# CHAPTER IV

# SUMMARY AND CONCLUSION

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## SUMMARY

Benzo(a)pyrene, an environmental carcinogen, undergoes oxidative metabolism by enzymes present mainly in the microsomes of target organs in susceptible species to form a multitude of metabolites. The predominant route of biological importance is the one in which BP is converted to proximate carcinogen BP-7,8-dihydrodiol which in turn is converted to ultimate carcinogen BP-7,8-dihydrodiol-9,10-epoxide (BPDE). This, then spontaneously generates carbocation at $C_{10}$ position. The highly electrophilic carcinogenic derivative binds to a variety of nucleophilic macromolecular targets in the cell. For obvious reasons, the reaction of carcinogen with DNA is believed to be of immense significance to the subsequent actions of the affected cell and its progeny. An immediate consequence of the interaction of carcinogen with genomic material would be altered gene expression and hence the fundamental objective of this study was to investigate the effect of BP on the process of transcription in rat liver during the early stages after administration of the carcinogen. The other objective, which is of more practical significance, was to study the ability of certain dietary factors to modulate the initial critical event of BP carcinogenesis i.e. the covalent interaction of BP with genomic

material. These studies were designed with a view to gain insight into the molecular actions of BP and to help in devising nutritional strategy to alleviate the carcinogenic challange.

The overall in vivo effect of BP on hepatic transcription may be summarized as follows:

1) Gross transcription measured as rapidly labeled RNA suffered sharp decline at 2 h and stimulation at 2 - 4 d followed by another decline at 7 - 14 d. The interesting 2 h inhibition could be due to decrease in synthesis of precursor rRNA or HnRNA (precursor mRNA).

2) When nuclei purified from BP (2 h)-treated animals were assayed in vitro for transcriptive ability in varying assay conditions it was observed that:

a) $Mg^{2+}$-dependent activity of osmotonic nuclei was not affected, while $Mn^{2+}$ + $(NH_4)_2SO_4$-dependent activity was inhibited. This could roughly be interpreted as inhibition of expression of polymerase II and not of polymerase I. The high salt condition may also reflect propagation of initiated chains of RNA. Thus, it could be interpreted as effect of BP on RNA chain propagation or that in a general background of propagation of all types of RNA chains, genuine inhibition of specific genes may be masked to some extent.

b) Hypertonic nuclei assayed under moderate conditions expressed 10% inhibition due to BP and when differentiated with α-amanitin the inhibition was mainly due to polymerase II and III, while I showed stimulation to the extent of 15%.

3) Nucleoli exhibited 10 - 18% inhibition due to BP. Since nucleolus is rich in polymerase I and rDNA, the template or enzyme confined to nucleolus may be inhibited due to BP but in total assay of nucleus this inhibition was not observed.

4) Both free enzyme fraction and engaged enzyme fraction (containing template and the enzyme) of nuclei showed inhibition due to BP.

5) When enzymes were solubilized and partially purified into three forms, the observations were as follows: a) Total enzymes: all three forms were inhibited. b) Free form: mainly II and I were inhibited while III was not affected. c) Engaged form: all three enzymes remained uninhibited, even though engaged fraction of nuclei was inhibited.

Thus, administration of BP strongly but reversibly inhibits transcription in liver at an early stage (2 h). This was observed to be due to effect of BP on both the template chromatin and free form of enzyme RNA polymerases. BP may also be inhibiting RNA chain propagation during transcription. Its effects on the nucleolar subfraction and on various factors other than the template or the enzyme may also influence the inhibition of the total process of transcription.

The effects of BP were sought to be recreated in a model system in vitro by reacting purified nuclei with BPDE I, the ultimate carcinogenic metabolite of BP. Using inhibitors like α-amanitin to differentiate the enzyme RNA polymerases and actinomycin D with synthetic template, poly[d(I-C)], to differentiate between effects on the enzyme or the template, it was observed that BPDE I inhibited nuclear transcription in vitro by inhibiting both the enzyme and the endogenous template. This is in close agreement with the observations regarding early in vivo inhibition of transcription by BP.

We have observed that nutritional factors like ascorbic acid and retinoids and non-nutritional factors like flavonoids and related phenolics are able to modulate the initial molecular actions of BP. Thus L-ascorbic acid inhibited adduct formation between BP and DNA at physiologically attainable concentrations. However the inhibition was limited in vitro in that significant inhibition (~50%) required very large concentrations of LAA ( > 10 mM). But in vivo, the inhibition obtained after administration of 300 mg $kg^{-1}$ was nearly 80%. These are indicative of a further action of LAA at levels other than the adduct formation. Role of LAA in decreasing metabolism or increasing detoxification, was apparent from its ability to reduce metabolic availability of activated intermediates of BP. Most important was its ability to intervene in the direct interaction between the ultimate

carcinogen BPDE I and DNA. This could be due to protection of DNA against attack by the carcinogen or due to scavenging of the electrophilic moiety of the ultimate carcinogen.

Retinoids showed inhibitory activity towards adduct formation in vitro to different extents in that aldehyde and esters were very effective inhibitors at concentrations ($< 0.1$ mM) far less than those of LAA. These compounds may have acted by direct intervention in the reaction between ultimate carcinogen BPDE I and DNA. However, their role in metabolic activation may also be important as they are known to be very active at the level of membrane-bound enzymes. The case of retinol and retinoic acid is most peculiar in that they were able to inactivate BPDE I very significantly but were unable to reduce the adduct formation between BP and DNA. This could be due to their inactivation or due to sequestering of these compounds by other components of the microsomes not directly involved in activation of BP.

Flavonoids also showed a range of response in the adduct formation assay. The effective flavonoids exerted their action at concentration well below the effective concentration of LAA. Some structural features which enhanced the inhibitory activity of flavone nucleus are the 2,3 double bond, OH group in 3 position and additional OH groups in A and B rings. The ineffectiveness of methylated hydroxyl group was easily demonstrable with some of the

polyhydroxy compounds. Flavan and isoflavones were ineffective and so were flavonoids with open ring structure. These studies might help in developing plans to either isolate naturally occurring flavonoid or make synthetic flavonoid which has all positive attributes of the inhibitory flavonoid. The ability of flavonoids to inhibit DNA adduct formation is primarily attributable to their direct inactivation of ultimate carcinogen BPDE I.

The significance of these observations in vivo can only be conjectured in light of all known facts about these compounds. The supporting evidence came from our work that LAA could inhibit the adduct formation between BP and DNA in vivo and, at the same time, it could decrease the inhibition in gene expression due to BP. This confirms effects of LAA not only on the critical molecular action of BP (binding to DNA) but also on the biochemical consequences of the adduct formed. Thus, early reversible inhibition of transcription in vivo and adduct formation between BP and DNA in vivo and in vitro serve as simple testing procedures which are sensitive to modulating effects of dietary factors.

So far, we have mainly screened those compounds with known anticancer effects (however controversial) and therefore it is easy to conclude that the proposed mechanism is the way by which these compounds prevent cancer. Our continued efforts, therefore, will be to extend the screening

to a vast variety of nutritional and non-nutritional factors. In absence of a prior knowledge regarding their antitumor effects, at least an inference can be drawn from such facile in vitro screening procedures regarding their potential to act as anticancer agents.

The earliest molecular action of the environmental carcinogen BP is to form adduct with DNA and a significant consequent biochemical change in the initial stages is inhibition of transcription in liver. Dietary modulators are able to decrease effect of BP on these processes. Factors of dietary origin are also able to reduce the metabolic activation of BP, or sequester the activated metabolite or protect the target DNA against the carcinogen.

## CONCLUSION

Chemicals which are carcinogenic are diverse group of compounds, having a wide variety of structures and properties. In spite of their diversity these carcinogens possess a common characteristic in being able to yield highly reactive species which covalently bind to cellular macromolecules. It seems axiomatic that the adverse biological effects of chemical carcinogens must ultimately be dependent upon initial reactions between these carcinogens and critical cellular targets. It is generally recognized that DNA is the ultimate

although not obligatory primary target of chemical carcinogens. Initial biochemical events, subsequent to these molecular interactions, should cast a significant impact on the eventual carcinogenic process that the cell undergoes. Animal studies have shown that several chemical carcinogens bring about profound changes in many biochemical processes in their own characteristic ways. It is also imperative that any measure which can arrest or slow down initial biochemical events that are elicited by chemical carcinogens will ultimately prevent carcinogenesis. This thesis presents results from a series of experiments conducted with a chemical carcinogen, benzo(a) pyrene, that have relevance to some of the points enumerated above.

Benzo(a)pyrene (BP), an environmental pollutant and a suspect human carcinogen, causes cancer in a variety of organs of experimental animals. The biochemical process by which BP brings about the transformation of a normal cell into a cancerous one is not well understood. In these studies the initial biochemical response to the administration of BP to rats has been observed to be an alteration of gene expression, grossly measured as incorporation of precursors into nascent RNA in liver nuclei. Although the inhibition was reversible it was considered significant in view of the reported observation that such early inhibition in transcription was observed in skin in response to carcinogenic BP but not in response to non-carcinogenic analog benzo(e)pyrene. Similar

results have also been observed with other carcinogens, and also in susceptible strain as compared to resistant one. Efforts were,therefore, directed to investigate various levels of organization of transcriptive process in order to determine the site(s) at which BP affected the transcription. Several lines of investigation conducted with nuclear subfractions and partially purified RNA polymerases have indicated that both the chromatin template as well as different RNA polymerases are affected by BP administration. It is envisaged that the carcinogen BP has the potential to alter gene expression. Regions of chromatin coding for mRNA in particular are most significantly affected. It is to be understood that such alterations are brought about after BP undergoes metabolic activation in the liver cells. Both microsomes and nuclear membrane mediate these activation reactions. It, therefore, remains to be proved that such alterations in transcription are indeed effected by the active metabolite of BP. It has been recognized that 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydro-BP (BPDE) is the ultimate carcinogenic metabolite of BP. Out of the two stereoisomeric bay-region diol epoxides BPDE I and BPDE II, it is the former that forms a major covalent adduct in vivo with macromolecules. In a further series of experiments using purified nuclei, actinomycin-D and an exogenous template poly[d(I-C)], it has been observed that BPDE I affects transcription at the level of chromatin as well as at the level of enzymes. This is in concurrence with the observations

made with nuclear subfractions and isolated enzymes from rats after administration of BP. Consequences of chromatin modification can easily be assumed with respect to carcinogenicity, while enzyme alteration may be a transient effect. These observations nevertheless open up areas which must be explored further.

Having recognized a significant molecular event elicited by the carcinogen BP, the thought naturally occurs as to how to prevent this event. If a way is found, it is expected that carcinogenesis will be prevented. The molecular event as recorded here is certainly due to an initial interaction of BPDE I with DNA and protein. Since reaction of a carcinogen with DNA is a critical event believed to be responsible for subsequent actions of the affected cell and its progeny, its modulation by any factor will reflect on the potential of that factor to modulate carcinogenesis. Several natural and synthetic factors have this potential that has been recognized from long-lasting and expensive animal studies. This thesis records a facile in vitro testing procedure which measures microsome-mediated adduct formation between BP and DNA. Employing this simple and reproducible test system it has been observed that ascorbic acid, retinoids and several plant flavonoids and related phenolic derivatives exert significant inhibitory response on DNA adduct formation. One advantage of the in vitro method is that it offers scope to study the mechanism of action of a particular substance. Such mechanistic

studies have revealed that majority of these substances act by direct interaction with BPDE I either forming inactive complexes or by accelerating its hydrolysis, although certain factors seem to inhibit the microsomal activating enzymes. In addition, dietary ascorbic acid has also been observed to partially prevent BP-induced inhibition of nuclear transcription, presumably an altered gene expression as a result of DNA adduct formation. These observations give rise to hope that may lead to discovery of many more factors, the application of which will create a hostile cellular environment for the carcinogens and thus may prevent the very crucial initial interaction which leads to cancer. Physiological and dietary factors are particularly important in this respect, and may play a significant role in the body's defence against carcinogenic onslaught. Such knowledge is thus vital in evolving a nutritional strategy to prevent cancer.