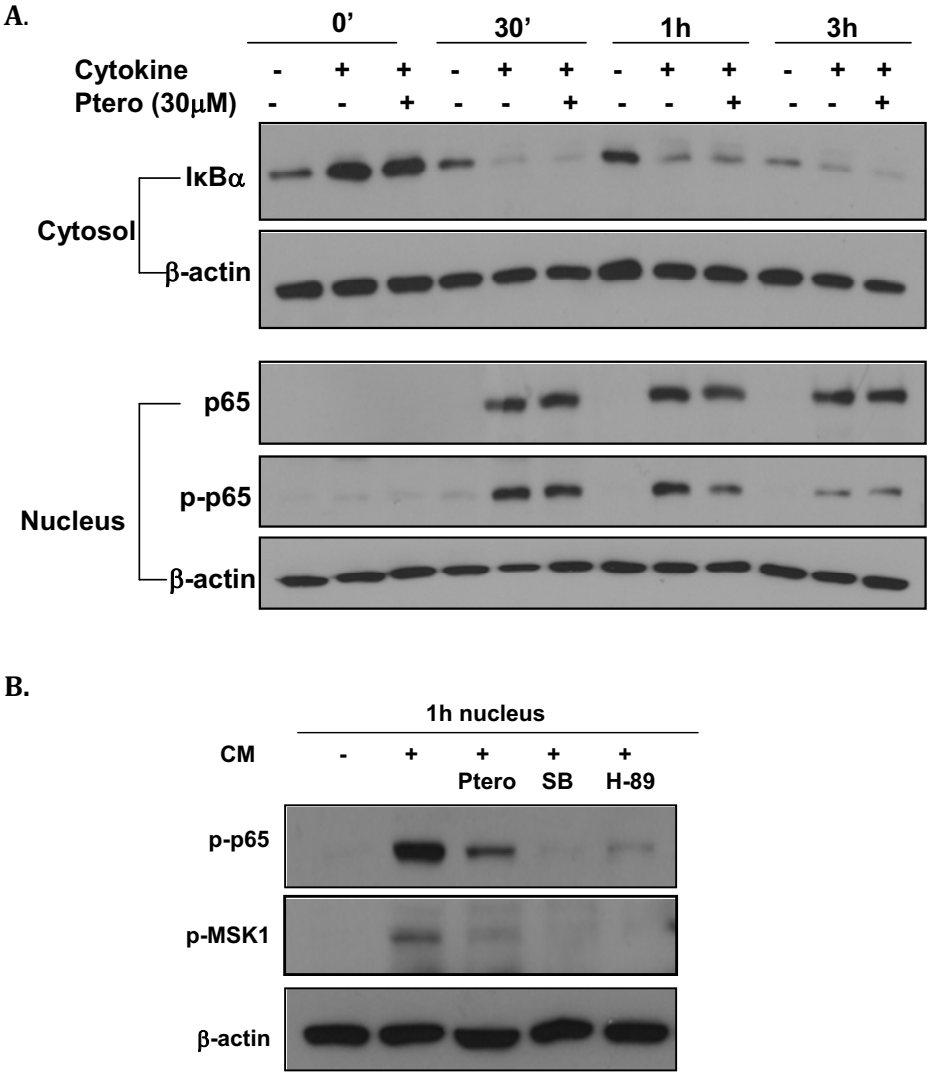


Fig. 2.10. Pterostilbene lowers the phosphorylation of p65 in the nucleus of HT-29 cells. A. HT-29 cells (1.5X 10^6 cells/ 100 mm dish) were incubated with a mixture of TNF-α, IFN-γ and LPS (each at 10 ng/ml) in the presence or absence of pterostilbene (30 μM) for indicated times, and nuclear protein fractions collected and immunoblotted. **B**. HT-29 cells (1.5X 10^6 cells/ 100 mm dish) were incubated with either a mixture of TNF-α, IFN-γ and LPS (each at 10 ng/ml) indicated as CM in the presence or absence of pterostilbene (30 μM), SB203580 (10 μM) or H-89 (10 μM) for 1 h, and nuclear protein fractions collected and immunoblotted.

PART III

Pterostilbene lowers the tumor burden in the azoxymethane (AOM)-induced colon carcinogenesis model in rats and inhibits cell proliferation in HT29 colon cancer cells

3.1. Introduction

In the previous section, we have demonstrated that pterostilbene lowered the expression of inflammatory enzymes, iNOS, COX-2, and down regulated the mRNA levels of proinflammatory cytokines such as, TNFα, IL-1β and IL-6 in HT-29 colon cancer cells [289]. Cyclin D1 is a protein which is required for the cell progression through G1-S phase and is overexpressed in patients with adenomatous polyps, primary colorectal adenocarcinoma [144] and familial adenomatous polyposis [145]. Cyclin D1 is a target gene of Wnt signaling pathway [148] and mutations in this pathway is responsible for causing approximately 90% of colorectal cancer [130]. Mutations in genes belonging to the Wnt pathway, such as inactivating mutations in the adenomatous polyposis coli (APC) gene or activating mutations in β-catenin result in the nuclear accumulation of β-catenin and subsequent complex formation with T-cell factor/lymphoid enhancing factor (TCF/LEF) transcription factors to activate gene transcription [132]. TCF/LEF binding sites on promoters of cell proliferation genes such as cyclin D1 and c-MYC [148,290] thus serve to transmit the aberrant mutations to tumorigenic signals within the colonic crypts.

Our present study was designed in the light of our earlier findings which proved pterostilbene effective in reducing the incidence and multiplicity of aberrant crypt foci in the colon of rats injected with the carcinogen, azoxymethane [248]. We had also observed a significant reduction in the markers for colonic cell proliferation and inflammation by pterostilbene in the colon crypts. With the promising results from the previous study at the ACF level (8 weeks of treatment with 40 ppm pterostilbene in the diet), our goal for the current investigation was to identify the chemopreventive potential of the stilbene with colonic tumor formation as the endpoint. Further, our objective was to evaluate the action of the stilbene in regulating the expression of key protein markers in the tumor tissue/mucosa, which are known to be critical in the process of colon carcinogenesis. Detailed mechanism based studies in a colon cancer cell line such as HT29 may provide further insights into the signaling pathways affected by pterostilbene for a few of the key markers found *in vivo*. The data on tumor incidence along with analysis of tumors for events such as cell proliferation and inflammation may provide rationale for further investigation and the testing of pterostilbene in clinical trials.

3.2. Results

3.2.1. Pterostilbene reduced the multiplicity of colonic tumors in AOM injected rats.

The effects of dietary administration of pterostilbene on AOM-induced colon tumorigenesis were evaluated, and the results are summarized in Table 3.1. Body

weights of the animals fed with 40 ppm pterostilbene throughout 45 weeks were comparable to those of the animals on the control diet. None of the rats in the saline group (without AOM injection, n=6) developed tumors when autopsied at week 45 (data not shown). At the termination of the study, the AOM-control rats and AOM-pterostilbene treated rats have tumor incidence of 87.5% and 67.8%, respectively. Histopathological analysis by hematoxylin/eosin (H & E) staining revealed approximately 95% of the tumors from the control group as adenocarcinomas (AC), and the remaining 5% were as carcinoma in situ (CIS). All tumors from the pterostilbene group were identified as ACs. In both the control and pterostilbene treated groups, approximately 90% of the total ACs belonged to the non-invasive adenocarcinoma (NIA) grade, while the remaining 10% was invasive adenocarcinoma (IA) (Table 3.1). Pterostilbene treatment reduced the number of NIA by 40.2% (p = 0.04). Also, the mean number of IA is decreased in pterostilbene group but there were not enough tumors per animals to achieve statistical significance. Since there is no report on serum or colon tissue levels of pterostilbene from long-term studies, we analyzed the serum and colon mucosa levels of pterostilbene in this study. As shown in Table 3.2, there was no detectable level of pterostilbene in the control group, whereas serum and colon mucosa levels in the pterostilbene-treated group were 48.0 ± 6.9 (ng/ml) and 10.9 ± 3.8 (ng/gram), respectively.

3.2.2. Pterostilbene lowered cell proliferation and expression of β-catenin and cyclin D1 in the colon adenocarcinoma tissue.

Proliferating cell nuclear antigen (PCNA) is a marker for cell proliferation and important for DNA synthesis during the S-phase in cell cycle [291]. Immunohistochemistry for PCNA of the colon tumor tissue from animals pterostilbene diet showed significant reduction in the expression of the proliferation marker, compared to that of the control group. PCNA positive nuclei was 71% in the control group compared to 38% in the pterostilbene treated rat colons and this lowering was significant ($p=0.02$) (Fig. 3.1).

β-Catenin was identified along the membrane of the epithelial cells in the control group (Fig. 3.2). The colonic crypt cells in the control group showed homogeneous and intense staining for β-catenin in the cytosol as well as in the membrane, with lower and scattered staining in the nucleus. In contrast, the tumors from the pterostilbene group had no observable nuclear staining (Fig. 3.2). The cytoplasmic expression of β-catenin was also significantly inhibited by the treatment with pterostilbene (Fig. 3.2).

Since overexpression of cyclin D1 is reported in patients with colorectal tumors and its lowering has therapeutic significance, we were interested to investigate whether pterostilbene reduced cyclin D1 levels in the colon. Results of cyclin D1 staining of colonic tumor tissue by immunohistochemistry are shown in Fig. 3.2. The colon from the control group rats had stronger staining for cyclin D1, compared to that of the colon from the rats fed pterostilbene diet. Positive brownish staining in the control group or pterostilbene fed group was predominantly localized in the cytoplasm.

3.2.3. Colon tumor tissues from pterostilbene fed animals showed reduced expression of inflammatory enzymes, iNOS and COX-2 and effectively reduced nuclear staining for p-p65 in the colon adenocarcinoma of pterostilbene fed rats.

Overexpression of inflammatory markers is a hallmark in colorectal tumors. This knowledge as well as our previous observations of the efficacy of pterostilbene against inflammation [248,289] led us to examine the effects of long term feeding of pterostilbene in the azoxymehane injected rats. As shown in Fig. 3.3, there was significant inhibition of the expression of iNOS and COX-2 proteins within the crypts in the adenocarcinomas from the pterostilbene group, compared to those from the control group. We next determined the effects of pterostilbene on a key NF-κB signaling molecule, p65, because NF-κB is a upstream factor of both iNOS and COX-2 transcription, and it is critical in the tumorigenesis where ablation of the proteins in this pathway caused the regression of tumors in animal models [292]. The activated form of NF-κB subunit p65, i.e. phospho-p65, is markedly reduced in the nucleus of the colon tumors from the pterostilbene group, when compared to those from the control group (Fig. 3.3).

3.2.4. Pterostilbene lowered mucosal levels of the inflammatory cytokines TNFα, IL-1β and IL-4.

Inflammatory cytokines are found to be present in human cancers including those of the colorectum, breast, prostate and bladder [42,53]. The action of

cytokines to facilitate carcinogenesis is multi-fold: DNA damage by ROS, RNS; inhibition of DNA repair by ROS; functional inactivation of tumor suppressor genes; tissue remodeling via activation of matrix metalloproteinases (MMPs); stimulation of angiogenesis and control of cell adhesion molecules [42]. ELISA conducted for inflammatory cytokines on mucosal scrapings derived from the AOM injected rats are shown in Fig. 3.4. The levels of all the cytokines were significantly lowered by pterostilbene feeding. Pterostilbene administration lowered the levels of TNFα by 51.0% ($p=0.009$), IL-1β by 47.7% ($p=0.008$) and IL-4 by 64.2% ($p=0.002$) in the colon.

3.2.5. Pterostilbene inhibited the proliferation of cultured colon cancer HT-29 cells.

We evaluated the effect of pterostilbene and resveratrol on the growth of cultured colon cancer HT-29 cells. The cells were incubated with different concentrations of pterostilbene for 1, 2 and 3 days, and cell proliferation was estimated by measuring [^{3}H] thymidine incorporated into DNA. The 3-day incubation gave the strongest growth inhibition, and there was a dose-dependent effect (Fig. 3.5.A.). As illustrated in Fig. 3.5.B, pterostilbene was a more potent inhibitor of proliferation (IC_{50} = 22.4 μM) when compared to resveratrol treatment (IC_{50} = 43.8 μM) under the same conditions. IC_{50} values were determined using TableCurve 2D® software (Ver. 5.01) from Systat. In order to evaluate whether pterostilbene potentiates cell cycle arrest or apoptosis in HT-29 cells, we examined

the effect of pterostilbene on proteins regulating the cell cycle/ apoptosis pathways. Pterostilbene was effective in reducing c-Myc and cyclin D1 levels, after a 9 h incubation (Fig. 3.5.C.). However, pterostilbene showed no induction of p21 and p27, which belong to the CIP-KIP family of cyclin dependent kinase inhibitors (data not shown). As a marker for the induction of apoptosis, we determined the level of cleaved PARP. Treatment with pterostilbene for 9 h or 18 h increased the level of cleaved PARP (Fig. 3.5.C.).

3.2.6. The effects of pterostilbene on growth inhibition are p38 MAPK independent.

Since pterostilbene was identified to lower p38 MAPK activation under inflammatory conditions, we were interested in determining whether this MAPK has a role in regulating cell proliferation. Towards this, we treated the cells with different concentrations of p38 MAPK inhibitor, SB203580, with or without pterostilbene. The results show (Fig. 3.6. A) that even in the presence of SB203580 which already suppresses p38 MAPK, pterostilbene lowers the amounts of cyclin D1 and c-MYC still further. This suggests that there exists some other mechanism to regulate cell proliferation in HT29 cells and p38 MAPK may not be relevant in this context. Also, we could not see any differences, neither increase nor decrease, in the protein expression level of cyclin D1 when the cells were treated with siRNA against p38α and p38β isoforms (Fig. 3.6.B). [^{3}H]thymidine incorporation studies to measure cell proliferation with siRNA against p38α and p38β also failed to show

any significant difference of cell proliferation on knocking down p38 isoforms (Fig. 3.6.C.).

3.2.7. Pterostilbene reduced the β-catenin protein levels and altered the cellular localization of β-catenin in HT29 colon cancer cells.

To investigate the effects of pterostilbene on β-catenin expression in colon cancer, we employed the HT-29 colon cancer cells, which are known to possess the wild-type β-catenin and truncated *Apc* gene [293]. As shown in Fig. 4A, we observed lowering of the protein levels of β-catenin after 30 min of treatment with pterostilbene. Cyclin D1 and c-MYC proteins, two well-known downstream targets of β-catenin, were decreased by pterostilbene at a later time point, 4 h (Fig. 3.7A). In confocal microscopy, the untreated cells showed intense staining for β-catenin predominantly in the cytoplasm and the membrane, and the treatment with pterostilbene markedly lowered the levels of β-catenin (Fig. 3.7B). The β-catenin transcriptional activity is regulated not only through the levels of protein degradation, but also through its nuclear localization [294]. In order to better understand the inhibitory effects of pterostilbene on the β-catenin pathway, we used a Wnt agonist that mimics the effect of Wnt in inducing β-catenin/TCF-dependent transcriptional activity. The Wnt agonist increased the expression of cyclin D1, c-MYC and β-catenin proteins, while co-treatment of the Wnt agonist with pterostilbene lowered the expression level of these proteins in the nucleus (Fig. 3.8A). In confocal microscopy, treatment with the Wnt agonist increased β-catenin

predominantly, and the co-treatment of Wnt agonist with pterostilbene lowered Wnt agonist-induced β-catenin in the membrane and nucleus (Fig. 3.8B).

Discussion

The present study is an extension of our previous work which identified pterostilbene as an effective agent in suppressing the formation of ACF in the colons of rats injected with the colon-specific carcinogen, azoxymethane [248]. The results from the current research conducted in same animal model of colon cancer, but with tumors as end point, reveal that dietary administration of pterostilbene reduces the colon tumor burden and regulates different intermediate signaling pathways that are relevant in the contexts of proliferation and inflammation in the colonic environment.

A comparison of tumor numbers across the different grades of tumor shows an overall reduction by the treatment with pterostilbene, although statistical difference was shown only with tumor multiplicity with non-invasive adenocarcinoma (40.2% reduction). The moderate reduction of tumor multiplicity and tumor incidence may be due to a low dose of pterostilbene used. In our study, pterostilbene was given at 40 ppm (0.004% in the diet), and approximately 40-50 ng/ml range of pterostilbene was detected in the serum from the animals. This number is low when considering that many of the reported studies on tumorigenesis with stilbenes have used much higher doses. Using colon adenoma as an end point, a study conducted by Sale *et al* indicated that resveratrol and its analogue DMU212 (3,4,5,4'-tetramethoxystilbene)

given as 0.2% in the diet significantly decreased the number of adenomas, whereas 0.05% of either stilbenes given in the diet did not [295]. Since pterostilbene at 0.004% dose shows reduction in tumor multiplicity, pterostilbene may be more effective than resveratrol in the inhibition of colon cancer, although dose-response studies are needed to fully understand and design effective chemoprevention strategies with stilbenes.

The canonical Wnt signaling pathway with β-catenin as the central effector molecule is mutationally activated in greater than 90% of all CRC [130]. Wnt signal induced by the ligands is transduced through the frizzled family of receptors that relieve the β-catenin from the degradation complex comprising of adenomatous polyposis coli (APC), axin and the glycogen synthase kinase, GSK3β. These result in the cytosolic accumulation and ensuing nuclear import of β-catenin and formation of heterodimeric complex of catenin and TCF that drives transcription of target genes [130]. A recent study purports that mutations or loss of APC gene along with deregulation of a transcriptional repressor, C-terminal binding protein-1 (CtBP1) contribute to colon adenoma initiation, while activation and nuclear accumulation of β-catenin by KRAS,RAC1 and RAF1 promotes adenoma to carcinoma progression [129].

AOM induced tumors result from mutations in the Wnt/ catenin pathway [296] as is the APC^{Min} model. However, unlike the APC^{Min} model, AOM induced tumors are caused by mutations in β-catenin gene [297,298]. These mutations result in β-catenin stabilization, loss of growth control and development of colorectal

adenomas [139]. Cyclin D1 is a very well known cell cycle protein targeted by β-catenin [148] and is known to be overexpressed in colonic tumors. c-MYC is yet another important protein for cell proliferation regulated by catenin and Wnt pathway [290]. Cooperative binding of β-catenin and another transcription factor, c-MYB, was recently shown to upregulate of c-MYC promoter activity and lead to an increase in intestinal adenoma formation in APC^{Min+} mice [156]. The deletion of MYC in adult murine small intestine with APC deficiency rescued the cells from perturbed proliferation, apoptosis, migration and differentiation. This indicated the critical role of MYC in transducing Wnt pathway signals following APC loss [299]. In our studies we identified pterostilbene to significantly lower the levels of cyclin D1 in the colon tumor tissues from rats induced for tumorigenesis with AOM injection (Fig. 3.2.). Also in experiments with cultured colon cancer cells pterostilbene significantly lowered the protein levels of cyclin D1 and c-MYC, both in unstimulated and in cells stimulated with a Wnt agonist (Fig. 3.7., Fig. 3.8.).

These observations on cyclin D1 and c-MYC were corroborated by the potency of pterostilbene to affect the β-catenin levels in the colon tumor tissue and in HT29 cells. Immunohistochemistry revealed abundance of β-catenin mostly in the cytoplasm and relatively lower nuclear staining in the adeocarcinomas of rats injected with AOM, while administration of pterostilbene markedly reduced the staining for β-catenin in both the cytoplasm and the nucleus (Fig. 3.2). HT 29 cells possess a Wnt activating mutation in the *APC* gene [293]. Pterostilbene was effective in reducing the abundance of β-catenin in the cytosol and to some extent in the

nucleus of HT-29 cells (Fig. 3.7). The effects by pterostilbene were better appreciated in the presence of a Wnt agonist, in which pterostilbene lowered the induced nuclear β-catenin (Fig. 3.8). Thus our present study on pterostilbene on colon tumorigenesis exposes the potency of stilbenes in targeting the Wnt pathway.

In addition to the effects on β-catenin and cell proliferation, our results indicate the anti-inflammatory property of pterostilbene. We noticed marked reduction in the staining intensities for iNOS, COX-2 and phospho-p65 in the colon tumors from the rats which were on pterostilbene diet compared to those from rats on control diet (Fig.3.3.). Also mucosal levels of inflammatory cytokines such as, TNFα, IL-1β and IL-4 were significantly down regulated by pterostilbene (Fig. 3.4.). In the previous section, we had discussed that in HT-29 cells, the phosphorylation of p65 subunit of NF-κB was down regulated by pterostilbene which probably leads to lower NF-κB binding potential (Fig. 2.9., Fig. 2.10.), which can eventually affect the transcription of a number of target genes, predominantly those involved in inflammatory cell responses. Several anti-inflammatory agents that target the nitric oxide or the prostaglandin pathway are reported to present chemopreventive action in the colon [117,300,301]. A clinical trial on celecoxib, the selective COX2 inhibitor, at a dose of 400 mg once daily, reduced advanced adenoma formation in the colon by almost 50% compared to the placebo through a 3-year treatment period [180]. Promising results with other agents such as the use of low concentrations of difluromethylornithine and sulindac as chemopreventive agents in colorectal cancer [301] highlight the undisputable role of inflammation in its pathogenesis. Since

pterostilbene at a low dose as 40 ppm in the diet was shown to lower the inflammatory markers based on our current and previous [248] studies, the development of this natural compound against colorectal cancer is not very far in the future.

Summary

Pterostilbene at a dose of 40 ppm in the diets of AOM injected rats was shown to lower the overall incidence of colon cancer and tumor multiplicity in pterostilbene fed group compared to the control group. Molecular markers, such as PCNA, β-catenin, cyclin D1, iNOS, COX-2, p-p65 and p-p38, were significantly down regulated in colon tumors collected from the animals which had pterostilbene mixed in their daily diet compared to those from animals which were on control diet. Pterostilbene feeding also resulted in marked reduction of the inflammatory cytokines in the colon mucosa. A detailed investigation in HT29 colon cancer cells on the effect of pterostilbene on the β-catenin signaling pathway showed that the stilbene affected β-catenin protein expression, which is an integral part of the Wnt signaling pathway, the pathway that is highly noteworthy in CRC.

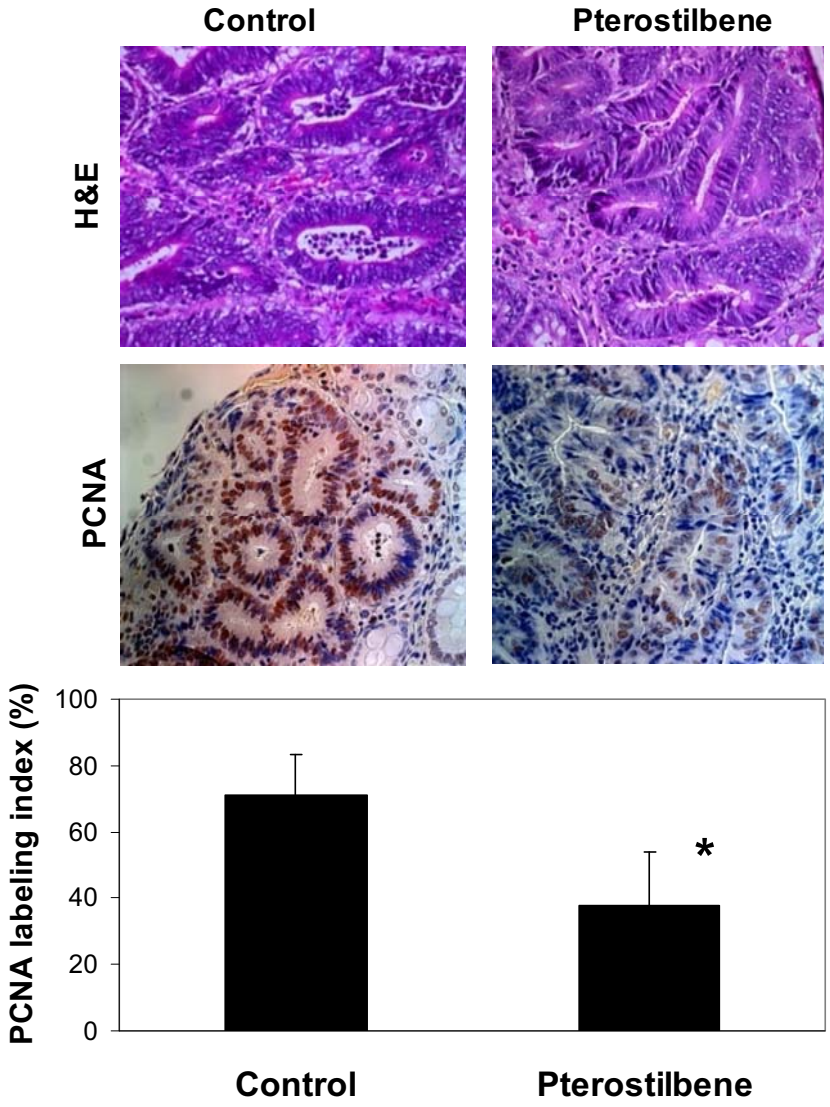


Fig. 3.1. H & E staining of the colon tumors (top panel) and PCNA staining and counting (bottom panels). Towards PCNA counting, four independent sections of the colon per animal with 3 animals per group were stained, and approximately 1000 cells were counted from each section in total. The PCNA labeling index (PI) was calculated as the [(number of positive cells) / (total number of epithelial cells)] x 100 for each field. These PI values for all the different colon sections from the animals belonging to same group were then averaged. Statistical significance of treatment between the groups was analyzed by Student's *t* test. * $p<0.05$.

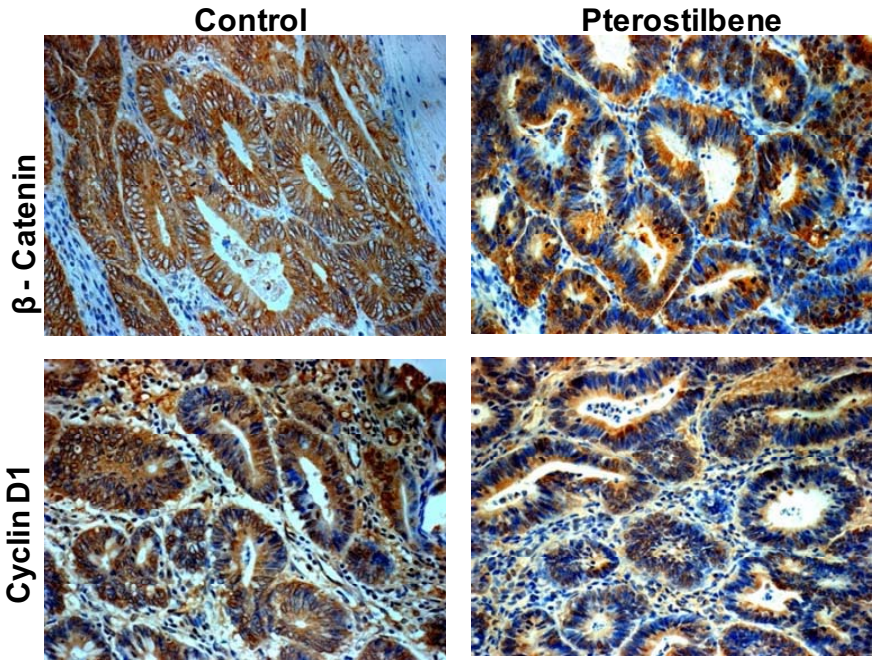


Fig. 3.2. Pterostilbene inhibits β-catenin and cyclin D1protein expression in the colon tumors. The colon tumor sections were processed and incubated with the respective primary antibodies as explained in the Materials and Methods section on immunohistochemistry. β-catenin and cyclin D1 staining was predominantly high in the cytosol and scattered in the nucleus to a lower extent. n=3 per group for each analysis. A representative section is shown. Image magnification 400X.

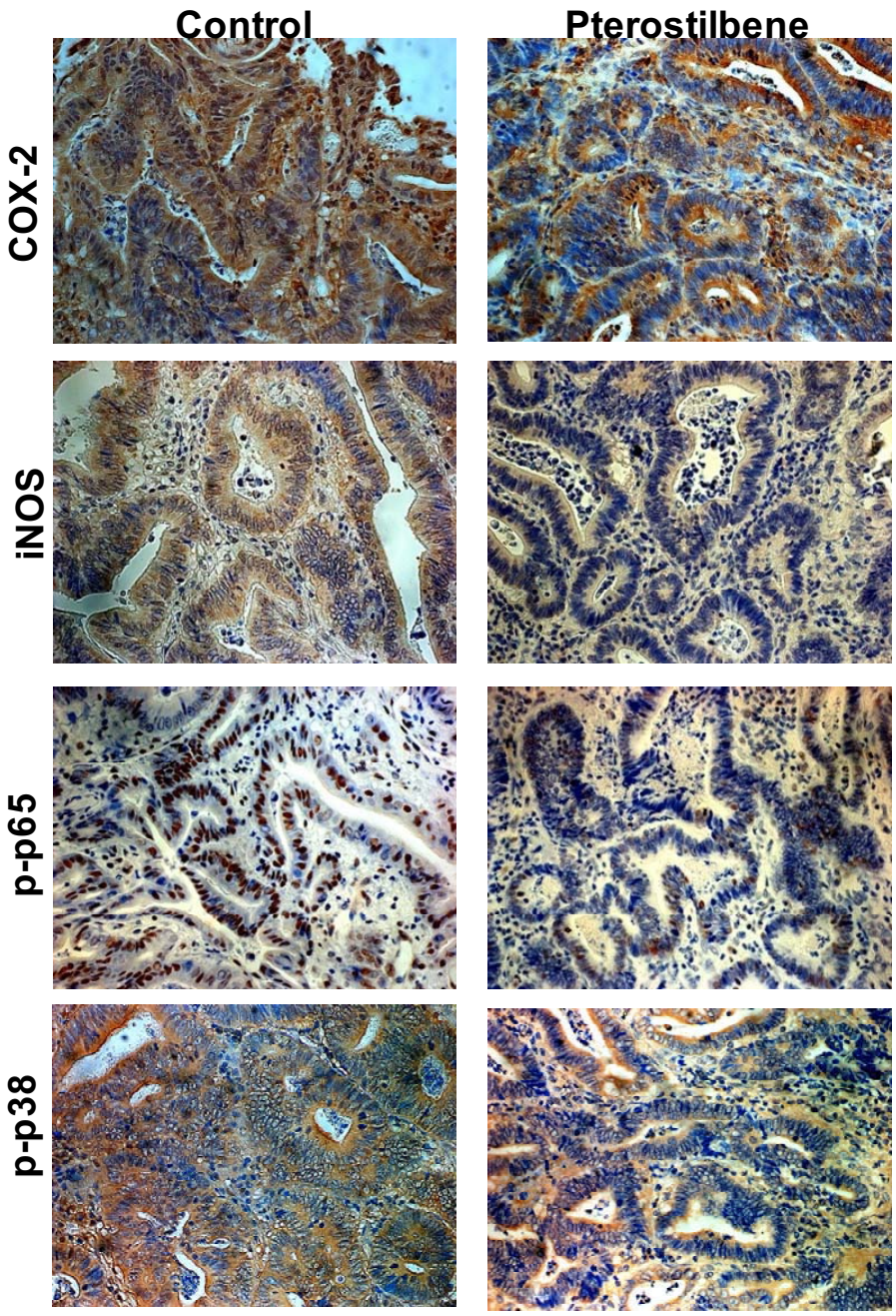


Fig. 3.3. Pterostilbene inhibits the expression of iNOS, COX-2, p-p65 and p-p38 in the colon tumors. The colon tumor sections were processed and incubated with the respective primary antibodies as explained in the Materials and Methods section on immunohistochemistry. iNOS and COX2 showed cytoplasmic staining, while nuclear staining was predominant with p-p65. n=3 per group for each analysis. A representative section is shown. Image magnification 400X.

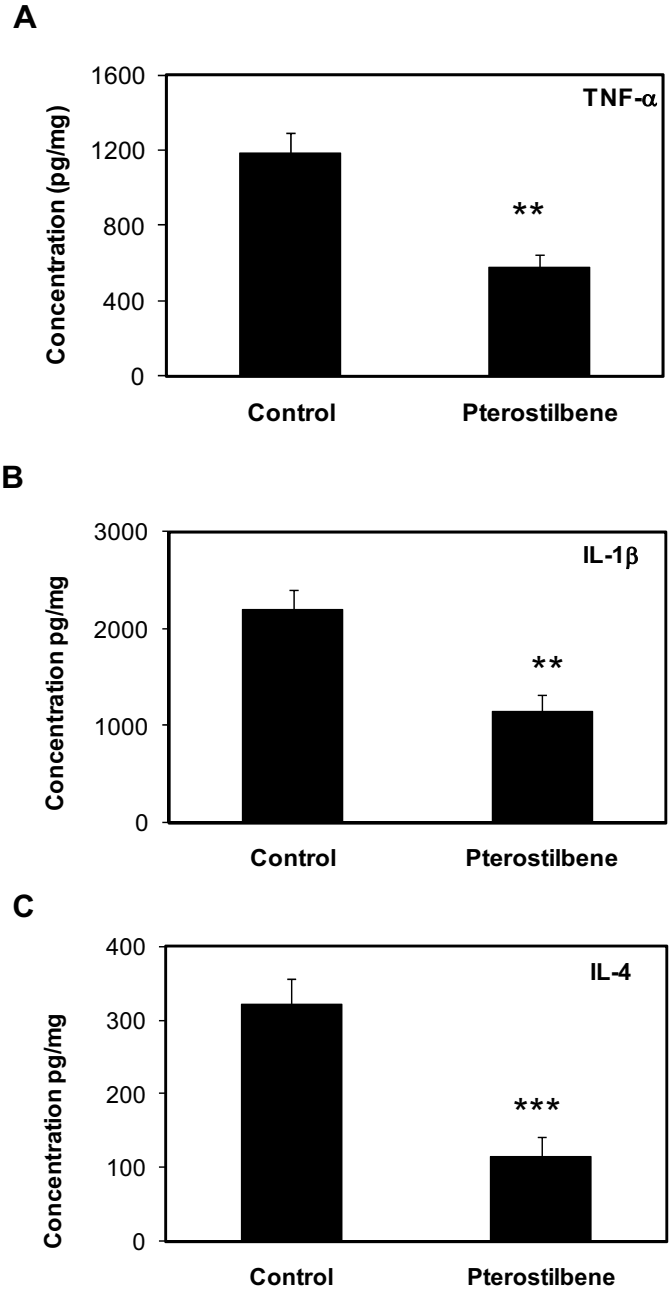


Fig. 3.4. Pterostilbene lowers the inflammatory cytokines, TNF-α (A), IL-1β (B) and IL-4 (C) in the colonic mucosa. The mucosa samples were homogenized and assayed by ELISA for the different cytokines as described under Materials and Methods section. Mean ± S.D. values are shown. n=6 per group. ** $p<0.01$, ***$p<0.005$.

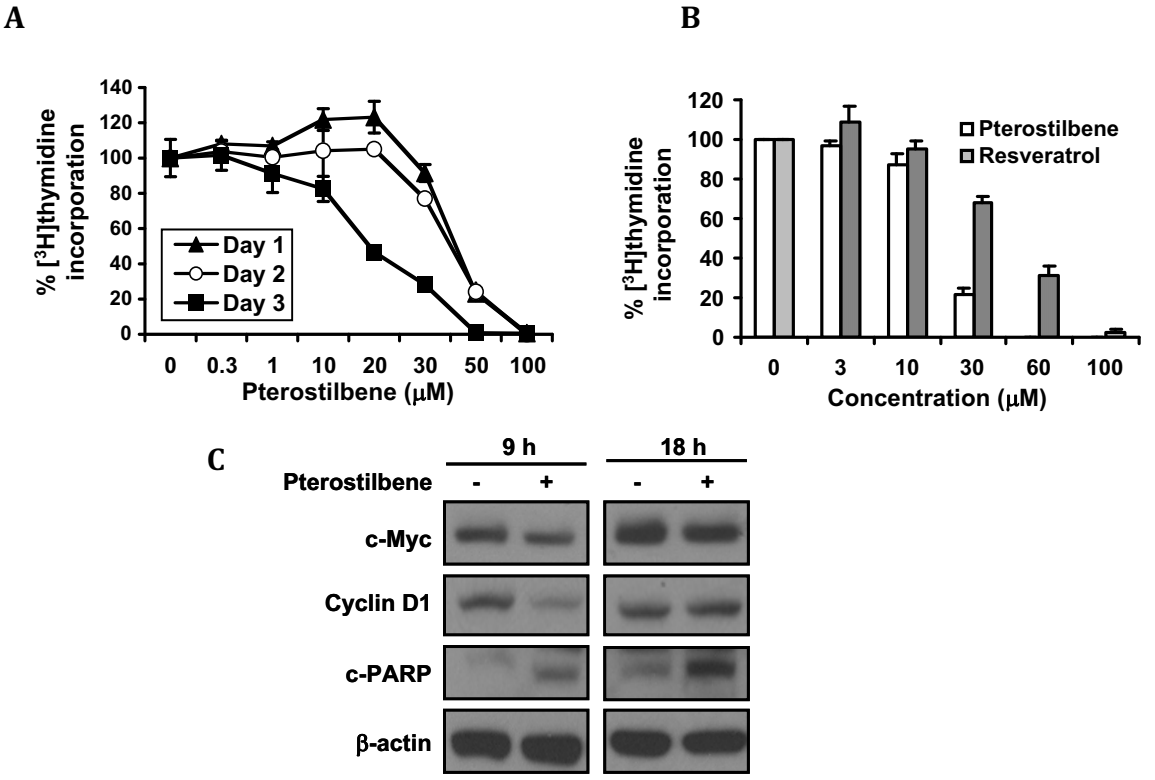


Fig. 3.5. Pterostilbene inhibits [^{3}H]thymidine incorporation into the DNA of HT-29 cells and modulates the level of proteins involved in cellular proliferation and apoptosis. A. HT-29 cells were seeded on a 24-well plate (20,000 cells/well) in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin. The cells were incubated with pterostilbene at different concentrations or with DMSO as the control vehicle for 1, 2 or 3 days. One μCi of tritium labeled thymidine ([^{3}H]thymidine) per well was added and the amount of incorporated radioactivity in DNA was measured using a liquid scintillation spectrometer. The experiment was repeated twice, with each experiment done in duplicates. The data are represented as mean ± standard deviation. **B.** Experiments were set up and conducted similar to the conditions as mentioned for Fig. 3.5.A with the exception that HT-29 cells were treated with pterostilbene, resveratrol or DMSO as control for 3 days before [^{3}H]thymidine incorporation. The experiment was repeated twice, with each experiment done in duplicates. The data are represented as mean ± standard deviation. **C.** HT-29 cells (1.5×10^6 cells/ 100 mm dish) were treated with pterostilbene at a concentration of 50 μM. The cells were harvested after 9 or 18 h of incubation with pterostilbene, and protein samples were analyzed by Western blotting.

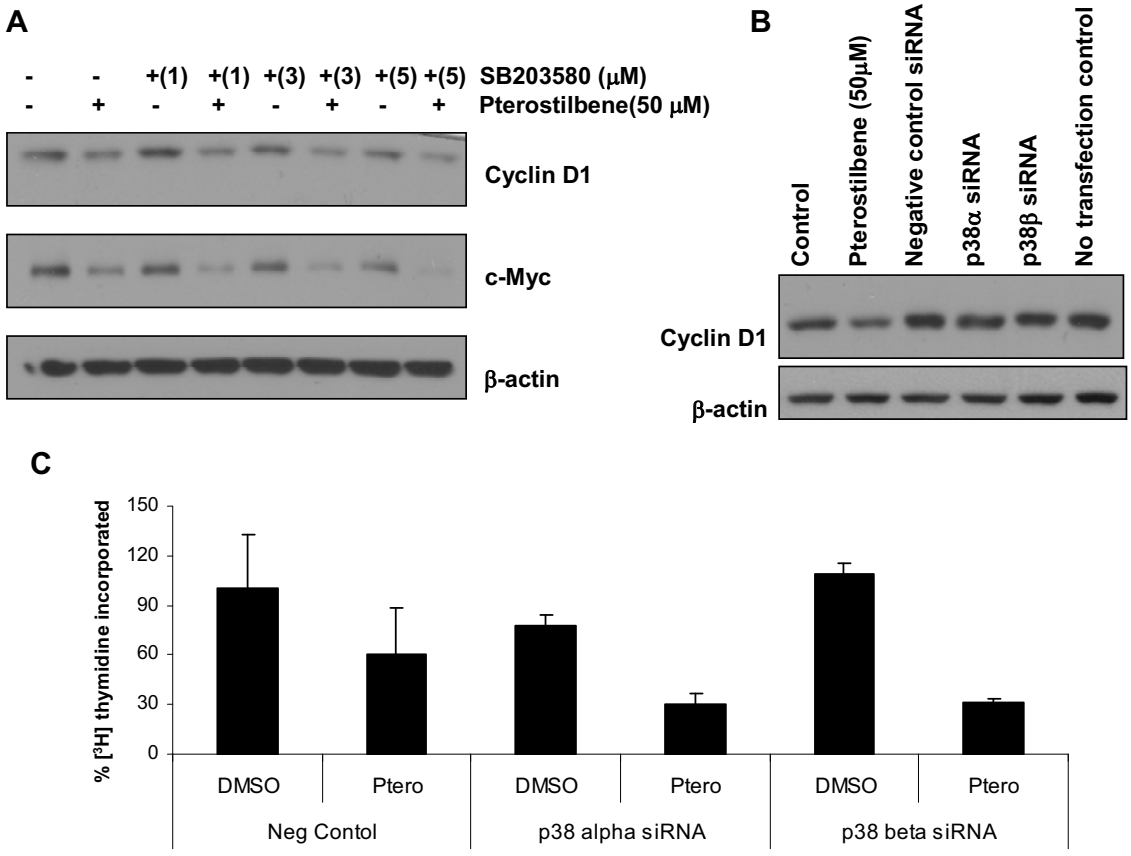


Fig. 3.6. Effects of pterostilbene on cell proliferation is p38 independent. A. HT-29 cells were seeded on 6-well plate (600,000 cells/well) in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin. The cells were incubated with pterostilbene (50 μM) with or without p38 inhibitor, SB203580, at different concentrations or with DMSO as the control vehicle for 9 h. **B.** HT-29 cells (150,000 cells per well in 6-well plate) were transfected with siRNA against p38α and p38β in serum free Accell delivery medium for 72 h. Medium was changed to regular medium with serum and cells were incubated with or without pterostilbene (30 μM) for additional 12 h. Cells were harvested for protein and the samples analyzed by western blotting. **C.** HT-29 cells (7,000 cells per well in 24-well plate) were transfected with siRNA against p38α and p38β in serum free Accell® delivery medium for 72 h. Medium was changed to regular medium with serum and cells were incubated with or without pterostilbene for 3 days. One μCi of tritium labeled thymidine ([^{3}H]thymidine) per well was added and the amount of incorporated radioactivity in DNA was measured using a liquid scintillation spectrometer. The data are represented as mean ± S.D.

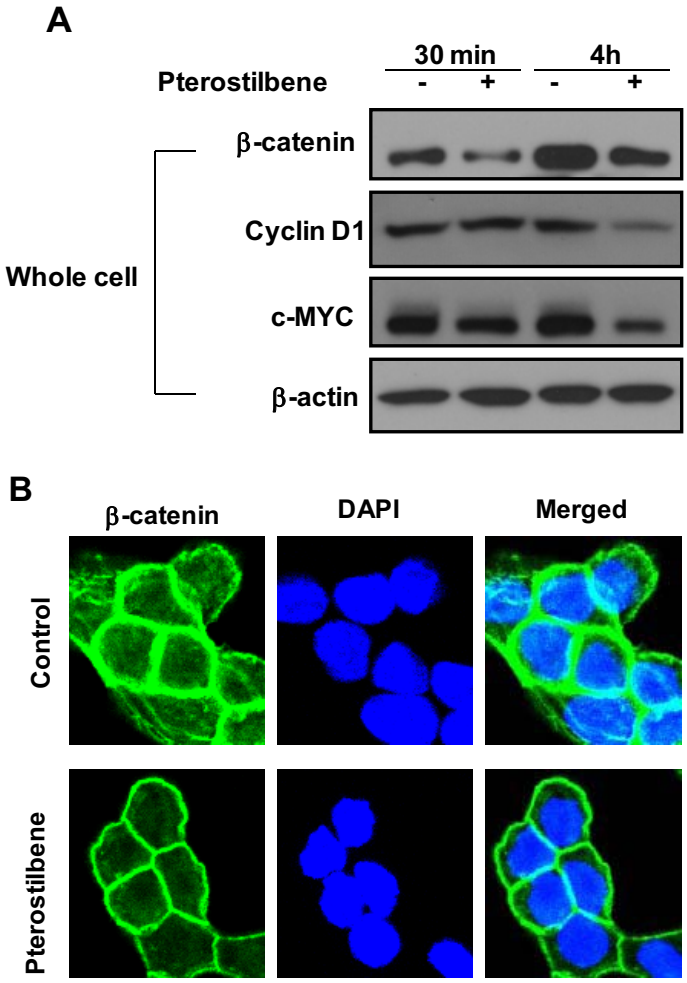


Fig. 3.7. Pterostilbene downregulates the expression of β-catenin and its downstream targets, cyclin D1 and c-MYC in HT-29 colon cancer cells. A. Effect of pterostilbene on whole cell protein levels of β-catenin, cyclin D1 and c-MYC. HT-29 cells (1.5 x 10^6/100-mm dish) were treated with pterostilbene (50 μM) for 30 min and 4 h. The cells were harvested for whole cell protein and samples were immunoblotted. **B**. Action of pterostilbene on cellular localization of β-catenin. HT-29 cells (30,000 per chamber in a 4-well chamber slide) were incubated with pterostilbene (50 μM) for 4 h. *Green*, staining for β-catenin; *blue*, nuclear staining by DAPI. Magnification, x63.

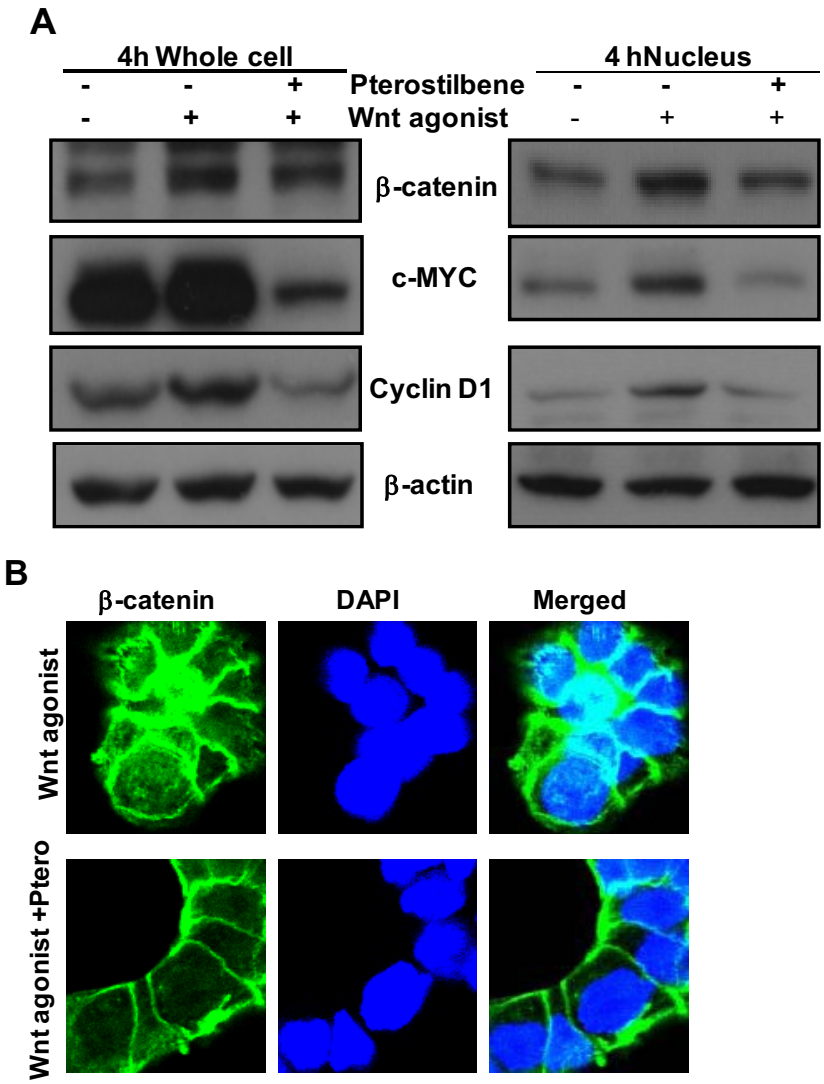


Fig. 3.8. Effects of pterostilbene in downregulating whole cell and nuclear Wnt signaling proteins in the presence of Wnt agonist. A. HT-29 cells (1.5 x 10^6/100-mm dish) were treated with a Wnt agonist (10 μM) with or without pterostilbene (50 μM) for 4 h. The cells were harvested for whole cell protein and nuclear protein samples were immunoblotted for β-catenin, cyclin D1, c-MYC and β-actin. **B.** Effects of pterostilbene on reducing nuclear localization of β-catenin induced by Wnt agonist. HT-29 cells (30,000 per chamber in a 4-well chamber slide) were incubated with Wnt agonist (10 μM) with or without pterostilbene (50 μM) for 4 h *Green*, staining for β-catenin; *blue*, nuclear staining by DAPI. Magnification, x63.

Table 3.1. Efficacy of pterostilbene in the azoxymethane (AOM)-induced colon cancer model in Fisher rats: Total Tumor Incidence and Total Tumor Multiplicity.

Experimental Group*	No. of animals at autopsy	Body weight at autopsy (Mean ± S.E.) (gram)	Tumor incidence** (Rats with Colon Tumors/ Total No. of Rats)	Tumor multiplicity*** (No. of non-invasive adenocarcinoma per rat) (Mean ± S.E.)	Tumor multiplicity*** (No. of invasive adenocarcinoma per rat) (Mean ± S.E.)
Control diet	24	439 ± 6.6	21/24 (87.5%)	1.79 ± 0.28	0.21 ± 0.08
Pterostilbene	28	445 ± 5.5	19/28 (67.8%)	1.07 ± 0.21 (p = 0.04)	0.07 ± 0.05

*Pterostilbene (40 ppm) was administered in the diet starting at one day after the second AOM treatment and continuously thereafter for 45 weeks.

**Tumor incidence was analyzed by two-tailed Fisher's exact probability test. No statistical significance was observed.

***Tumor multiplicity was analyzed by the Student's *t*-test.

Table 3.2. Serum and colon mucosa levels of pterostilbene in AOM-treated F344 male rats.

Experimental Group*	Serum level (Mean ± S.E.) (ng/ml)	Colon mucosa level (Mean ± S.E.) (ng/g)
	Pterostilbene	Pterostilbene
Control diet	N.D.**	N.D.**
Pterostilbene	48.0 ± 6.9	10.9 ± 3.8

*Pterostilbene (40 ppm) was administered in the diet starting at one day after the second AOM treatment and continuously thereafter for 45 weeks. Samples were randomly selected from the control (serum, n=10; colon mucosa, n=10) or pterostilbene fed groups (serum, n=8; colon mucosa, n=6) for analysis.

**N.D.: Not detectable.

PART IV

Evaluation of resveratrol and other stilbene analogs for their therapeutic efficacy against colon cancer

4.1. Introduction

Resveratrol and pterostilbene are natural phenolic stilbenes found in food of easy access to Americans such as grapes, berries and peanuts as well as in wine. The antiproliferative activity of resveratrol was observed in several human cancer cell lines, mainly by disturbing progression through S and G2 phases of the cell division cycle [302]. Cell cycle regulation and induction of apoptosis are key check points in the attempts aimed to control tumorigenesis. Naturally occurring stilbene analogs, resveratrol, piceatannol and pterostilbene has been identified to elicit these effects in several human cancer cells *via* a number of mechanisms which includes its ability to cause G1, S phase arrests in cell cycle, modulate the levels of cyclins and the cyclin dependent kinases and increase the cyclin dependent kinase inhibitor proteins of the Cip-Kip family [222,228,241,303,304].

There are numerous studies investigating the potential anticancer activity of resveratrol and its analogs against cancers of the colon. Resveratrol exhibits proapoptotic activities in several cell lines including human leukemia [305] and breast cancer [306]. Resveratrol significantly suppressed colon crypts in azoxymethane-induced aberrant colon crypt model [307]. Methoxylation has been suggested to improve the anti-tumor potential of compounds significantly. The more

the number of methoxy groups, the better the anti-tumor activity of the compound [308]. The synthetic stilbene analog *trans* 3,4,5,4'-tetramethoxystilbene (DMU-212) exhibited superior availability than resveratrol in the colon [309] and effectively reduced adenoma load in $Apc^{Min/+}$ mice [295]. 3,5,4'-trimethoxystilbene was identified to present greater tumor anti-tumor activity than the parent compound, resveratrol, in COLO 205 tumor xenografts [310]. From the foregoing results and because of our interest in discovering new natural product based anticancer agents, a comprehensive study was undertaken to evaluate the activity of a wide range of stilbenes with different side chains which included amino, nitro, methoxy, hydroxyl, halogen modifications. *Cis*-isomers were compared alongside their *trans* counterparts. Screening of the compounds for the potency was conducted in human HT-29 and Caco-2 colon cancer cell lines. In the light of the data collected from *in vitro* assay, a few analogues were selected to study their effects in HT-29 xenograft tumor growth in immunodeficient mice. Overall the present study was aimed in identification of pharmacologically active chemopreventive agents for the treatment of colon cancer.

4.2. Results

4.2.1. Pterostilbene exhibits greater effects against inflammation and cell proliferation compared to resveratrol in HT29 cells, and effects of the two stilbenes are mediated by separate pathways

Since pterostilbene is a naturally occurring analog of resveratrol, we first compared the inhibitory effects of pterostilbene and resveratrol against the induction of iNOS and COX-2 protein in this condition. At the concentration tested (30 μM), pterostilbene showed better inhibitory effect against induction of iNOS and COX-2 proteins than resveratrol (Fig. 4.1.). Also, the close structural similarity between the compounds leads us to believe that similar molecular pathways may be targeted by these compounds. We investigated whether that is the case here and so we analyzed the effect of pterostilbene and resveratrol on different pathways critical during inflammation (Fig. 4.2.). Interestingly, pterostilbene lowered p38 MAPK, and resveratrol at the concentrations used (30 μM) showed little activity against this pathway. Moreover, resveratrol was effective against p-STAT3, one of the molecules whose overexpression is known to be critical in CRC (Fig. 4.2.). On the other hand, pterostilbene did not lower the levels of p-STAT3 suggesting the significance and differential effects of substituent modifications to stilbene structure on its activity *per se*.

Against cell proliferation as discussed earlier, pterostilbene displayed greater potency with IC_{50} = 22.4 μM, when compared to resveratrol treatment (IC_{50} = 43.8

μM) under the same conditions (Fig. 3.5.B). Also pterostilbene lowered the levels of cyclin D1 to a greater extent than resveratrol at the same concentration of 50 μM (Fig. 4.3.). In case of c-MYC, another oncogenic protein involved in cell proliferation, we found that the activity of both the stilbenes were almost similar at 50 μM (Fig. 4.3. left panel). However, at 30 μM concentration, pterostilbene displayed superior inhibitory action against c-MYC compared to resveratrol (Fig. 4.3. right panel), suggesting that pterostilbene has greater inhibitory action than resveratrol on a specific pathway/pathways governing cell proliferation.

4.2.2. *In vitro* activity against HT-29 and Caco-2 colon cancer cells

Stilbenes **1**-**27** (Fig. 4.4.) were synthesized via Wittig reaction of different phosphonium salts and aromatic aldehydes, as reported elsewhere [311,312]. The activity of stilbenes **1-27** against HT-29 and Caco-2 cells is shown in Table 4.1. While most studies on stilbenes have focused on the *trans* isomers, it was interesting to observe that in this present study the *cis* isomers were, in general, the most active *in vitro* (Table 4.1). The stilbenes showed similar activity against HT-29 and Caco-2 cells except for **6,** which was very active against HT-29 cells (IC_{50} = 0.2 μM) but was weakly active against Caco-2 cells (IC_{50} = 14.71 μM). The *cis* trimethoxy stilbene derivative **10**, which has been reported as a naturally-occurring compound, was the most inhibitory among all the stilbenes tested (IC_{50} = 0.04 and 0.08 μM in HT-29 and Caco-2 cells, respectively). The majority of the compounds showed better activity than resveratrol (**17**) and pterostilbene (**16**), both of which

have been reported to prevent colon cancer development in animals. With the exception of the carboxylic acids **7** and **8**, desoxyrhapontigenins **13** and **14**, and fluorides **21** and **22**, where both isomers showed weak activity, the *cis* analogs of the diastereomeric pairs (**1** and **2**, **3** and **4**, **5** and **6**, **9** and **10**, **19** and **20**, **23** and **24**, **25** and **26**) had greater activity than the *trans* analogs. The compounds with dimethoxy substitution at C-3 and C-5 (**9**, **10** and **16**) had better inhibitory activity than the corresponding analogs with hydroxyl substitution at the same positions (**13, 14** and **17,** respectively). Notably, while dimethoxy substituted analog **10** was very potent against HT-29 and Caco-2 cells the hydroxylated analog **14** had relatively weak activity.

4.2.3. Anti-inflammatory action of stilbene analogs in HT29 cells

Figure 4.5. shows the expression levels of iNOS and COX-2 after cotreatment of HT-29 cells with cytokine mixture and stilbene analogs. Figure 4.5.A represents a set of compounds that were selected based on initial screening with western blot analysis. Among the different compounds tested **(1-14)**, stilbene compounds **3, 6,** and **10** lowered the iNOS and COX-2 induction and more importantly the *cis*–ester derivative **6** and *cis*-trimethoxy derivative **10** were very potent against COX-2 induction. A few studies have however shown better COX-2 inhibition for *trans* resveratrol compared to the *cis* isomer [313]. For iNOS inhibition, the presence of the dimethoxy group in the A ring has been observed to be crucial. Also, quinoline substitutions in the B ring showed better activity than pterostilbene in a study by Meng *et al.* [314]. Additional methoxy groups in stilbene structure increased the

activity against iNOS and COX-2 induction in our study, as observed for the most active trimethoxy derivative **10**. This may be mediated by increase in lipophilicity and corresponding bioavailability with increasing methoxy groups. We observed a dose-dependent inhibition for compounds **6** and **10** (Fig 4.5.B). The improved activity of **3, 6** and **10** against inflammatory enzymes is consistent with the growth inhibition data by [^{3}H]thymidine incorporation.

4.2.4. Anti-tumorigenic activity of stilbene analogs *in vivo* against HT-29 xenograft tumor growth

Based on results from the *in vitro* assays **4**, **6**, and **10** were selected for *in vivo* testing against HT-29 xenograft tumor growth in severe combined immunodeficiency (SCID) mice. Furthermore, in our interest to determine whether *cis-trans* isomerization occurs *in vivo*, the corresponding *trans* isomers (**3**, **5**, and **9**, respectively) were also tested in SCID mice. The amino derivatives **3** and **4** showed the best activity, resulting in mice with the lowest tumor weight and tumor volume. Both compounds decreased tumor weight by 40% after 3 weeks (Table 4.2). The ester derivatives **5** and **6** did not demonstrate antitumor effects against HT-29 xenografts. Compound **9** had better tumor inhibitory effect than its *cis* isomer **10**. Tumor weight was 21% lower and tumor volume was 45% lower in animals treated with **9** compared to the control. Tumor weight of animals treated with **10** was 15% lower than the control, but this effect was found to be not statistically significant.

4.2.5. Serum levels of the stilbenes

GC-MS analysis of the serum from mice treated with the *cis*-amino analog **4** revealed that this analog isomerized to **3**, based on the retention time (10.0 min) and mass spectrum (*m/z* 270, $[M]^+$) of the peak displayed, which was the same as that for the *trans*-amino analog **3**. The serum levels of **4** and **3** were also similar (Table 4.3). Stilbenes **5**, **6**, **9**, and **10** retained their configurations in the serum, as determined from their retention times and mass spectra, compared to standards. Stilbenes **6** and **10** demonstrated strong activity *in vitro*; however, both compounds did not demonstrate activity in SCID mice. This may be due to low levels of **6** and **10** in the serum (18.8 and 15.5 ng/mL, respectively). Stilbene **9** was found at a higher level in the serum (69.9 ng/mL). Thus, while only moderately active *in vitro*, this level may have been a sufficient concentration to provide tumor growth inhibition in SCID mice. Stilbene **5**, although found in relatively high levels in the serum was not active. This is observation is consistent with *in vitro* results, which showed only moderate inhibition. A higher serum concentration of **5** may be needed for an effective dose.

4.2.6. Effects of the stilbenes on cell proliferation and cell cycle proteins

Analysis of the tumors was conducted for the stilbenes that demonstrated reduction in tumor growth (i.e., **3, 4** and **9**). Figures 4.6 and 4.7 represent the effects of these stilbenes on the markers of cell proliferation by western blotting and immunohistochemistry, respectively. proliferating cell nuclear antigen (PCNA)

levels were lowered by **3, 4,** and **9**. p27 expression levels were upregulated by all the treatments as shown by western and immunohistochemical analysis and their presence was predominant in the nucleus. However, markers of apoptosis such as caspase-3 and PARP were unaltered by the compounds (data not shown).

Discussion

The present study was able to identify a few stilbenes that possess superior growth inhibition than either resveratrol or pterostilbene against HT-29 and Caco-2 colon cancer cells. Barring a few exceptions, the *cis* analogs demonstrated greater activity than the *trans* isomers *in vitro*, as indicated by the IC_{50} values. Compounds with amino, ester, and methoxy substitutions at the 4′ position of the B ring exhibited good anti-proliferative effects *in vitro*. However, the tumor growth inhibitory effect *in vivo* was different from *in vitro* observations for the six compounds tested in SCID mice. The *cis*-amino analog **4**, which showed only moderate activity *in vitro*, had the same effect as its *trans*-isomer **3** *in vivo*. This was because **4** isomerized to **3**, as shown from the analysis of the serum of mice administered with **4**. Compounds **3** and **4** were also detected at high levels in the serum, thus for these analogs *cis*-*trans* isomerization and bioavailability appeared to be important factors for *in vivo* activity. Stilbenes **5**, **6**, **9**, and **10** retained their configuration in the serum. The *cis* isomers **6** and **10** demonstrated strong activity *in vitro*, but did not inhibit tumor growth *in vivo*. The lack of activity may be due to low bioavailability, thus low levels of **6** and **10** in the serum. The serum levels of **9** (69.9 ng/mL) were higher than that of its *cis* isomer **10**; thus while **9** was only

moderately active *in vitro*, it demonstrated good activity *in vivo*. Stilbene **5**, although it was found in relatively high levels in the serum, did not show *in vivo* activity, consistent with effects observed *in vitro*. A higher serum level of **5** may be needed for *in vivo* activity. These data suggest that for **5**, **6**, **9**, and **10**, bioavailability of the compounds may have influenced their *in vivo* activity. While there is a discrepancy between *in vivo* and *in vitro* results, it must be noted that our *in vitro* study first provided leads for the selection of the compounds to be investigated *in vivo*. The *in vivo* studies have discovered **3** and **4** with better anticancer activity than **9**, which has been previously reported to have activity *in vivo* using COLO 205 tumor xenograft [310]. It is also worth noting that Pan et al. [310] used 50 mg/kg dose, while we showed that **9** reduced tumor growth at the dose of 10 mg/kg body weight. Moreover, although **3** and **4** have been evaluated *in vitro* against leukemia cell lines HL-60 [238] and nasopharyngeal carcinoma cell lines CNE-1 and CNE-2 [315], this is the first study to report the *in vitro* and *in vivo* activity of **3** and **4** against cancer of the colon.

Tumor analysis by Western blotting and immunohistochemistry suggests that the antitumor activity of the active stilbenoids **3, 4** and **9** may be associated with the effects predominantly on cell proliferation, as noted by lower expression of PCNA and cyclin D1 with significant increase in p27 protein. PCNA, being an auxiliary protein of DNA polymerase δ required for DNA synthesis during S-phase is a useful cell proliferation marker [291]. All the active compounds were shown to lower the % of PCNA labeled cells, although **4** treatment failed to show statistical

significance, the stilbene analogs proved to be effective in lowering cell proliferation. Cell cycle progression involves different proteins such as cyclins, cyclin dependent kinases (CDK) and the kinase inhibitors and the events that down regulate their expression or disrupt their interaction has significance in the regulation of cell cycle. p27, also known as kinase inhibitory protein (Kip 1) and related CDK inhibitor, p21 (CDK2 inhibitory protein 1 or CIP 1), regulate the Go-S transition in cell cycle [316]. These regulatory proteins when upregulated under certain conditions block the activation of CDKs by cyclins or can promote the assembly and nuclear import of cyclin D-CDK complexes [317]. Fig. 4.6. and Fig. 4.7. show that the treatment of the compounds **3, 4** and **9** increased the levels of these cell cycle inhibitors, and in particular very pronounced effects were observed with p27 expression. Immunohistochemistry shows nuclear localization and the compounds significantly increased the % of nuclei stained with p27. One of the earlier studies have identified p27 as an independent prognostic marker, particularly in stage 2 colon tumors [318]. Survival amongst patients with colorectal adenocarcinoma was up by approximately 60% for p27/Kip 1 positive patients compared to those who were negative [319].

Summary

Overall, in the present study on the different stilbenes, we screened various natural and synthetic analogs for their anti-proliferation effects against two different colon cancer cell lines. The *in vivo* administration of a few of these

stilbenes in immunodeficient mice with HT-29 xenograft tumors, further identified the more potent ones, **3, 4** and **9**. Interestingly, we also observed difference in efficacies between *in vitro* and *in vivo* systems. In summary, the significant lowering of the molecular markers involved in cell proliferation by the treatment with stilbenes, **3**, **4** and **9** underscores the relevance of further research of these interesting compounds and their consideration for treatment of human colorectal cancer.

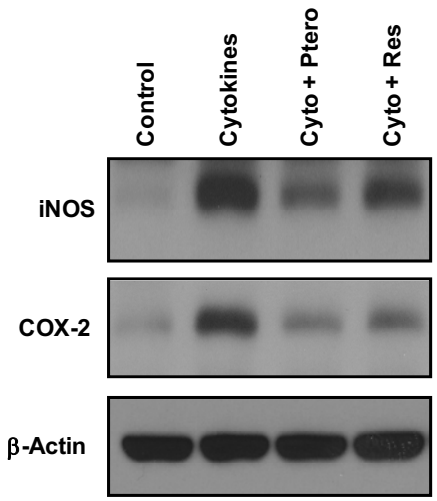


Fig. 4.1. Pterostilbene exhibits greater inhibition on the iNOS and COX-2 than resveratrol in HT-29 colon cancer cells. HT-29 cells (1.5×10^6 cells/ 100 mm dish) were treated with a mixture of TNF-α, IFN-γ and LPS (each at 10 ng/ml) (cytokines, cyto) in the presence or absence of different concentrations of pterostilbene (Ptero, 30 μM) and resveratrol (Res, 30 μM) for 15 h. The protein levels of iNOS and COX-2 were determined by Western Blot analysis.

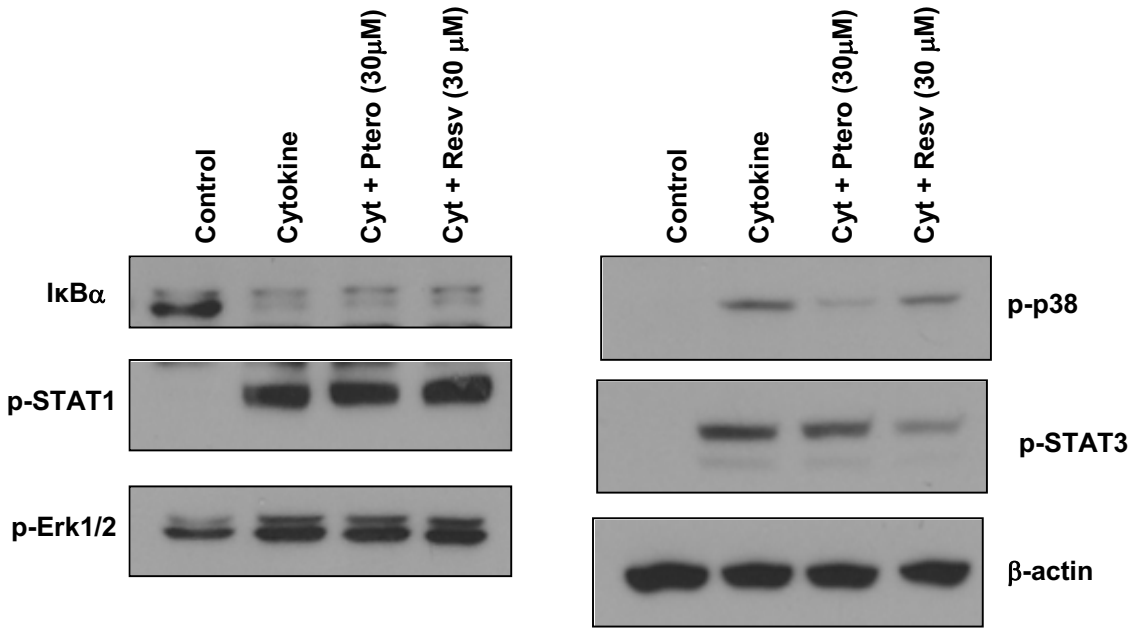


Fig. 4.2. Pterostilbene and the structurally similar resveratrol may act through different mechanisms in eliciting anti-inflammatory response. HT-29 cells (1.5×10^6 cells/ 100 mm dish) were treated with a mixture of TNF-α, IFN-γ and LPS (each at 10 ng/ml) (cytokines, cyt) in the presence or absence of pterostilbene (Ptero) and resveratrol (Resv) at 30 μM for 15 min. The cells were harvested, protein collected and immunoblotted.

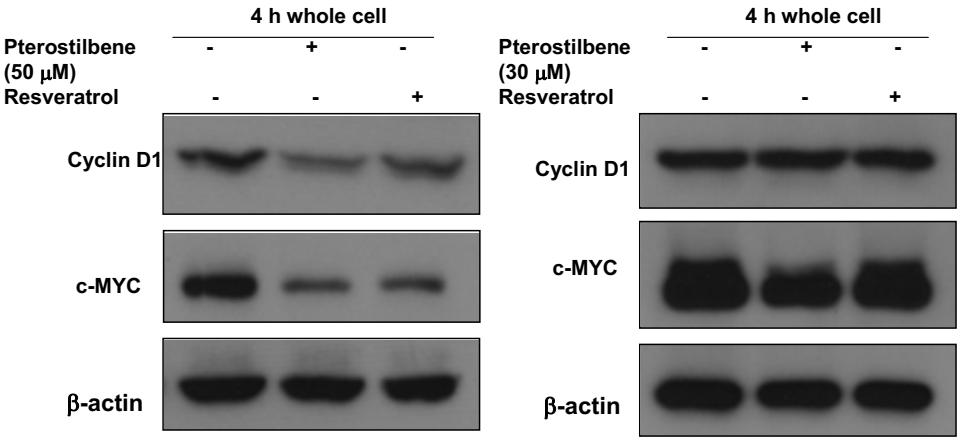
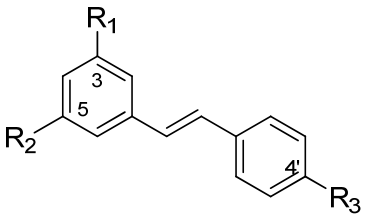
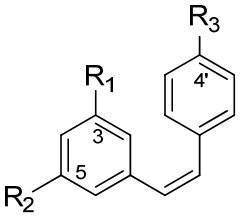


Fig. 4.3. Pterostilbene shows greater potential in lowering cyclin D1, c-MYC than resveratrol in HT-29 colon cancer cells. HT-29 cells (1.5×10^6 cells/ 100 mm dish) were treated with or without pterostilbene and resveratrol (Res) for 4 h. The concentration of pterostilbene and resveratrol used in the left panel is 50 μM, while 30 μM is used for the ones on the right hand side.





1 R_1= R_2= OMe, R_3= NO_2
3 R_1= R_2= OMe, R_3= NH_2
5 R_1= R_2= OMe, R_3= COOMe
7 R_1= R_2= OMe, R_3= COOH
9 R_1= R_2= R_3= OMe
11 R_1= OH, R_2= OMe, R_3= OH
12 R_1= R_2= OMe, R_3= OH (saturated on allylic position)
13 R_1= R_2= OH, R_3= OMe
15 R_1= R_2= OMe, R_3= OPO_3H
16 R_1= R_2= OMe, R_3= OH
17 R_1= R_2= R_3= OH
18 R_1= R_2= OMe, R_3= $OC_6H_{11}O_5$
19 R_1= R_2= OMe, R_3= Cl
21 R_1= R_2= OMe, R_3= F
23 R_1= R_2= OMe, R_3= Br
25 R_1= R_2= OMe, R_3= CF_3
27 R_1= R_2= OMe, R_3= SH

2 R_1= R_2= OMe, R_3= NO_2
4 R_1= R_2= OMe, R_3= NH_2
6 R_1= R_2= OMe, R_3= COOMe
8 R_1= R_2= OMe, R_3= COOH
10 R_1= R_2= R_3= OMe
14 R_1= R_2= OH, R_3= OMe
20 R_1= R_2= OMe, R_3= Cl
22 R_1= R_2= OMe, R_3= F
24 R_1= R_2= OMe, R_3= Br
26 R_1= R_2= OMe, R_3= CF_3

Fig. 4.4. Chemical structure of the stilbene analogs

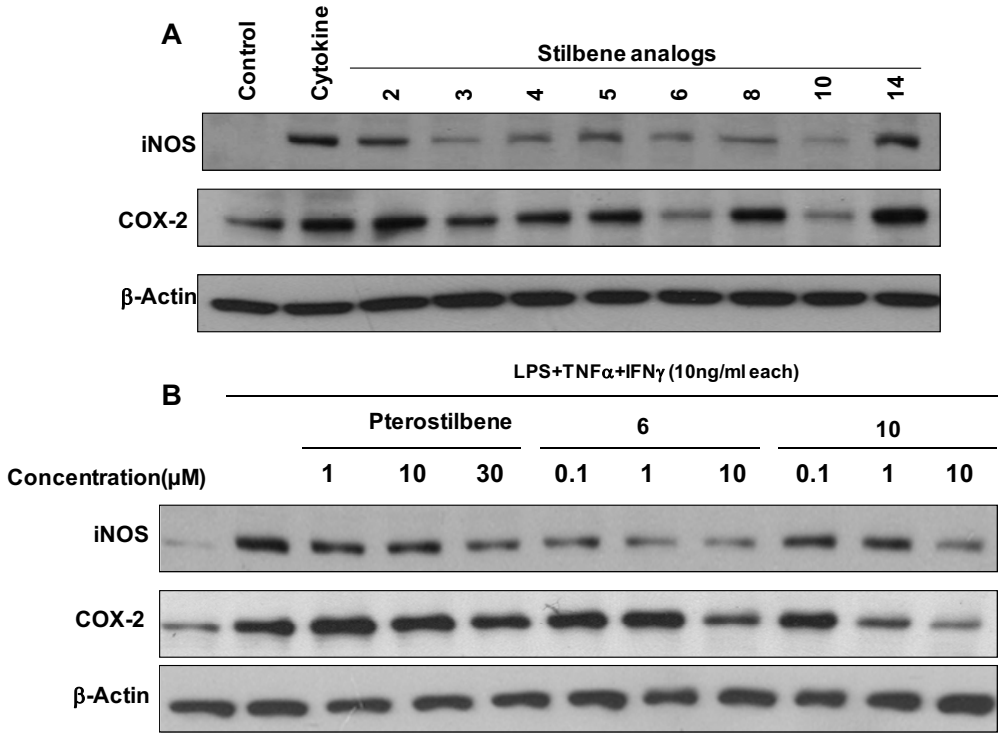


Fig. 4.5. Stilbene analogs exhibited greater anti-inflammatory action than pterostilbene against HT-29 colon cancer cells. A. Effects of analogs against iNOS and COX-2. HT-29 cells (1.5×10^6 cells/ 100 mm dish) were treated with a mixture of TNF-α, IFN-γ and LPS (each at 10 ng/ml) in the presence or absence of different concentrations of stilbene analogs for 15 h. The protein levels of iNOS and COX-2 were determined by Western Blot analysis. B. Pterostilbene, stilbene 6 and stilbene 10 blocks the induction of iNOS and COX-2 in a dose-dependent manner. HT-29 cells ($1.5X10^6$ cells/ 100 mm dish) were treated with a mixture of TNF-α, IFN-γ and LPS (each at 10 ng/ml) in the presence or absence of different concentrations of pterostilbene, #6 and #10 for 15 h. The protein levels of iNOS and COX-2 were determined by Western Blot analysis.

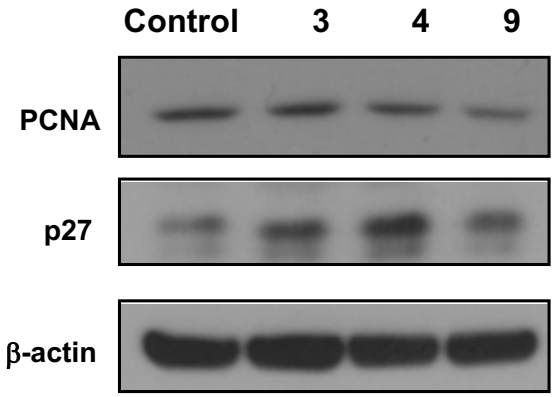


Fig 4.6. Effect of stilbenoids on molecular markers in HT-29 colon xenograft tumors. Tissue homogenates from individual mice in each treatment group were pooled together(n=6) and analyzed by western blotting for PCNA, p27 and β-actin was used as the loading control.

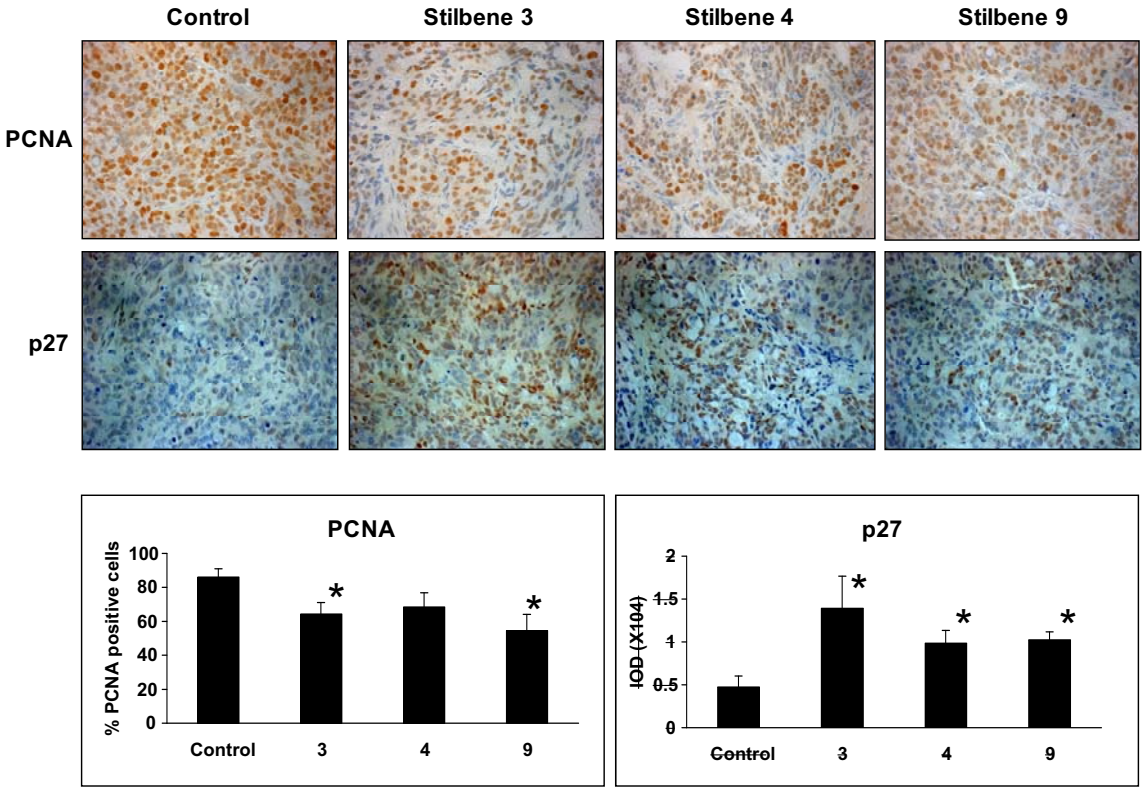


Fig. 4.7. Immunohistochemical staining of molecular markers in colon tumors and its quantification of staining intensity. A representative section of tumor tissue from the control group or groups treated with **3, 4** and **9** is shown a. PCNA: Positive cells in the nucleus (*brown*) and PCNA-negative cells (*blue*). Quantification to obtain PCNA labeling index (PI) was done as described under Materials and Methods. Data are represented as ± SE. Data are represented as ± SE. b. p27: Positive brown staining in the nucleus. * represents $p<0.05$

Table 4.1. IC_{50} values of stilbene analogs against HT-29 and Caco-2 colon cancer cells

Compound	IC_{50} (μM) against HT-29 cells	IC_{50} (μM) against Caco-2 cells
1	>30	22.87 ± 0.0
2	10.1 ± 0.0	18.43 ± 5.5
3	12.7 ± 2.0	19.57 ± 4.8
4	3.63 ± 0.8	2.61 ± 1.0
5	33.4 ± 4.6	42.64 ± 5.8
6	0.2 ± 0.05	14.71 ± 1.2
7	>30	>30
8	>30	>30
9	16.1 ± 5.0	11.95 ± 2.9
10	0.04 ± 0.01	0.08 ± 0.02
11	33.5 ± 2.4	19.21 ± 2.9
12	>30	>30
13	24.5 ± 5.5	>30
14	>30	>30
15	>30	19.59 ± 3.2
16	23.8 ± 3.1	14.46 ± 1.2
17	45.3 ± 4.4	24.35 ± 0.2
18	>10	>10
19	>10	>10
20	2.2 ± 1.0	7.1 ± 0.2
21	>10	>10
22	>10	>10
23	>10	>10
24	0.6 ± 0.01	0.59 ± 0.01
25	>10	>10
26	3.2 ± 0.7	2.95 ± 0.7
27	>10	>10

HT-29 or Caco-2 cells were seeded on a 24-well plate (20,000 cells/well) in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin. The cells were incubated

with stilbene analogs at different concentrations or with DMSO as the control vehicle for 3 days. One μCi of tritium labeled thymidine ([^{3}H]thymidine) per well was added and the amount of incorporated radioactivity in DNA was measured using a liquid scintillation spectrometer. The experiment was repeated twice, with each experiment done in duplicates. The IC_{50} values are represented as mean ± standard deviation.

Table 4.2. Tumor inhibitory effect of stilbene analogs against HT-29 xenograft in SCID mice

Treatment*	No. of animals	Body weight at autopsy (grams) (mean ± S.E.)	Tumor weight (grams) (mean ± S.E.)	Tumor volume (mm^3) (mean ± S.E.)
Control	10	20.4 ± 0.4	0.72 ± 0.1	1087.0 ± 138.9
3	8	19.4 ± 0.6	0.43 ± 0.0 (p=0.011)‡	661.4 ± 76.0 (p=0.020)‡
4	8	20.0 ± 0.5	0.44 ± 0.1 (p=0.015)‡	592.7 ± 84.5 (p=0.010)‡
5	4	19.5 ± 0.7	0.78 ± 0.1	1321.5 ± 275.9
6	8	20.0 ± 0.8	0.76 ± 0.1	1148.9 ± 95.5
9	8	19.5 ± 0.7	0.57 ± 0.1	590.3 ± 87.2 (p=0.014)‡
10	8	19.8 ± 0.3	0.61 ± 0.1	1023.6 ± 199.4

*HT-29 colon cancer cells were suspended in DMEM at a density of 10^7 cells/ml in a 1:1 vol/vol mix of DMEM Matrigel (BD Biosciences, Bedford, MA). Female SCID mice (7-8 week old) were inoculated subcutaneously in the hind flank with 0.1 ml (10^6 cells/animal) of cell suspension. Beginning the day after injection of the cells, the mice were treated with stilbenes intraperitoneally once daily for 3 weeks. The size of palpable lesions was measured twice a week with calipers. Tumors at autopsy were measured and weighed.

‡p is the value for the comparison of tumor weight and tumor volume in mice treated with stilbene analogs to the tumor measurements in control mice.

ξ Compounds **5**, **6**, and **10** were not detected in the sera.

Table 4.3. Serum levels of stilbenes in SCID mice[a].

Compound name (no.)	**Serum levels (ng/mL) means ± st. dev**
Control	0
(*E*)-4-(3,5-dimethoxystyryl)aniline **(3)**	99.0 ± 7.8
(*Z*)-4-(3,5-dimethoxystyryl)aniline **(4)**	109.1 ± 11.6[b]
(*E*)-methyl 4-(3,5-dimethoxystyryl)benzoate (**5**)	54.6 ± 2.4
(*Z*)-methyl 4-(3,5-dimethoxystyryl)benzoate (**6**)	18.8 ± 5.3
(*E*)-1,3-dimethoxy-5-(4-methoxystyryl)benzene **(9)**	69.9 ± 0.3
(*Z*)-1,3-dimethoxy-5-(4-methoxystyryl)benzene **(10)**	15.5 ± 3.4

[a] n = 4 for **4**, **5**, **6**, **9**, and **10**; n = 5 for **3**; n = 8 for control.

[b] Value represents that of the *trans* isomer (**3**). No *cis* isomer (**4**) was detected in the serum.

CONCLUSION

The project was able to identify the anti-tumorigenic action and the mechanisms involved in the observed activity of pterostilbene and also a few stilbene analogs against colorectal carcinogenesis in human colon cancer cells and in animal models for CRC. Using azoxymethane (AOM)-induced colon cancer model in rats, we found that pterostilbene diet at 40 ppm level significantly lowered the numbers of aberrant crypt foci (ACF) compared to the control diet. The stilbene downregulated the markers for cell proliferation and inflammation in the normal colonic crypts and in the ACF. A detailed mechanism based study in HT29 cells revealed the p38αMAPK pathway as a critical pathway in mediating the anti-inflammatory action of pterostilbene. A long-term study was conducted to identify the chemopreventive potential of the stilbene in reducing colorectal tumors. We observed that in azoxymethane injected rats fed pterostilbene in the diet at a very low dosage as 40 ppm of the diet lowered the tumor multiplicity. We observed marked reduction in β-catenin, cyclin D1, iNOS, COX-2 and p-p65 protein expression in the colons of rats from the pterostilbene group compared to those from the control group. Studies in HT29 cells showed that the stilbene lowered cellular β-catenin levels, which may account for, at least in part, to its anti-proliferation effects. We also identified a few stilbene analogs with amino and methoxy modifications to exert tumor suppressive action against the colon cancer cells and in the HT29 xenograft model in SCID mice. Overall, the data shows that pterostilbene and several

stilbene analogs hold great promise in the field of chemoprevention of CRC by dietary agents.

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RESEARCH PUBLICATIONS:

1. **Paul, S.**, Rimando, A. M., Lee, H. J., Ji, Y., Reddy, B. S., and Suh, N. Anti-inflammatory Action of Pterostilbene Is Mediated through the p38 Mitogen-Activated Protein Kinase Pathway in Colon Cancer Cells. Cancer Prev Res, *2:* 650-657, **2009**.
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