CHAPTER 2
STUDY RATIONALE, AIMS, OBJECTIVES AND WORKING HYPOTHESIS

RATIONALE FOR THE PRESENT STUDY

Human papillomavirus infections underlie substantial human morbidity and mortality throughout the world. Current epidemiological studies suggest that the prevalence of HPV infection in developed countries may be increasing dramatically (Phelps and Alexander, 1995). Conventional therapy (Surgery, radiotherapy and chemotherapy) and immunotherapy (interferon α) have been used in the treatment of HPV associated diseases. Such methods are expensive, painful and are associated with high recurrence rate. So far, no available drug therapy can effectively eliminate HPV infection or prevent the associated malignant progression (Abdul karim and Bourhis, 2001). Recent available knowledge of molecular mechanism of HPV oncoproteins has allowed new drugs design to suppress infections by HPV, so as to prevent the development of cervical cancer (Beerheide et al, 1999). The development of inhibitory compounds targeting E6 and E7 oncoproteins via prevention of DNA virus replication would be useful (Bourhis and Abdul Karim, 2001). It has been reported that antisense oligonucleotides can inhibit tumor cell growth in vitro and in vivo by downregulation of E6 and E7 gene expression (Tan et al, 1995; Steele et al, 1993). Stable transfection of cervical tumor cell lines with antisense or ribozyme expression vectors can also inhibit in vitro cell growth and the efficiency of colony formation and increase serum requirements (Steele et al, 1992;
Watanabe et al, 1993; Hamada et al, 1996) and the tumorigenicity in vivo (Hamada et al, 1996; Chen et al, 1996). Therefore, an in vitro screening system for development of drugs to treat HPV infection is primarily required to search compounds with desirable properties. Successful development of antiviral therapies for HPV infections will continue to be important both for clinical relief to the many infected persons and for the epidemiologic control of the transmission of a human tumor virus (Phelps and Alexander, 1995).

OVERALL AIMS OF THE STUDY

Human papillomavirus (HPV) infection plays a key role in the development of cervical cancer. Strong association of cervical cancer with high-risk HPV infections underlines the importance of developing rational antiHPV therapy for treatment of cervical cancer. Oncogenic HPV E6 and E7 proteins interact with cellular growth-regulatory proteins, p53 and Rb, abrogating their normal function, which may lead to the development of cervical cancer. Any compound inhibits E6 or E7 expression could potentially treat HPV induced tumors. The overall aim was thus to elucidate the molecular pharmacology of curcumin and Cleistanthin A including its effects on cell proliferation, apoptosis, apoptotic pathways, activation of NFκB and AP-1 transcription factors, expression of cyclooxygenase-2 and HPV associated viral gene expression.
SPECIFIC OBJECTIVES

➢ Bioassay directed column chromatography to identify the active principle, Cleistanthin A from Cleistanthus collinus.

➢ Analyze the cytotoxic activity of Curcumin and Cleistanthin A in cell lines known to harbor HPV.

➢ Assessment of apoptosis induction in cells treated with these two compounds.

➢ Analyze the expression of apoptosis regulatory molecules (Bax, Bcl2) in cells after treatment with curcumin and cleistanthin A.

➢ Evaluation of possible antiviral actions of curcumin and cleistanthin A in terms of changes in the expression of viral proteins E6 and E7.

➢ To evaluate the role of these compounds in regulating NFκB, AP-1 and COX2 expression.

WORKING HYPOTHESIS

Research for the past few decades has substantiated the role of human papillomavirus (HPV) in oncogenesis of tumors in the uterine cervix and upper aerodigestive tract. Increase in frequency of HPV infection has been reported globally and in India as well. The biological behavior and molecular manifestations of HPV has been studied extensively. However, translation of these studies to the management of this disease has not materialized. HPV infection has been found to be alarmingly high in both oral and cervical cancers reported at the Regional Cancer Center, Thiruvananthapuram. In this context, we have elucidated various cellular and molecular manifestations of HPV infection in
lesions of the oral cavity and uterine cervix including the development of a "condemned mucosa syndrome". The present study sought to understand these observations at the molecular level in relation to the mode of action of natural compounds with possible anti HPV action. India being immensely rich in traditional practice of medicine for centuries has vast resources of medicinal plant species. Natural products have proved to be an infinite source for remedies over the ages. The concept that certain diet-derived substances can be used to prevent cancer or postpone its onset is currently eliciting considerable interest. Several plant-derived compounds are currently successfully employed in cancer therapy. The search for new chemopreventive and antitumor agents that are more effective and less toxic has kindled great interest in phytochemicals. Compounds known to have antitumor/ antiviral properties reported in traditional medicine and previous studies were screened for anti-papilloma viral activity in established cell lines harboring HPV infection. This study selected two compounds- Curcumin, isolated from *Curcuma longa* and Cleistanthin A, isolated from *Cleistanthus collinus*. When the cytotoxicity of Cleistanthin A was compared to five anticancer drugs, it was found to be more effective for the oral carcinoma cell line, KB and cervical carcinoma cell line, SiHa, both harboring HPV. This study will thus provide valuable data on response of cells infected with HPV to anti-viral compounds at the cellular and subcellular level. With the high turnover of HPV associated cancer patients in India, it is essential that such a data be available.
PLANT DERIVED COMPOUNDS

The use of medicinal plants for the treatment of many diseases is described in folk medicine from different parts of the world. Natural products from plants, fungi, bacteria and other organisms, continue to be used in pharmaceutical preparations either as pure compounds or as extracts. There are a great variety of such compounds that can be extracted and characterized from plants (Araujo and Leon, 2001). Recently, emphasis has focused upon a variety of clinical and basic studies of chemoprevention using naturally occurring substances that are found in normal diets, since they might provide useful strategies to inhibit cancer with minimal toxicity (Greenwald and Kelloff, 1996). Epidemiological studies suggest that dietary manipulations play an important role in reducing the cancer death rate as much as 90% (Plummer et al, 1999). Large number of minor food components and chemically related compounds block different stages of the carcinogenic process in animal models and some of these substances partially prevent or delay cancer formation in some high risk human populations (Blot et al, 1993; Hong et al, 1990; Kraemer et al, 1988).

CURCUMIN

Phenolic antioxidants are a class of dietary compounds that possess anti-inflammatory properties (Kagan and Tyurina, 1998). Curcumin (diferuloyl methane) is a representative phenolic antioxidant found in the dietary spice turmeric. It is derived from the ground rhizome of Curcuma longa L., belonging to the Zingeberaceae family. The major constituent, curcumin is the most important fraction of Curcuma longa L. and it's chemical structure (Figure1) was
determined by Roughley and Whiting (1973). It melts at 176-177°C and forms red-brown salts with alkalis.

**FIGURE : 1 STRUCTURE OF CURCUMIN**

Curcumin is soluble in ethanol, alkalis, ketone, acetic acid and chloroform; and is insoluble in water. In the molecule of curcumin, the main chain is aliphatic, unsaturated and the aryl group can be substituted or not (Araujo and Leon, 2001). The degradation kinetics of curcumin under various pH conditions and the stability of curcumin in physiological matrices have been established (Wang et al, 1997).

Current traditional Indian medicine uses curcumin for biliary disorders, anorexia, cough, diabetic wounds, hepatic disorders, rheumatism and sinusitis (Jain and DeFilipps, 1991). This nonnutritive phytochemical is pharmacologically safe, considering that it has been consumed as a dietary spice, at doses upto 100mg/day for centuries (Ammon and Wahl, 1991). Recent phase I clinical trials indicate that people can tolerate a dose as high as 8g/day (Cheng et al, 2001). Numerous reports suggest that curcumin has chemopreventive and chemotherapeutic effects. It's anticancer potential in various systems was

Mechanisms that suppress tumorigenesis often involve modulation of signal transduction pathways, leading to alterations in gene expression, arrest of cell cycle progression or apoptosis. Apoptosis is a mode of cell death used by multicellular organisms to eradicate cells in diverse physiological and pathological settings. Several studies have demonstrated that apoptosis may be involved in cell death induced by chemotherapeutic agents, including cisplatin, camptothecin, etoposide, etc. There is accumulating evidence that the efficiency of antitumor agents is related to the intrinsic propensity of the target tumor cells to respond to these agents by apoptosis (Villunger et al, 1997). Recent evidence also shows that suppression of apoptosis by tumor promoting agents in pre-neoplastic cells is thought to be an important mechanism in tumor promotion (Shibata et al, 1996).

Compounds that block or suppress the proliferation of tumor cells have potential as anticancer agents. Curcumin has been shown to inhibit the proliferation of a wide variety of tumor cells, including B cell and T cell leukemia (Abe et al, 1999;
Han et al., 1999; Piwocka et al., 1999; Kuo et al., 1996), colon carcinoma (Chen et al., 1999) and epidermoid carcinoma cells (Korutla et al., 1994). It has also been shown to suppress the proliferation of various breast carcinoma cell lines in culture (Ramachandran and You, 1999; Simon et al., 1998; Mehta et al., 1997). Additionally curcumin exhibits antimetastatic activity (Menon et al., 1999). It has also been shown to inhibit the growth of endothelial cells (Singh et al., 1996), suppresses angiogenesis in vivo (Arbiser et al., 1998), abrogate FGF-2-induced angiogenic response and matrix metalloprotease (MMP)-9 expression (Mohan et al., 2000), block expression of adhesion molecules (Kumar et al., 1998). Overexpression of cyclooxygenase-2 (COX-2) has been shown to be associated with a wide variety of cancers, including colon (Fournier and Gordon, 2000), lung (Hida et al., 1998) and breast (Harris et al., 2000) cancers. Several groups have shown that curcumin downregulates the expression of COX-2 protein in different tumor cells (Chen et al., 1999; Plummer et al., 1999) most likely through the downregulation of NFκB activation (Plummer et al., 1999), which is needed for COX-2 expression.

NFκB is a nuclear transcription factor required for the expression of genes involved in cell proliferation, cell invasion, metastasis, angiogenesis and resistance to chemotherapy (Baldwin, 2001). This factor is activated in response to inflammatory stimuli, carcinogens, tumor promoters and hypoxia, which is frequently encountered in tumor tissues (Pahl, 1999). Studies have shown that various tumor promoters, including phorbol ester, TNF and H₂O₂ activate NFκB and that curcumin can downregulate this activation (Singh and Aggarwal, 1995).
Curcumin has been shown to induce downregulation of NFκB through suppression of IkBα kinase activation (Jobin et al, 1999; Plummer et al, 1999). AP-1 is another transcription factor that has been closely linked with proliferation and transformation of tumor cells (Karin et al, 1997). Curcumin has been shown to inhibit the activation of AP-1 induced by tumor promoters (Huang et al, 1991). This observation is of considerable significance to this study. Suppression of AP-1 would lead to downregulation of the oncogenic HPV transforming gene E6/E7 expression. This would then disrupt the process of transformation and tumor progression induced by high risk HPVs. Eight different studies of the safety and efficacy of curcumin in humans have been reported. A short-term, double blind, cross over study was performed in 18 patients to compare the antirheumatic activity of curcumin and phenylbutazone. They administered 1200mg curcumin/day or 300mg phenylbutazone/day for two weeks. Curcumin was well tolerated, had no side effects and showed comparable antirheumatic activity (Deodar et al, 1980). The toxicology, pharmacokinetics and biologically effective doses of curcumin in humans were studied (Cheng et al, 2001). The study demonstrated that curcumin is not toxic to humans at doses up to 8000mg/day when taken orally for 3 months suggesting a biologic effect of curcumin in the chemoprevention of cancer.

**CLEISTANTHUS COLLINUS**

Herbal remedies used in the traditional folk medicine provide an interesting and still largely unexplored source for the creation and development of potentially new drugs for chemotherapy, which might help to overcome the growing problem
of resistance and also the toxicity of currently available commercial antibiotics. The traditional medicinal methods, especially the use of medicinal plants, still play a vital role to cover the basic health needs in the developing countries (Ali et al, 2001). *Cleistanthus collinus*, a shrub of the Euphorbiaceae family is abundant in many parts of India, Malaysia and Africa. All parts of the plants are poisonous, but the leaves are used as poison in the form of decoction (Chopra et al, 1965). Various parts of the plant are used as folk medicine in acute gastrointestinal disorders and as abortifacient. The main chemical components of the leaves of *Cleistanthus collinus* are cleistanthin A, cleistanthin B, collinusin, diphyllin and elagic acid (Govindachari et al, 1969). When the alcoholic extract of the whole plant was tested for anticancer activity, using cells of human epidermoid carcinoma of nasopharynx in culture, Walker carcinoma in rats and lymphoid leukemia in mice, the extract was found to be effective only against the epidermoid carcinoma cells (Pradheepkumar and Shanmugam, 1999). In vitro screening with a variety of tumor cell lines and normal cells showed that this compound is selectively more cytotoxic towards tumor cells than to the normal cells. Of the various cancer cell lines, the oral carcinoma cell line KB showed maximum sensitivity to cleistanthin A followed by cervical carcinoma cell line, SiHa (Pradheepkumar and Shanmugham, 1999). This observation was particularly interesting to the present study, since both these cell lines carry the human papillomavirus. The potential of Cleistanthin A to arrest growth of tumors was evaluated in vivo (Pradheepkumar and Shanmugham, 1999). They observed significant tumor regression within 2 weeks of treatment at high doses. This
compound also increased the life span of animals bearing ascites tumors (Pradheepkumar and Shanmugham, 1999). Cleistanthin A is a novel anticancer agent because it did not cause significant reduction in body weight and did not decrease the blood cell counts in treated animals (Pradheepkumar and Shanmugham, 1999). It has been shown to cause neutrophilic granulocytosis in rats, mice, cats and monkeys (Rao and Nair, 1970). It also prevented granulocytopenia induced by intraperitoneal injection of cyclophosphamide (Rao and Nair, 1971). Cleistanthin A has been shown to cause significant chromatid abberations in CHO cells with increase in exposure time and dose of the compound. It has been shown to induce DNA ladder formation characteristic of apoptosis in K562 cells and HeLa cells (Pradheepkumar et al, 2000). Preliminary experiments showed that Cleistanthin A caused lipid peroxidation and oxidative stress both in vivo and in vitro (Pradheepkumar et al, 2000). K562, cells deficient in p53 function, underwent apoptosis upon treatment with Cleistanthin A and to a lesser extent with Cleistanthin B. These results indicate that cleistanthins may be useful as effective chemotherapeutic agents even in tumors with p53 mutations. Cleistanthin B is also an anticancer agent isolated from *Cleistanthus collinus*. However, anticancer potential of this compound is relatively lower than that of Cleistanthin A. It has been shown to be clastogenic and induced micronuclei formation and chromosomal aberrations. It induced apoptosis in cervical carcinoma (SiHa) cells (Pradheepkumar et al, 1998). Cleistanthin A has been shown to inhibit DNA synthesis in synchronized CHO cells. It caused 50% inhibition in thymidine incorporation into DNA in comparison to the untreated
cells. Cleistanthin B stopped entry of cells into S phase and subsequently drove them to apoptosis (Pradheepkumar et al, 1998). Studies have shown that Cleistanthin A inhibited MMP-9, a 92kDa matrix metalloprotease responsible for the remodeling of the extracellular matrix throughout the body (Meenakshi and Shanmugham, 2000).