Investigation of Cancer Chemopreventive Activity of Polymeric Black Tea Polyphenol

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Title:

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Abstract:

Polymeric black tea polyphenols (PBPs) are the most abundant polyphenols present in black tea. We examined mechanisms of PBPs-mediated anti-initiating effects through modulation of transcription of CYP1A in mouse skin epidermis employing benzo(a)pyrene [B(a)P] as a model carcinogen. Topical pre-treatments with PBP-3 and PBP-mix resulted in significant inhibition of B(a)P-induced enzyme activity, mRNA and protein levels of CYP1A1/1A2 in mouse skin epidermis, whereas PBP-5 was not effective. Although topical pre-treatments with PBP-3 and PBP-mix alone did not alter the basal levels of aryl hydrocarbon receptor (AhR), they significantly decreased the B(a)P-induced AhR protein levels, its phosphorylation, nuclear translocation, heterodimerization with AhR nuclear translocator and subsequent binding to DNA, thereby diminishing the transactivation of CYP1A. In agreement with the observed effects on B(a)P-induced phase I enzyme, significant reduction of B(a)P-induced DNA adduct was observed. To conclude, PBP-3 and PBP-mix exhibit anti-initiating action via modulating the transcriptional regulator of Phase I enzymes in mouse skin.

Key Words: Aryl hydrocarbon receptor, Benzo(a)pyrene, cytochrome P450 1A, DNA adducts, Polymeric black tea polyphenols.

Brief description on the state of the art of the research topic:

Cancer is a complex group of diseases characterized by the loss of control of cellular growth leading to uncontrolled and abnormal proliferation of cells with potential to spread to other organs. Agents inducing cancer are known as carcinogens.

Cancer is a growing health problem around the world and major cause of death worldwide. Majority of human cancers are caused or mediated by the complex interactions between exogenous (environmental) and endogenous (genetic, hormonal and immunological) factors. Despite the improvements of medical technologies and therapeutic approaches, the mortality rates for cancer have not declined in the past 50 years (Harris, 1983; Tsao et al., 2004). Efforts to eliminate known human carcinogens like tobacco from the environment and current cancer treatment approaches have met with limited success. Based on the experience with some infectious and cardiovascular diseases, prevention of disease appears to be one of the achievable, cost effective and attractive approaches. Chemoprevention is thus, gaining considerable attention as a promising alternative strategy for the control of cancer (Cummings & Bingham,1998; Patel et al., 2007).

Cancer chemoprevention can be defined as the use of natural or synthetic compounds to prevent, suppress/delay, or reverse the develoPBP-mixent of invasive carcinoma (Gescher et al., 2001; Kumar et al., 2010). The scientific rationale for the use of cancer chemoprevention is based on the fundamental concept of multi-step carcinogenesis. Chemopreventive agents potentially act by inhibiting various stages of carcinogenesis like initiation, promotion and progression (Smith et al., 2005; Maru et al., 2016).

Amongst various chemopreventives, use of plant-derived anti-oxidants is fast becoming lucrative choice for cancer chemoprevention because of their low toxicity and high tolerability (Patel et al., 2007; Maru et al., 2016). Based on experimental and epidemiological evidences, chemoprevention or dietary intervention especially employing herbal anti-oxidant is receiving increasing attention as they have shown anti-initiating and/or anti-promoting activities in experimental system (Surh, 2003). Number of plant derived extracts like Epigallocatechingallate (green tea catechin), Curcumin, Resveratrol etc. have shown chemo-protective activities in vitro as well as in vivo (Azuine & Bhide, 1992; Thapiyal & Maru, 2001; Aziz et al., 2003; Kumar et al.,2010).

Tea is fast emerging as a potential chemopreventive beverages and green tea is now a well-established chemopreventive beverage with proven anti-initiating, anti-promoting and anti-progression activities, which are attributed to certain active flavanols like epigallocatechingallate (EGCG) etc. In contrast with green tea polyphenols, similar evaluations for polymeric black tea polyphenols (PBPs), the most abundant polyphenol present in black tea are limited partly due to their poor structural characterization (Kumar et.al., 2010).

The chemopreventive potential of PBPs have been established at both initiation and promotion stages of carcinogenesis. Their anti-initiation activities have been proven by its ability to protect against benzo[a]pyrene (B[a]P)-induced DNA adduct formation in vitro; 7,12-dimethylbenz[a]anthracene-induced DNA adducts in mouse skin (Krishnan and Maru 2004; 2005) and 1,2-dimethylhydrazine-induced colorectal carcinogenesis (Patel et al., 2005). But mechanisms of its anti-initiation actions are still not well elucidated. The effect of PBPs on carcinogen induced changes on phase II enzyme and its transcriptional factors in lung and liver of mice has been studied but its effect on phase I enzyme and its transcriptional factor is still remained unclear (Patel et al., 2008).

Definition of the problem:

The mechanism(s) of chemopreventive actions of tea-derived compounds are being extensively studied worldwide. Studies to understand the mechanisms of chemopreventive actions and commonality and/or differences in the observed mechanisms were planned and undertaken.It has been shown that PBPs act by decreasing activity of phase I enzyme and inducing Phase II enzyme. Nevertheless, it remained unclear as to how PBPs modulates phase I/II enzymes in the course of its action as an anti-initiating agent.

CYP450 1A isozymes and some detoxifying enzymes, in general, are regulated by a basic helix-loop-helix cytosolic protein, aryl hydrocarbon receptor (AhR). Upon ligand binding, AhR translocates to the nucleus (Garg et al., 2008), where it heterodimerizes with AhR nuclear translocator protein and binds to the xenobiotic response element (XRE) flanking CYP1A1 gene, thereby activating its transcription, which results in CYP 1A metabolism. The electrophilic intermediate formed as a result of metabolic activation of carcinogen interacts with cellular

biomolecules. This interactions leads to DNA damage, DNA-adduct formation and stress produced by DNA damage results in inflammation.

Hence, in the present study we aimed to delineate in vivo anti-initiating mechanisms of PBPs in skin by investigating the effects of PBPs on xenobiotic metabolizing enzymes such as CYP450 1A1/1A2 and detoxifying enzymes such as GST & NQO1 and their probable mechanism of action in mouse skin employing B(a)P as carcinogen. Further, effect of PBPs pre-treatment on DNA adduct formation, Inflammation and DNA damage was also exploited.

Scope of research work:

Dietary agents or herbal anti-oxidants due to low toxicity and relative safety have emerged as promising chemopreventive agents. These agents after emerging successful through a series of *in vitro* and *in vivo* assay enter clinical trials. However, in clinical trials these compounds have met with limited success.

This emphasizes the need for further detailed research on the mechanisms of observed chemoprevention and choice, dose, duration and bioavailability of chemopreventive agent used. Chemoprevetive agents act by inhibiting process of carcinogenesis at initiation and/or promotion/progression stage. Cellular metabolism of carcinogen entering cellular environment plays a very critical role in the process of initiation during carcinogenesis (Maru et al., 2016) Xenobiotics are metabolized by Phase I and Phase II enzymes. Benzo[a]pyrene (B[a]P), a well-known ubiquitous carcinogen belonging to polycyclic aromatic hydrocarbons group of compounds, is metabolically activated by CYP1A class of cytochrome P450 (CYP450) enzymes to form a highly mutagenic reactive electrophile, benzo[a]pyrenediol-epoxide (BPDE) (Niranjan et al., 1984). Though phase II enzymes catalyze the detoxification of BPDE, some of the reactive electrophile interacts covalently with DNA to form adducts. Unrepaired/ misrepaired adduct leads to mutation in genes involved in proliferation, growth and apoptosis and finally to a disease condition like cancer. This implicates that phase I and II enzymes play an important role in carcinogen metabolism and hence could be an important target for chemoprevention. Thus, any agent, which can block pro-carcinogen activation and induce detoxification of carcinogen in course of their mechanism of action, can act as promising chemopreventive agent. PBPs, are structurally

and chemically ill-defined heterogeneous polymers of flavan-3-ols and flavano-3-ol gallates with di- and tribenzotropolone skeletons (Haslam, 2003; Krishnan & Maru, 2006). This study was undertaken to delineate anti-initiation mechanism of PBPs.

Goal and Objectives:

Hence, in the present study we aimed to investigate the effects of pre-treatment with topical application of chemopreventive doses of PBPs on xenobiotic metabolizing enzymes such as CYP450 1A1 and 1A2 as well as detoxifying enzyme like GST, NQO1 and their probable mechanism of action in mouse skin employing B(a)P as a model carcinogen.

(1) Standardization of Dose and Duration of the application of Carcinogen for the induction of Aryl hydrocarbon Receptor (AhR) in mouse skin.

(2) Isolation and fractionation of Polymeric black tea polyphenols (PBPs) by using soxhlet continuous extraction.

(3) To study the efficacy of Polymeric black tea polyphenol mixture in Benzo (a) pyrene treated mouse skin.

(4) To study the effect of Various PBPs and PBP mixture on transcriptional regulators of phase I enzyme in Benzo (a) pyrene treated mouse skin.

(5) To study the effect of Various PBPs and PBP mixture on phase II enzyme in Benzo (a) pyrene treated mouse skin.

(6) To study the effect of Various PBPs and PBP mixture on DNA adduct formation, inflammation related bio-markers and different MAP kinases in Benzo (a) pyrene treated mouse skin.

Methodology of research, Results/Comparisons:

(1) Standardization of Dose and Duration of the application of Carcinogen for the induction of Aryl hydrocarbon Receptor (AhR) in mouse skin.

Methodology:

All animal studies were conducted after approval from the Institutional Animal Ethics Committee as per the Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India guidelines. S/RV/Cri-ba or ‘‘Swiss bare’’ mice-hairless mutants that are highly susceptible to skin tumorigenesis (Bhisey et al., 1987) – were obtained from the animal colony of the Advanced Centre for Treatment, Research and Education in Cancer (India). Swiss bare mice (6-8 weeks old) were randomized into 3 groups. In Group 1, 2 and 3 100 µl of acetone was applied on dorsal skin of mice for 3 days. In group 2, on 3rd day 1mg B(a)P in 100 µl acetone was applied after 20 mins of acetone application, whereas, in group 3 1mg B(a)P in 100 µl acetone was applied for 3 consecutive days after 20 mins of acetone application. After 24 hour of last application animals were sacrificed and skin was collected. Total cell lysates were prepared from the epidermis by cell fractionation protocol as previously described (Afaq et al., 2004). Protein levels of AhR were measured by immunoblotting using anti-AhR.

Result:

Animals challenged with single dose of B(a)P 1 mg in 0.1 ml acetone on 3rd day of experiment showed significant induction of AhR protein as compared to vehicle treated animals. There was no significant induction of AhR found in mice treated with B(a)P for 3 consecutive days as compared to vehicle control mice. SO Animals were treated with single application of 1 mg B(a)P in 100 µl acetone after 20 mins of acetone application on 3rd day of experiment for further study.

(2) Isolation and fractionation of Polymeric black tea polyphenols (PBPs) by using soxhlet continuous extraction.

Methodology:

Five PBP fractions were isolated from a popular brand of black tea powder (Brooke Bond Red Label, India) in our laboratory, employing a Soxhlet extractor (Krishnan and Maru, 2006). Briefly, black tea powder was serially extracted in a Soxhlet extractor with chloroform, ethyl acetate and n-butanol. The ethyl acetate extract yielded PBP-1, whereas the n-butanol extract yielded PBP-2 and PBP-3 after treatment with methanol and acetone, respectively. The tea powder residue was then brewed with distilled water, the resultant solution acidified with sulfuric acid and extracted with n-butanol to obtain PBP-4 and PBP-5 after treatment with methanol and acetone, respectively. The PBP fractions were confirmed to be free from other biologically active black tea derived contaminants such as caffeine, catechin(s), and theaflavins by using thin layer chromatography.

Result:

The yields of PBP-1, PBP-2, PBP-3, PBP-4 and PBP-5 were 2.68%, 3.79%, 1.34%, 2.20% and 0.32%, respectively. PBP fractions did not show any presence of other known biologically active, such as free catechins, theaflavins or caffeine, known to be present in black tea, and PBPs were retained at the origin in thin layer chromatography (TLC) analysis, showing strong solid matrix reactivity. All the fractions were mixed in the same ratio in which they obtained to prepare PBP-mixture, which was used for further studies. From previous studies, established by Kumar et al., 2012 in TPA-induced skin carcinogensis model, PBP-3 being most effective and PBP-5, being least effective were selected to carry out further experiment for better comparison of results.

(3) To study the efficacy of Polymeric black tea polyphenol mixture in Benzo(a)pyrene treated mouse skin.

Methodology:

All animal studies were conducted after approval from the Institutional Animal Ethics Committee as per the Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India guidelines. S/RV/Cri-ba or ‘‘Swiss bare’’ mice-hairless mutants that are

highly susceptible to skin tumorigenesis (Bhisey et al., 1987) - were obtained from the animal colony of the Advanced Centre for Treatment, Research and Education in CancerMumbai (India). Swiss bare Mice (6–8 weeks old) were housed in polypropylene cages, maintained under standard conditions of 22 ± 2°C, 45 ± 10% relative humidity and 12 h light–dark cycles and were provided with standard pelleted diet and plain drinking water ad libitum. All animals were randomized into 8 different treatment groups (each group with at least five animals) . Animals from group 1 were treated topically onto the dorsal skin with 0.1 ml acetone (Vehicle) alone per day for consecutive 3 days and group 2, 3 and 4 with PBP-5, PBP-3 and PBP-mix (200 µg) in 0.1 ml acetone respectively served as the respective controls. Animal belonging to group 5 were topically pre-treated with 0.1 ml acetone alone for 3 days but on 3rd day 20 mins after application of 0.1 ml Acetone, B(a)P (1 mg) in 0.1 ml acetone was applied. Mice in group 6, 7 and 8 were topically pre-treated with PBP-5, PBP-3 and PBP-mix (200 µg) in 0.1 ml acetone and on 3rd day B(a)P (1 mg) in 0.1 ml acetone was applied after 20 mins of PBP-5, PBP-3 and PBP-mix application (Fig. 1). Mice were sacrificed by cervical dislocation after 24 hrs. of last application and their dorsal skin was excised. A part of the skin was fixed in 10% buffered formalin for histological analysis. Histopathological analysis was performed on formalin fixed, paraffin-embedded 5 µm tissue sections and stained with haematoxylin and eosin.

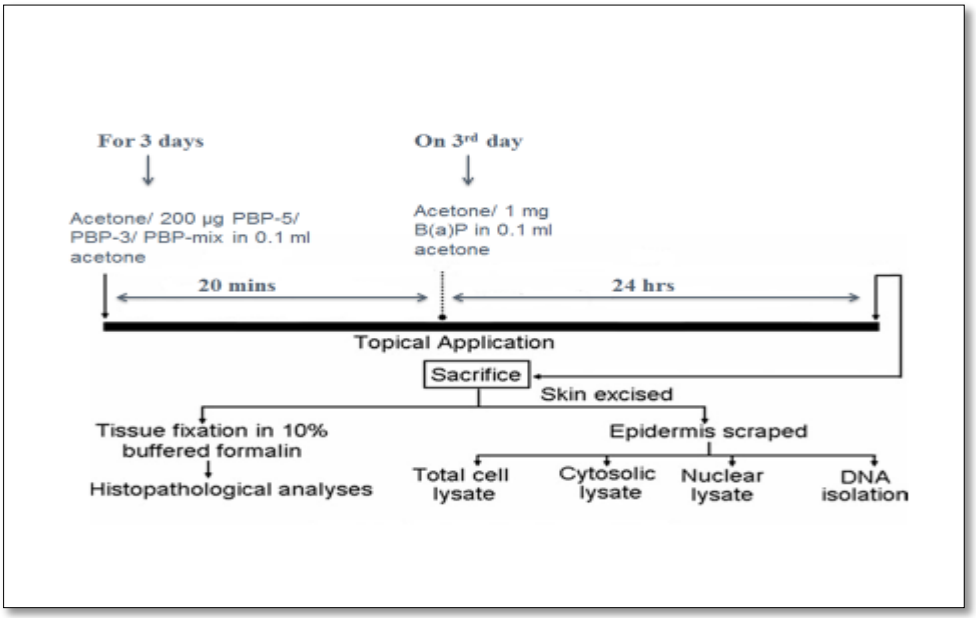


Figure 1. Experimental Plan

Result:

Histopathological observations indicated that although the topical applications of PBP-5, PBP-3 and PBP-mix alone did not induce significant epidermal hyperplasia in mouse skin, their pre-treatments, markedly prevented the B(a)P-induced hyperplasia as seen by relatively less number of cell layers when compared to B(a)P treated skin.

(4) To study the effect of Various PBPs and PBP-mixture on transcriptional regulators of phase I enzyme in Benzo(a)pyrene treated mouse skin.

Methodology:

Mice were divided into 8 different groups and treated as described in (3). Mice were sacrificed by cervical dislocation after 24 hrs. of last application and their dorsal skin was excised. A part of the skin was fixed in 10% buffered formalin for histological analysis, whereas rest of the tissue was stored at -80°C. Tissues of animals belonging to the various treatment groups were washed with acetone since this facilitates the removal of maximal color of PBPs from the skin before separation of the epidermis. This was done to avoid color interference in protein determination and further study. The epidermis was gently separated from the skin using a Watson skin grafting knife with suitably adjusted cutting angle. The separated epidermal tissue was used to prepare different cell fraction lysates by homogenization.

Densitometry and quantitative analysis of images were performed using Image J 1.43 (NIH) software. Statistical analysis was performed using Graph Pad Prism 5.3. Data are presented as mean ± SE. Differences among groups were determined by one-way ANOVA followed by Bonferroni's test. $p \leq 0.05$ was considered statistically significant.

4 (a) Effect of topical pre-treatments with PBP-3, PBP-5 and PBP-mix. on B(a)P-induced activities and levels of Phase I enzyme (CYP 1A1/1A2) in mouse skin epidermis.

Methodology:

The effect of topical pre-treatments with PBP-3, PBP-5 and PBP-mix was studied on B(a)P-induced Phase I CYP450 isozymes CYP1A1 and CYP1A2, which are involved in the metabolic activation of polycyclic aromatic hydrocarbons (PAHs).were measured as resorufin formed from

isozyme specific probe drugs-ethoxyresorufin and methoxyresorufin by spectrofluorimetry. Microsomal and cytosolic fractions of skin epidermal cells prepared by differential centrifugation were used for measuring activities of CYP1A1 and 1A2 employing isozyme-specific probe drugs-ethoxyresorufin and methoxyresorufin, respectively, as detailed (Pohl et al., 1980). Activity measurements were further confirmed at the protein levels by immunoblotting using isozyme-specific antibodies and at mRNA levels by RT-PCR using specific primer sequences, wherein β-actin was used as the housekeeping gene. In absence of a well-accepted standard internal control quantitative analysis of CYP1A1/1A2 immunoblot were done by normalizing the band intensity with a prominent band visualized on membrane after fast green staining of transferred blot.

Result:

CYP 450 isozymes 1A1/1A2 catalyzes the oxidative metabolism of xenobiotics (Patel et al., 2007). Importantly, topical pre-treatments with PBP-mix and PBP-3 showed significant decrease in B(a)P-induced activities of CYP 1A1/1A2 in mouse skin epidermis, whereas PBP-5 did not show any significant effect. However, Topical treatments with PBP-3, PBP-5 and PBP-mix alone did not alter the basal activities of CYP 1A1/1A2 in mouse skin epidermis. The observed PBP-mix and PBP-3 mediated decrease in activity was further complemented by the decrease in B(a)P-induced protein as well as mRNA expressions of CYP 1A1/1A2 in mouse skin epidermis upon topical pre-treatments with PBP-mix and PBP-3. These observations thus suggest that topical PBP-mix and PBP-3 mediates inhibition of carcinogen-induced CYP450s at the transcriptional level. Thus, PBPs decreases the metabolism of B(a)P as previously seen in lung and liver of mice (Krishnan et al., 2005)

4 (b) Effect of topical pre-treatments with PBP-3, PBP-5 and PBP-mix. on the transcriptional regulation of Phase I enzyme (CYP 1A1/1A2) in mouse skin epidermis.

Methodology:

Total cell, cytosolic and nuclear extracts were prepared from the epidermis by cell fractionation protocol as previously described (Afaq et al., 2004). Transcriptional regulation of CYP 1A class of phase I enzyme is mediated by a ligand-dependent transcription factor Aryl hydrocarbon receptor (AhR) via xenobiotic response element (XRE) (Patel et al., 2007). Hence, to investigate the mechanism(s) of observed PBPs-mediated inhibition of B(a)P-induced CYP1A1/1A2, effects

of topical pre-treatments with PBP-3, PBP-5 and PBP-mix were studied on the DNA-binding ability of their transcription regulator (AhR) in mouse skin epidermis nuclear extracts, by electrophoretic mobility shift assay (EMSA). In addition, the effect(s) of topical pre-treatments with PBP-3, PBP-5 and PBP-mix were studied on the cellular distribution of AhR by assaying its protein levels in total cell, cytosolic and nuclear extracts from mouse skin epidermis. β-actin, Tubulin and Histone H1 were used as loading controls in each of the respective cellular compartments. Furthermore, the effect(s) of topical pre-treatments with PBP-3, PBP-5 and PBP-mix on protein levels of AhR related proteins like HSP90 and XAP-2 were also studied in total cell extract by immonublotting. Levels of AhR phosphorylation was measured in mouse skin epidermis by immunoprecipitating the p-serine/p-threonine residues in total cell lysate and subsequently, analyzing the levels of phosphorylated AhR by immunoblotting using anti-AhR. Effect of topical pre-treatments with PBP-3, PBP-5 and PBP-mix on binding of AhR:ligand complex to AhR nuclear transporter (Arnt) was measured in mouse skin epidermis by immunoprecipitating Arnt in nuclear extract and subsequently, analyzing the levels of AhR bound to Arnt by immunoblotting using anti-AhR.

Result:

To further delineate the mechanism of observed PBPs-mediated decrease of B(a)P-induced CYP1A1/1A2 , effect(s) of topical pre-treatments with PBP-3, PBP-5 and PBP-mix were studied on their transcriptional regulator AhR. Significant decrease in B(a)P-induced AhR-DNA binding was observed in nuclear extracts of mouse skin epidermis upon topical pre-treatments with PBP-mix and PBP-3, employing EMSA. Importantly, topical pre-treatments with PBP-mix and PBP-3 also resulted in significant decrease in B(a)P-induced new synthesis of AhR protein as well as AhR nuclear translocation and binding of AhR:ligand to Arnt, thus accounting for the observed PBP-mix and PBP-3 mediated decrease in AhR-DNA binding and subsequent CYP1A transcription/ transactivation. PBPs did not show any significant effect on levels of HSP90 and XAP-2 in mouse skin epidermis. It was interesting to observe that topical-pre-treatment with PBP-mix and PBP-3 significantly decreased the B(a)P-induced AhR phosphorylation in mouse skin epidermis, an event essential for the transformation of liganded AhR to a DNA-binding form. However, PBP-5 was observed to be ineffective at all the stages.

Previous studies have demonstrated that green and black tea extracts suppressed AhR transformation in vitro (Fukuda et al., 2004; Fukuda et al., 2005). This is the first report showing that PBPs suppresses AhR transformation in vivo.

(5) To study the effect of Various PBPs and PBP- mixture on phase II enzyme in Benzo(a)pyrene treated mouse skin.

Methodology:

Effect(s) of topical pre-treatments with PBP-3, PBP-5 and PBP-mix on the activity and levels of Phase II enzymes such ass Glutathione-S-Transferase (GST) and NAD(P)H quinone oxidoreductase-1 (NQO1) in mouse skin epidermis. Activities of GST and NQO1 were measured spectrophotometrically (Habig et al., 1974) in cytosols prepared from mice belonging to the different treatments groups as in (4). Activity measurements were further correlated with the protein levels, measured in cytosols by SDS-PAGE and immunoblotting employing ECL detection kit followed by quantitation; β-actin was used as loading control. GST and NQO1 induction was also confirmed at RNA level via RT-PCR analysis with specific primer sequences. β-actin was employed as the housekeeping gene.

Result:

During the course of xenobiotic metabolism, Phase I enzymes predominantly CYP450 metabolize the xenobiotics such as B(a)P to more reactive electrophilic moieties, which in turn are detoxified by Phase II enzymes. Results in the present study showed that topical pre-treatments with PBP-3 and PBP-5 did not show any significant effect on activities and protein levels of GST and NQO1. Whereas, PBP-mix significantly increases the enzyme activities and protein levels of GST and NQO1 in mouse skin epidermis as compared to controls. PBP-mix –mediated enhancement of GST and NQO1 activities and protein expressions were also confirmed at the mRNA levels by RT-PCR analysis in mouse skin epidermis. Results thus, suggest the transcriptional regulation of Phase II enzymes by PBP-mix.

(7) To study the effect of Various PBPs and PBP mixture on DNA adduct formation, inflammation related bio-markers and different MAP kinases in Benzo (a)pyrene treated mouse skin.

Methodology:

Effect(s) of topical pre-treatments with PBP-3, PBP-5 and PBP-mix on B(a)P-induced DNA adducts, Oxidative damage, Stress responsive MAP kinases and inflammatory markers in mouse skin epidermis.B(a)P derived DNA adducts were analyzed by immunohistochemical staining in formalin-fixed, Paraffin embedded 5µm thick sections of mouse skin [treated as in (4)] using monoclonal antibody recognizing BPDE-DNA adducts. BPDE-DNA adducted nuclei (indicated by brown colour) were counted in 5 different randomly selected fields of epidermis in tissue section. Besides, B(a)P-induced damage was measured in terms of oxidative lesion, 8-OH-dG, in DNA isolated from mouse skin epidermis belonging to various treatment groups by ELISA. Likewise, the B(a)P-induced inflammation was determined by measuring levels of PGE@ by enzyme-immuno assay and also protein levels of COX-2 were measured in total cell extract by immunoblotting. Furthermore, levels of stress-responsive MAP kinases (ERK, JNK and PBP-38) were studied in total cell extracts prepared from mouse skin epidermis, by immunoblotting using antibodies specific to total form as well as phosphorylated form of these kinases.

Result:

Topical pre-treatments with PBP-mix and PBP-3 resulted in significant decrease in B(a)P-derived DNA adducts in mouse skin epidermis. Furthermore, Topical pre-treatments with PBP-mix and PBP-3 significantly decreased the B(a)P-induced oxidative damage in mouse skin epidermis. This could be either due to the observed PBPs-mediated decrease in B(a)P-induced CYP450s or free radical scavenging activity of PBPs. In addition, Topical pre-treatments with PBP-mix and PBP-3 showed significant decreases in B(a)P-induced PGE2 levels as well as COX-2 expression in mouse skin epidermis, suggestive of decreased inflammation, usually associated with stress and DNA damage. Topical pre-treatments with PBP-mix and PBP-3 also resulted in significant abrogation of B(a)P-induced levels of phosphorylated MAP kinases (ERK, JNK, PBP-38) in mouse skin epidermis although the extent of decrease varied for each of the kinases.

Achievements with respect to Goal and Objectives:

Topical Pre-treatment of mice with PBP-3 and PBP-mix showed significant inhibition of B(a)P-induced-enzyme activity, protein level and mRNA levels of cytochrome P450 1A1 and 1A2 They significantly decreased the B[a]P-induced AhR protein synthesis, nuclear translocation and subsequent binding to DNA and Arnt. Pre-treatment of PBP-3 and PBP-mix also significantly inhibit B(a)P-induced DNA adduct formation, level of PGE2, COX-2 and 8-oh-dG in mouse skin. Interestingly PBP-mix also significantly induced enzyme activity, protein levels and mRNA levels of detoxifying enzyme GST and NQO1

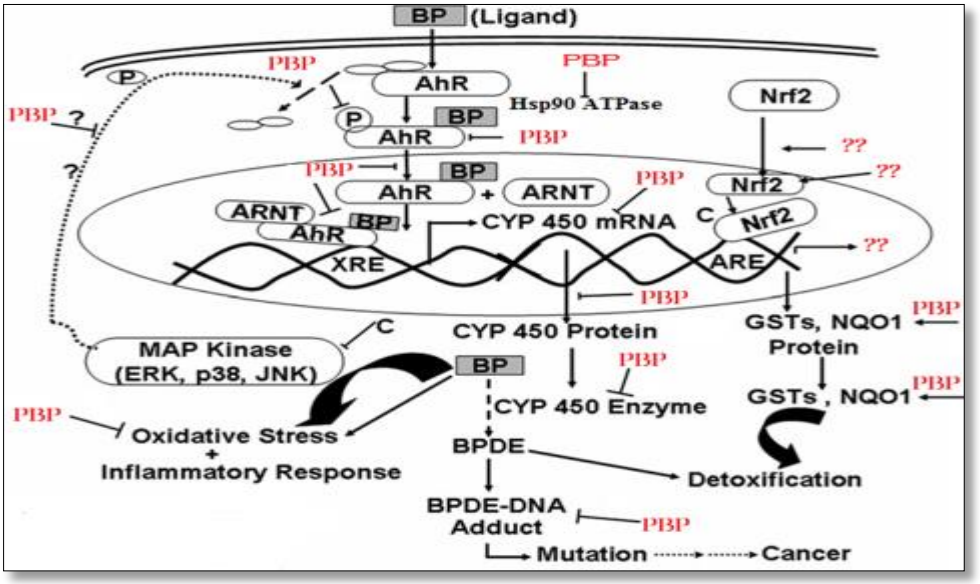
Discussion:

It has been shown that phase I and II enzymes play an important role in carcinogen metabolism and hence could be an important target for chemoprevention. In this study it has been reported that PBP-3 and PBP-mix inhibit B(a)P-induced activity, protein and mRNA levels of CYP1A1/1A2. Previous studies have demonstrated that green and black tea extracts suppressed AhR transformation in vitro (Fukuda et al., 2004; Fukuda et al., 2005). It has also been reported that Polyphenols derived from plant foods, such as flavonoids including catechins, curcumin, and resveratrol, suppress the transformation in vitro (Fukuda et al., 2004; Ciolino et al., 1998; Ciolino & Yeh, 1999). In present study, we observed that topical pre-treatments with PBP-mix and PBP-3 decreased B(a)P-induced AhR Transformation by inhibiting nuclear translocation of AhR, AhR-Arnt binding and phosphorylation of AhR. This decreased AHR-ligand binding to Arnt and decreased nuclear translocation of AhR may be responsible for the observed inhibitory effect of PBP-mix and PBP-3 on AhR–DNA binding and subsequent CYP1A transactivation. PBP-mix significantly induced levels of GST and NQO1, which supports observed decrease in B(a)P-induced DNA adduct formation. Further, Several lines of evidence indicate that phosphorylation of AhR is one of the crucial events required in transformation of liganded AhR to DNA-binding form (Pongratz et al., 1991). Results herein indicate reduction in B(a)P-induced AhR phosphorylation by topical pre-treatments with PBP-mix and PBP-3 in mouse skin epidermis. It has been previously shown that topical application of black tea polyphenols consisting of theaflavine gallates and EGCG, inhibits TPA-induced epidermal hyperplasia and inflammatory

markers COX-2 and PGE2 in SENCAR mice skin (Katiyar et al., 1997). In our study, we observed inhibition of B(a)P-induced hyperplasia by topical pre-treatments with PBP-3 and PBP-mix in mouse skin epidermis. Topical pre-treatments with PBP-mix and PBP-3 also diminished B(a)P-induced levels of COX-2 and PGE2. In present study, topical pre-treatments with PBP-mix and PBP-3 significantly abrogated the B(a)P-induced levels of 8-OH-dG in mouse skin epidermis, thus perhaps protecting against B(a)P-induced electrophilic/oxidative stress. These results collectively suggest that decreased inflammation usually associated with stress and DNA damage. To summarize, topical pre-treatments with PBP-mix and PBP-3 inhibit carcinogen-induced CYP450 isozymes transcription by modulating the transcriptional regulator of CYP1A and decrease the carcinogen induced DNA damage (BPDE adduct and oxidative DNA lesion), thereby further reducing the carcinogen-induced inflammatory and stress response to exhibit its anti-initiating activity.

Conclusions:

Polymeric Black Tea polyphenols showed significant Chemopreventive activity. They exhibit their anti-initiation activity via modulation of AhR, which is transcriptional regulator of phase-I enzyme and subsequently by decreasing DNA adducts, inflammation and oxidative damage.



Schematic presentation of possible steps at which PBPs exhibits anti-initiating effects.

References:

Afaq F., Saleel M., Aziz M. H., Mukhtar H. Inhibition of 12-O-tetradecanoylphorbol-13-acetate-induced tumor promotion markers in CD-1 mouse skin by oleandrin. *Toxicology and applied pharmacology.,* 2004, **195;** 361-369.

Aziz M.H., Kumar R. and Ahmad N. Cancer chemoprevention by resveratrol: in vitro and in vivo studies and the underlying mechanisms. *Int. J. Oncol*., 2003, 23;17-28

Azuine M. A., Bhide S. V. Chemopreventive effect of turmeric against stomach and skin tumors induced by chemical carcinogens in Swiss mice. *Nutrition and cancer*.,1992, **17;** 77-83.

Bhisey R. A., Veturkar P. L., Borges A. Comparison of sensitivity of Swiss albino and hairless Swiss bare mice to two stage skin tumorigenesis. *Indian J Exp Biol.,* 1987, **25;** 90-96.

Ciolino H.P., Daschner P.J., Wang T.T.Y. and Yeh G.C., Effect of curcumin on the aryl hydrocarbon receptor and cytochrome P450 1A1 in MCF-7 human breast carcinoma cells. *Biochem. Pharmacol*., 1998,**26**; 1754-1760.

Ciolino H.P. & Yeh G.C., Inhibibition of aryl hydrocarbon-indued cytochrome P-450 enzyme activity and CYP1A1 expression by resveratrol. *Mol.Oharmacol*., 1999,**56**; 760-767

Cumming J.H. and Bingham S.A. Diet and prevention of cancer. *Br. Med. J.,* 1998, **317**; 1636-1640.

Fukuda I., Sakane I., Yabushita Y., Kodoi R., Nishiumi S., Kakuda T., Sawamura S., Kanazawa K. and Ashida H., Pigments in green tea leaves (Camellia sinesis) suppress transformation of the aryl hydrocarbon receptor induced by dioxin. *J. Agric. Food Chem*., 2004, **52**; 2499-2506.

Fukuda I., Sakane I., Yabushita Y., Kodoi R., Nishiumi S., Kakuda T., Sawamura S., Kanazawa K. and Ashida H.,Black Tea Theaflavins suppress dioxin-induced transformation of the aryl hydrocarbon receptor. *Biosci. Biotechnol. Biochem.,* 2005, **69(5)**; 883-890

Garg R., Gupta S., Maru G. B. Dietary curcumin modulates transcriptional regulators of phase I and phase II enzymes in benzo[a]pyrene-treated mice: mechanism of its anti-initiating action. *Carcinogenesis.,* 2008, **29;** 1022-1032.

Gescher A.J., Sharma R.A. and Steward W.P. Cancer chemoprevention by dietary constituents: a tale of failure and promise. *Lancet Oncol.,* 2001,2;371-379.

Habig W.H., Pabst M.J. and Jakoby W.B., Glutathione S-transferase. The first step in mercapteuric acid formation. *J. Biol. Chem*., 1974, **249**; 7130-7139

Harris C.C. Concluding remarks: role of carcinogens, co-carcinogens and host factors in cancer risk, in human carcinogenesis, eds. By Harris CC and Autrup H, Academic press, New York, 1983, pp. 941-970.

Haslam, E. Thoughts on thearubigins. *Phytochemistry, 2003,* **64;** 61-73.

Katiyar S., Mukhtar H. Tea in chemoprevention of cancer. *International journal of oncology*, 1996, **8;** 221-238.

Krishnan R., Maru G. B. Inhibitory effect(s) of polymeric black tea polyphenol fractions on the formation of [(3)H]-B(a)P-derived DNA adducts. *Journal of agricultural and food chemistry,* 2004, **52;** 4261-4269.

Krishnan R., Maru, G. B. Inhibitory effect(s) of polymeric black tea polyphenols on the formation of B(a)P-derived DNA adducts in mouse skin. *J Environ Pathol Toxicol Oncol.,* 2005, **24;** 79-90.

Krishnan R., Maru G. B. Isolation and analyses of polymeric polyphenol fractions from black tea. *Food Chemistry., 2006,* **94;** 331-340.

Kumar G., Pillare S. P., Maru G. B. Black tea polyphenols-mediated in vivo cellular responses during carcinogenesis. *Mini reviews in medicinal chemistry.* 2010, **10;** 492-505.

Kumar G., Dange P., Kailaje V., Vaidya M.M., Ramchandani A.G., Maru G.B. Polymeric black tea polyphenols modulate the localization and activity of 12-O-tetradecanoylphorbol-13-

acetatemediated kinases in mouse skin: mechanisms of their anti-tumorpromoting action. *Free Radic Biol Med*, 2012, **53**; 1358-1370.

Laemmli U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.,* 1970, **227;** 680-685.

Maru G.B., Hudlikar R.R., Kumar G.,Gandhi K. and Mahimkar M.B. Understanding the molecular mechanisms of cancer prevention by dietary phytochemicals: From experimental models to clinical trials. *World J Biol Chem*., 2016, **7(1**); 88-99.

Niranjan B.G. Wilson N.M., Jefcoate C.R., Avadhani N.G. Hepatic mitochondrial cytochrome P-450 system. Distinctive features of cytochrome P-450 involved in the activation of aflatoxin B1 and benzo(a)pyrene. *J Biol Chem*. 1984, **259**; 12495–1250.

Patel R., Garg R., Erande S., Maru G.B. Chemopreventive herbal anti-oxidants: current status and future perspectives. *Journal of clinical biochemistry and nutrition, 2007,* **40;** 82-91.

Patel R., Ingle A., Maru G. B. Polymeric black tea polyphenols inhibit 1,2-dimethylhydrazine induced colorectal carcinogenesis by inhibiting cell proliferation via Wnt/beta-catenin pathway. *Toxicology and applied pharmacology., 2008,* **227:**136-146.

Patel R., Krishnan R., Ramchandani A., Maru G. Polymeric black tea polyphenols inhibit mouse skin chemical carcinogenesis by decreasing cell proliferation. *Cell Prolif., 2008,* **41;** 532-553.

Patel R., Maru GB. Polymeric black tea polyphenols induce phase II enzymes via Nrf2 in mouse liver and lungs. *Free Radic.Biol.Med.*,2008, **44**; 1897–1911.

Pohl R.J. A rapid method for assaying the metabolism of 7-ethoxyresorufin by microsomal subcellular fractions. *Anal. Biochem*.,1983,**107**; 150–155.

Pongratz I. et al. Inhibition of the specific DNA binding activity of the dioxin receptor by phosphatase treatment. J. Biol. Chem., 1991, **266**, 16813–16817.

Smith J.J., Tully P. and Padberg R.M. Chemoprevention: A primary cancer prevention strategy. *Semin. Oncol. Nurs*., 2005, **21**; 243-251.

Surh, Y. J. Cancer chemoprevention with dietary phytochemicals. *Nat. Rev. Cancer., 2003,* **3 ;**768-780.

Thapliyal R., Maru G. B. Inhibition of cytochrome P450 isozymes by curcumins in vitro and in vivo. *Food Chem Toxicol.,* 2001, **39;** 541-547.

Tsao, A. S.; Kim, E. S.; Hong, W. K. Chemoprevention of cancer. *CA Cancer J. Clin.* 2004,**54;** 150-180.

Papers Presented:

Presented Paper entitled 'Modulation of Transcriptional Regulator of Phase I enzyme as a Potential Mechanism of Polymeric Black Tea Polyphenols for Anti-initiating Action' at 47th annual conference of the Indian Pharmacological Society IPSCON-2014 held at Guawahati, between 28th-30th December 2014.

Oral Presentation was given entitled 'Polymeric black tea polyphenols inhibit aryl hydrocarbon receptor mediated activation of CYP 1A1 and CYP 1A2 transcription and DNA adduct formation in mouse skin: Mechanism of their anti-initiation action'at 3rd international conference and exhibition on pharmacognosy, phytochemistry & Natural product held at Hyderabad between 26th October to 30th October 2015.

Papers Published:

Maru Girish B., Hudlikar Rasika R., Kumar Gaurav.,**Gandhi Khushboo**. and Mahimkar Manoj .B. Understanding the molecular mechanisms of cancer prevention by dietary phytochemicals: From experimental models to clinical trials. *World J Biol Chem*., 2016, **7(1**); 88-99.

Girish B. Maru, Khushboo Gandhi, Asha Ramchandani, Gaurav Kumar. Role of inflammation in Skin Cancer. Inflammation and Cancer, Advances in Experimental Medicine and Biology 816. Published by Springer Basel AG. B. B. Aggarwal et al. (eds.), 2014. Pp: 437-469; DOI: 10.1007/978-3-0348-0837-8_17.

Papers under communication:

Tejal Gandhi, **Khushboo Gandhi**, Kinnar Monpara. Anti-cancer activity of Boswellic acid and Montelukast sodium against human prostate cancer cell line PC- 3. (accepted in Iranial Journal Of Pharmaceutical Science).

Khushboo Gandhi, Tejal Gandhi, Girish B. Maru. Black Tea Polyphenol modulates transcriptional regulators of phase I in benzo[a]pyrene-treated mice Skin: mechanism of its anti-initiating action (MS under preparation).

Gaurav Kumar, Pooja Tajpara, **Khushboo Gandhi**, Amirali B. Bukhari, Arvind Ingle, Sadhna Kannan, Abhijit De, Asha G. Ramchandani, Girish B. Maru. Dietary curcumin inhibits B(a)P-induced lung tumors by modulating apoptosis and cell proliferation-related markers in A/J mice (submitted to Anti-cancer drugs).