DEVELOPMENT OF BIOASSAYS FOR ENDOCRINE DISRUPTORS SCREENING AND STUDY THEIR MODE OF ACTION

A THESIS

Submitted in partial fulfilment of the requirements for the award of the degree of

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in

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by

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CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **DEVELOPMENT OF BIOASSAYS FOR ENDOCRINE DISRUPTORS SCREENING AND STUDY THEIR MODE OF ACTION** in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy and submitted in the Department of Biotechnology of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during the period from July 2005 to November 2008 under the supervision of Dr. Partha Roy, Assistant Professor, Department of Biotechnology and Dr. C.B. Majumdar, Assistant Professor, Department of Chemical Engineering, Indian Institute of Technology Roorkee, Roorkee, India.

The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other institute.

G. Viswanath
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This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

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The PhD Viva-Voce Examination of **Mr. Gunda Viswanath** Research Scholar has been held on 3/4/09

Partha Roy 3/4/09
Signature of Supervisors

3/4/09
Signature of External Examiner

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G. Viswanath
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LIST OF ABBREVATIONS

DNA	DeoxyNucleic Acid
RNA	RiboNucleic Acid
hAR	Human Androgen Receptor
hPR	Human Progesterone Receptor
hER	Human Estrogen Receptor
LBD	Ligand Binding Domain
DBD	DNA Binding Domain
PRE	Progesterone Response Element
ARE	Androgen Response Element
ERE	EstrogenResponse Element
EDC	Endocrine Disrupting Chemical
PCR	Polymerase Chain Reaction
RT-PCR	Reverse Transcriptase- Polymerase Chain Reaction
cDNA	Complemantary DNA
mRNA	Messenger Ribonucleic acid
rRNA	Ribosomal Ribonucleic acid
RNase	Ribonuclease
DNase	Deoxyribonuclease
ppm	Parts Per Million
T_m	Melting Temperature
U	Units
v/v	Volume/Volume
w/v	Weight/Volume
K_D	Dissociation Constant
EC_{50}	Half-Effective Concentration
IC_{50}	Half-Inhibition Concentration
α	Alpha
β	Beta
D	Dalton

kD	KiloDalton
m	Meter
°C	Degree Centigrade
%	Percentage
~	Approximately
bp	Basepair
ng	Nanogram
µg	Microgram
mg	Milligram
g	gram
kg	Kilogram
µl	Microliter
ml	Milliliter
l	Liter
M	Molar
mM	MilliMolar
µM	Micromolar
nM	NanoMolar
pM	PicoMolar
h	Hour
min	Minute
sec	Second
Fig.	Figure
GC-MS	Gas Chromatography Mass Spectrometry
NCBI	National Centre for Biotechnology Information
LH	Lutenizing Hormone
FSH	Follicle Stimulating Hormone
3β-HSD	3 Beta Hydroxysteroid Dehydrogenase
17β-HSD	17 Beta Hydroxysteroid Dehydrogenase
StAR	Steroidogenic Acute Regulatory protein
P450scc	P450 Side Chain Cleavage enzyme

P450C-17	P450 C-17 Enzyme
cAMP	Cyclic Adenosine Mono Phosphate
NFkB	Nuclear Factor of kB
AP1	Activator Protein 1
CBP	CREB Binding Protein
CRE	cAMP response element

CHAPTER 1

INTRODUCTION

CHAPTER 1

1.1 General Introduction

Endocrine system and nervous system are two major modes of communication that coordinate and control different body functions. While the dynamics of nervous system are maintained by conduction of electrical impulses through the complex circuits of nervous system, the messengers of the endocrine system are hormones that are synthesized and excreted at very low quantities from specialized glands and transported to the target organ(s) via the bloodstream. A tuned functioning of endocrine orchestra is necessary for sustained maintenance of different pivotal functions in human or animal body like reproduction and development, growth and maturation, energy production, electrolyte balance, etc. However, increasing scientific evidences depict the existence of a newly defined category of environmental contaminants which may have diverse chemical structures and can alter the normal functioning of the endocrine and reproductive systems (Cargouët et al., 2004). These chemicals mimic/inhibit the actions of endogenous hormones or modulate the synthesis of latter and have been named as "endocrine disrupting chemicals (EDC)" (Sonnennschein & Soto, 1998). In this way EDC may interfere with the usual hormonally regulated biological processes and thus, may adversely affect the development and reproductive function in wildlife, experimental animals, and humans (Satoh et al., 2001). In general EDC have been defined as-

"An exogenous agent that interferes with the synthesis, storage/release, transport, metabolism, binding, action or elimination of natural blood-borne hormones responsible for the regulation of homeostasis and of developmental processes".

Depending on the steroid with which the EDC interferes, steroid EDCs can be broadly categorized as (anti)androgenic, (anti)estrogenic and (anti)progestagenic chemicals. They may enter the body of animals/humans through the diet, contaminated water or occupational exposure, and then may lead to the generation of an agonistic or antagonistic effect (Kumar et al., 2008). Once inside the physiological system the EDC may exert their effect by targeting any of the following steps: (i) through arylhydrocarbon receptor (AhR) (Indarto & Izawa, 2001) (ii) direct binding of these chemicals to steroid receptors, steroidogenic enzymes and proteins associated with steroidogenesis (like StAR protein) (Walsh et al., 2000; Sanderson & Vanden Berg, 2003; Rice et al., 2006), and (iii) increasing the stability of transcripts and transcriptional rate of the promoter of steroidogenic enzymes (Lin et al., 2006; Lyssimachou et al., 2006).

An increasing body of evidences reveals the association between various therapeutic/environmental compounds that act as EDC and many sex hormone-sensitive disease/disorders (Colborn & Clement, 1992; Satoh et al., 2001; Sone et al., 2005; Guillette, 2006; Massart et al., 2006; Chen et al., 2007). The possibility of various diseases like reduced fecundity, abnormal fetal development, delayed onset of puberty, cryptorchidism, abnormal lactation, testicular dysfunction and even various types of cancers due to the exposure to EDCs have been reported (Sharpe & Irvine, 2004; Roy et al., 2005; Buck et al., 2006; Darbre, 2006; Guillette, 2006; Maffini et al., 2006).

In the past decade, the utilization of many chemicals (including pesticides and persistent organic pollutants) has been limited worldwide due to their hazardous potential. However, the chemicals having endocrine disruption (ED) potential are being used directly or indirectly for various purposes in the daily life leading to a chronic

exposure to them. These chemicals range from the simple household utilities like detergents, cosmetics and toilet utilities to specialized applications viz. pharmaceuticals, insecticides and pesticides. Therefore there is a continuous discharge of these EDCs from various sources - household utilities, industrial and agricultural applications etc. into the environment without our knowledge of their toxicological potential. Once these chemicals reach the environment, they become the part of the food chain finally reaching the animal/human systems. Although the carcinogenic potential of these compounds are evaluated by routine mutagenicity testing or biophysical tests, the concentrations necessary to disrupt endocrine regulation may be much lower than that required to act as a carcinogen. Chronic exposure even in very low doses of these compounds may disturb the delicate hormone balance and compromise the reproductive health of many species (Ralph et al., 2003). EDCs have already been reported to be in the food chain and different strata of environment in various forms like persistent organic pollutants (POPs) such as the insecticide dichlorodiphenyl-trichloroethane (DDT) and its metabolites, the industrial by-product dioxins, the industrial compounds polychlorinated biphenyls (PCB), several agrochemicals, pesticides and biocides (e.g. chlorinated insecticides, organotins, imidazoles, triazoles, etc.) and other industrial compounds (several phenol compounds such as bisphenol A) (Mantovani et al., 1999). According to one of the environmental scientists (Trivedi, 2007) -

"***Today and every day, you can expect to be exposed to some 75,000 artificial chemicals. All day long you will be breathing them in, absorbing them through your skin and swallowing them in your food. Throughout the night they will seep out of carpets, pillows and curtains, and drift into your lungs. Living in this chemical soup is***

an inescapable side effect of 21st-century living. The question is: is it doing us any harm?"

Once used in various applications and activities, different types of chemicals and their byproducts are discharged finally to sewage water making it a complicated broth of chemicals having diverse structures and different effects on biological organization including endocrine system (Darbre, 2006; Heidler et al., 2006; Sarmah et al., 2006). Thus sewage water receives a number of chemicals which can be potent EDCs, however, the rigorous treatment process that is followed in the sewage treatment plant for the removal of harmful contaminants is found to be ineffective with regard to the EDCs (Kumar et al., 2008). Majority of the adverse physiological observations in the aquatic environment concerning the reproductive system, for instance, the feminization of male fish with sewage treatment plant effluents, are attributed to the presence of EDC in these discharged effluents (Sumpter & Jobling, 1995; Sumpter, 1998; Ternes et al., 1999). It has been hypothesized that the statistical decrease in sperm counts over the last decades, increasing incidents of testicular cancer and other disorders regarding male infertility might have been caused by the intake of these chemicals via food or drinking water (Sharpe & Skakkebaek, 1993).

1.2 Aims and Objectives

The present work endeavored to adopt a holistic approach to understand the mode of action of some EDCs. Initially the androgen and progesterone receptor ligand binding domains were cloned into bacterial expression vectors and the recombinant proteins were purified and used for the screening of EDCs by competitive receptor binding assay. This was followed by the development of luciferase reporter based stable cell lines with

NIH3T3 cells for screening of androgen and progesterone EDCs. These cell lines were optimized for their high sensitivity with low background screening conditions and utilized for the screening of a large array of different classes of compounds for their endocrine disruption. Some of the test compounds which showed high (anti)androgenicity and (anti)progestagenicity were further tested in *in vivo* rat and rabbit systems, respectively, to understand their biological effects. It was especially focused to understand their interference in steroidogenesis both in *in vivo* system and *in vitro* Leydig cell culture. These compounds were then studied for their mode of action in terms of co-factor (CBP) requirement and crosstalk with other secondary signaling pathways. Overall, this study attempted to achieve the following objectives-

1. Recombinant protein production of androgen and progesterone ligand binding domains (LBD) and screening for EDCs by competitive receptor binding studies using these receptor proteins.

2. Development of stable cell lines expressing steroid receptor (AR and PR) and steroid response element (Probasin and PRE) driving the luciferase reporter gene. Screening for EDCs using these cell based bioassays.

3. *In vivo* characterization of (anti)androgenic and (anti)progestagenic activity of some selected EDCs in rodent and rabbit models, respectively.

4. To elucidate the molecular mechanism of action of some EDCs and further study their interference with steroidogenesis in *in vitro* Leydig cells.

CHAPTER 2

REVIEW OF LITERATURE

CHAPTER 2

2.1 The Endocrine System

The endocrine system can be defined as a highly organized system composed of different types of ductless glands, called endocrine glands, which synthesize and release specific chemical messengers or hormones into the body fluids which transport them to their respective target organs in the body where they exert their specific physiological effects. The endocrine system maintains homeostasis in the body by responding to signals originating from other organs and enabling a dynamic coordinated response with the help of hormones.

It is the second great controlling system of the human body. Along with the nervous system, it coordinates and directs the activity of the body's cells. However, these two regulating systems differ in their pace of response. The slower acting endocrine system uses chemical messengers called hormones, which are released into the blood to be transported throughout the body. The major processes controlled by hormones are reproduction, growth and development, mobilizing body defenses against stress, maintaining electrolyte, water and nutrient balance of the blood and regulating cellular metabolism and energy balance.

2.1.1 The Chemistry of Hormones

Hormones may be defined as chemical substances that are secreted by cells into the extra cellular fluids and regulate the metabolic activity of the cells in the body. Although many different hormones are produced, nearly all of them can be classified as either amino acid-based molecules (including proteins, peptides and amines) or steroids. Steroid hormones (made from cholesterol) include the sex hormones made by

the gonads and the hormones produced by the adrenal cortex. All others are non-steroidal amino acid derivatives. A third class of hormones, are present, called prostaglandins made of highly active lipids found in plasma membranes.

2.1.2 Mechanism of Hormone Action

Although the blood borne hormones circulate to all the organs of the body, a given hormone affects only certain tissue cells or organs, referred to as its target cells or target organs. In order for a target cell to respond to a hormone, specific protein receptors must be present on its plasma membrane or in its interior to which the hormone can bind. Hormones bring about their effects on the cells primarily by altering cellular activity – that is, by increasing or decreasing the rate of a normal, or usual, metabolic process rather than by stimulating a new one. The precise changes that follow hormone binding depend on the specific hormone and the target cell type, but typically one or more of the following occurs:

1. Changes in plasma membrane permeability or electrical state.
2. Synthesis of proteins or certain regulatory molecules (such as enzymes) in the cell.
3. Activation or inactivation of enzymes.
4. Stimulation of mitosis.

2.2 Reproductive hormones

In the current thesis, major emphasis was laid on the reproductive system due to its relevance to the study. In both male and female, reproduction and all events related to it are maintained by an appropriate level of steroid hormones. All steroids hormones are derived from a single precursor, cholesterol, using a complicated

enzymatic cascade and hence they are structurally related. The main male reproductive hormones are considered to be testosterone and its derivative, 5α–dihydro-testosterone (Michal, 1998; Rang & Dale, 2000) and they are also known as androgens. As described by Michal. (1998), estrogens (C-18 steroids) control the development of the reproductive system and its functions in female vertebrates. For instance, estrogens act on the ovaries, promoting the development of small groups of follicles producing an ovum. 17β–Estradiol constitutes the major female reproductive hormone. In mammals, progesterone (a C-21 steroid) plays an essential role as the active gestagen. It is produced mostly in the corpus luteum of the ovaries during the second half of the menstrual cycle and in the placenta during pregnancy. Its functions include preparation of the uterus for implantation of the fertilized ovum, preservation of the mucous layer of the uterus during pregnancy, prevention of further ovulations and formation of lactating alveoli in the breasts (synergistically with estrogens) (Michal, 1998). In addition, progesterone plays an important role in bone metabolism and neurotrophic functions as well (González et al., 2004).

2.2.1 Steroid Biosynthesis

Numerous organs are known to possess the capacity to synthesize biologically active steroids- adrenal gland, testis, ovary, brain, placenta, and adipose tissue. Three endocrine organs that specialize in *de novo* steroid production include the adrenal gland, testis and ovary. Steroids are synthesized through a shared pathway in which cholesterol (containing 27 carbon atoms) provides the basis for the different steroid structures (Fig 2.1). Cholesterol is transported into the mitochondria with the help of StAR (Stocco, 2000, 2001) and its associated proteins. P450ssc, 3β-HSD and 17β-

HSD are the important rate limiting enzymes in steroidogenesis. However StAR expression and P450scc are dependent on the cAMP triggered by LH receptor stimulation. Progesterone (C21), testosterone (C19) and estradiol (C18) are formed as intermediates in this pathway. The schematic representation of the steroid biosynthesis is as follows-

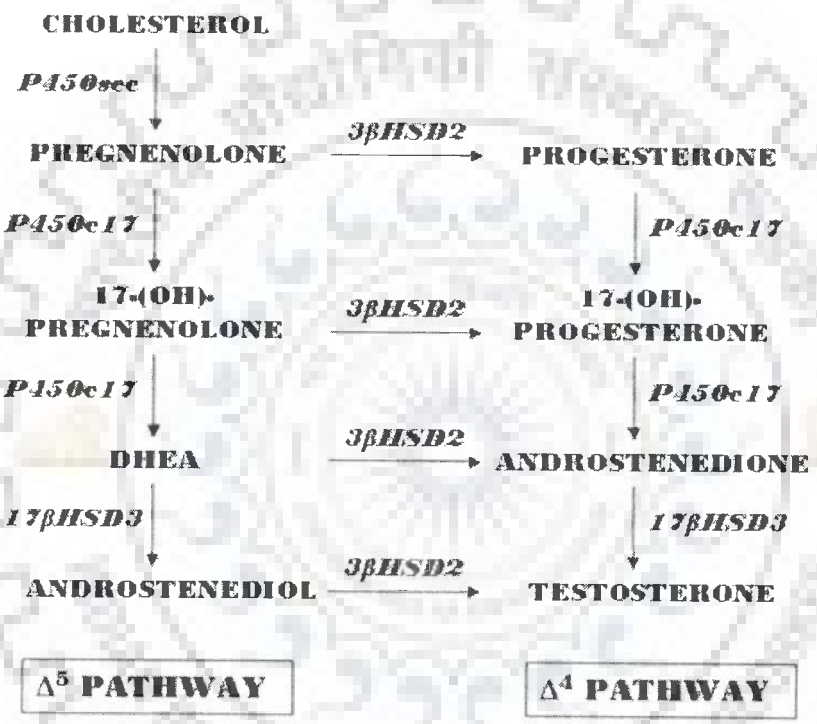


Fig 2.1 Biochemical pathway for the synthesis of steroid hormones

2.2.2 Regulation of steroid hormones

2.2.2.1 Hypothalamus Pituitary Gonadal Axis

The Hypothalamus-Gonadal axis consists of three parts – the GnRH neurons projecting from the hypothalamus of the brain, gonadotropes in the anterior pituitary gland (adenohypophysis), which secrete the gonadotropins LH and FSH, and the somatic cells of the gonads (theca and granulosa cells in the ovary, Leydig and Sertoli

cells in the testis). The terminals of GnRH neurons secrete GnRH in pulses (Kimura & Funabashi, 1998; Terasawa et al., 1998; Tanriverdi et al., 2003). GnRH induces the secretion of LH and FSH from gonadotropes which further act on theca/Leydig cells and granulosa/Sertoli cells respectively.

LH stimulates the secretion of testosterone and estradiol while FSH stimulates the protein hormone inhibin, which is in turn released into the blood stream. Sex steroids inhibit LH secretion while inhibin selectively inhibits FSH by a feedback inhibition on the hypothalamus and primary gonadotropes reducing the secretion of GnRH as well as LH and FSH (Crowley et al., 1991). The feedback mechanism is depicted in the Fig 2.2.

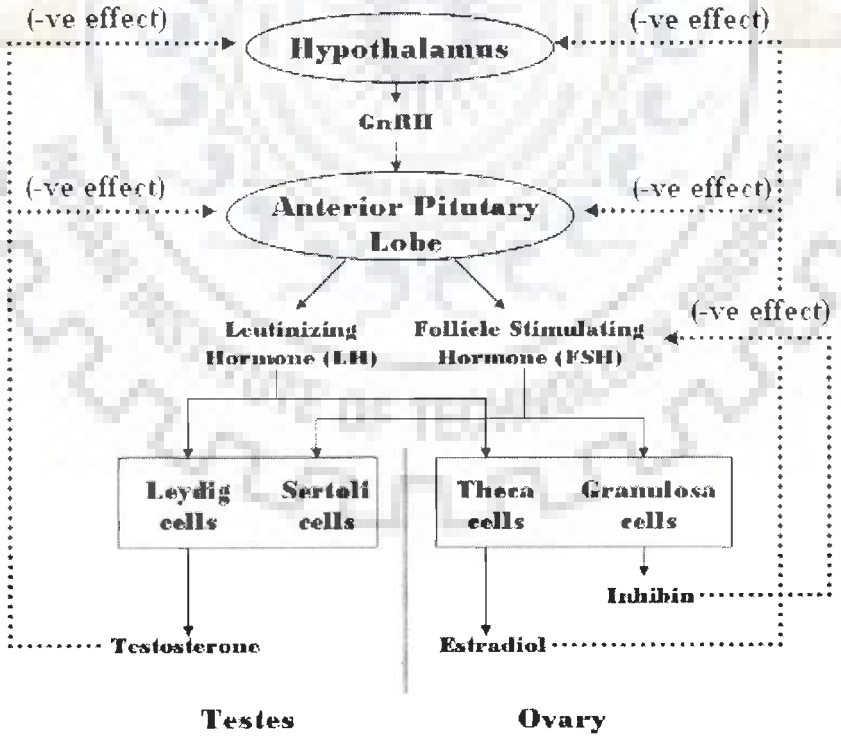


Fig 2.2 Hypothalamus- pituitary-gonadal axis

However, sex steroids (primarily testosterone in the male, E2 in the female) also effect FSH secretion by a negative feedback mechanism. Unlike these steroids, inhibin exhibits selective inhibition of FSH secretion only. GnRH shows a marked difference in the stimulation of LH and FSH, for instance, LH secretion is stimulated acutely in pulses by the GnRH while FSH secretion is extremely slow and takes many hours after the stimulation of the gonadotropes by GnRH. (Crowley et al., 1991; Bousfield et al., 1994). This difference is due to the variability in GnRH-induced synthesis, packaging and release of LH and FSH.

2.2.2.2 Sensitivity of the target cell to the hormone

Gonadotropes and the gonads do not always respond similarly to GnRH and gonadotropes respectively. They exhibit a varied sensitivity to acute and chronic stimulation. When the gonadotropes are exposed to chronic GnRH pulses or its high doses, these cells become resistant or less sensitive to the stimulation by GnRH by down regulating the number of surface receptors of this hormone. This is an extremely quick process and leads to the "desensitization" of the GnRH receptors thereby effecting the second messenger signaling mechanism stimulated by these receptors. Thus, the process completely reduces the overall response of the gonadotropes to its target hormone even in the presence of high concentration of GnRH (Erickson & Schreiber, 1995; Jameson, 2004). A similar mechanism where the target cell controls its own responsiveness to the stimulus is also exhibited by the gonadal cells. Cross talk between neighboring cells of the gonads controlling the stimulus of these cells to the gonadotropic hormones was suggested by Leung et al. (1992). This was supported by Sharpe et al. (1993), who showed that the Sertoli cells in the testes affect the

number of LH receptors and the steroidogenesis (by altering the expression of steroidogenic enzymes) in the neighbouring Leydig cells. This group also showed the paracrine effect exerted by the Leydig cells on the Sertoli cell responsiveness.

2.2.2.3 Endocrine Hormones metabolism

Another aspect of HPG axis which can be modulated physiologically is by the metabolism of hormones. The half-life of a hormone can undergo a significant change without any alteration in its level of secretion, by means of increased or decreased catabolism. For example, FSH is metabolized more slowly resulting in its longer half-life which is one of the reasons why changes in FSH levels are slower than changes in LH levels (Bousfield et al., 1994). Proteins that bind the sex steroids play an important role in the metabolism of hormones and they include albumin and AFP in the fetus/neonate and most important, Sex hormone binding globulin (SHBG) in humans. In the circulating steroids found in the blood, about 98 % of the testosterone and estradiol are complexed with SHBG while the remaining 2% is only biologically available (Moore & Bulbrook, 1988). This leads to the increase in the half life of the sex steroids and also indirectly affects the available circulating sex steroid levels by modulating the SHBG secretion by the liver (Bataille et al., 2005).

2.3 Steroid hormone receptor

Nuclear receptors (NR) are a class of ligand-regulated and DNA-sequence specific intracellular receptors that control the activity of genetic networks in response to diverse signals (Glass & Rosenfield, 2000; McKenna & O'Malley, 2002). They can be classified as steroid receptors, non-steroid receptors and orphan receptors. Steroid

receptors, which are categorized into type I class within the NR superfamily, are a group of structurally and functionally related proteins that include estrogen receptor (ER), androgen receptor (AR), progesterone receptor (PR), glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) (Shiau et al., 2001; Schulman & Heyman, 2004; Germain et al., 2006; Lu et al., 2006).

2.3.1 Steroid receptor structure and function

It has been suggested by several biochemical studies that steroid hormone receptors are structurally organized into different domains (Wrange & Gustafsson, 1978; Carlstedt- Duke et al., 1987). The results of cDNA cloning and the comparison of the corresponding amino acid sequence of different hormone receptors, have confirmed this prediction (Rusconi & Yamamoto, 1987).

All nuclear receptors including steroid receptors comprise the following regions:

a variable NH_2-terminal region (A/B), a conserved DNA-binding domain (DBD) or C region, a hinge D region, a conserved E region that accommodates the ligand-binding domain (LBD) (Fig 2.3) (Claessens et al., 2008). The schematic representation of androgen receptor gene was depicted in Fig 2.4. In some NRs, a COOH terminal F region of unknown function is present. The hypervariability of NH2-terminal region or A/B region with respect to both size and sequence is due to alternative splicing and differential promoter usage.

Steroid hormone receptors consist of two transcription activation function domains – AF1 and AF2 that mediate their transactivational activity. AF1 is located in the N-terminal region of many receptors and AF2 is a short amphipathic α-helix

located in the C-terminus of the ligand-binding domain. AF1 has an independent activation function that contributes to constitutive ligand-independent activation by the receptor whereas AF2 is strictly ligand dependent and is conserved among members of the nuclear receptor (NR) superfamily. A third activation function termed AF3 is present at the extreme N-terminus in the human PR-B form making it unique (Sartorius et al., 1994).

The C-terminal domain appears to be complex structurally and functionally, because in addition to its role in hormone binding and nuclear translocation, it may also perform transactivation and dimerization functions.

The DBD which is the most conserved region of the NRs, spans a total of 60-70 residues and it consists of two zinc finger motifs containing amino acids required for the dimerization and recognition of specific target DNA motifs. An array of cysteine residues can be seen in the central domain, compatible with the formation of two zinc fingers (Miller et al., 1985) each of them with a zinc atom tetrahedrically coordinated to four cysteines. This structure, *in vitro* binding studies and functional evidence make it highly probable that the DBD is responsible for the DNA binding activity of the receptors (Evans & Hollenberg, 1988). Interestingly, two separate exons of the receptor gene codes for each of the zinc fingers (Ponglikitnongkol et al., 1988).

The third domain, the hinge or D region is quite variable within the NR superfamily. It functions as a bridge between the DBD and ligand-binding domain (LBD), enabling the NR to adopt different conformations by means of rotation and it might also include localization signals that determine the intracellular localization of the NR (Gronemeyer & Laudet, 1995). This sequence is similar to those found in

SV40 T antigen (Kalderon et al., 1984)

Ligand binding domain (LBD) interacts chiefly with the steroid molecule. Affinity labeling studies of GRs and PRs by Stromstedt et al. (1990) and Carlstedt-Duke et al. (1988) confirmed that the steroid interacts with amino acids that are more than 100 residues apart in the primary structure. Binding of hormone initiates a series of events that result in activation or repression of target genes and it has been demonstrated that hormone binding changes the conformation of the receptor. Fritsch et al. (1992) showed that ligand binding changed the hydrophobicity of the ER, implying a major conformational change. The three-dimensional structure of the ligand-binding domain of the retinoic acid receptor (RAR) bound to ligand, when compared with the RXR in the absence of ligand suggested that a helical region moves to cover the ligand upon binding (Bourguet et al., 1995; Renaud et al., 1995). The activation function AF-2 domain, which is harbored by the LBD, generates ligand-dependent transcriptional activity by the NR. This domain also has many other functions, including interaction with heat-shock proteins, mediation of homo and hetero dimerization (Fawell et al., 1990) and often a transcriptional repression function (Tsai & O'Malley, 1994; Moras & Gronemeyer, 1998). Region F is present in the C-terminal of the ligand-binding domain in some steroid NRs, but this region is not well conserved and its function is unclear.

Antagonists whose mechanism of action has been of interest both mechanistically and clinically for many years are either steroidal or non-steroidal compounds that compete with agonists for binding and prevent activation of receptors. Although it was initially assumed that the binding sites for agonists and antagonists

are the same, evidence points towards the fact that they can be different, although overlapping. A human PR mutant lacking the C-terminal 42 amino acids no longer bound R5020, but still bound the antagonist RU486. Moreover, RU486 acted as an agonist for this mutant. Vegeto et al. (1992). Allan et al. (1992) compared partial protease patterns of agonist bound versus antagonist-bound PRs to reveal that a smaller limit fragment lacking the C-terminal amino acids was produced when antagonist was bound. The antibody C262, does not interact with agonist-bound receptor but interacts with antagonist-bound receptor, suggesting that different conformations are induced (Weigel et al., 1992). Most antagonists induce dissociation from heat-shock proteins and binding to DNA; in some cases these compounds act as partial agonists. The conformation of the antagonist-bound receptor, blocks productive interaction with one or more proteins needed to induce transcription and the likely candidates for this function are the recently discovered co-activators. A second class of antagonists, (e.g. ZK98299, a PR antagonist, and flutamide, an AR antagonist), fail to promote DNA binding efficiently (Beck et al., 1993). ICI 164,384, an antagonist, seems to function in part by reducing the stability of the ER (Montano et al., 1996). Thus there appear to be multiple mechanisms by which antagonists can inhibit receptor action. All antagonists identified to date are synthetic compounds.

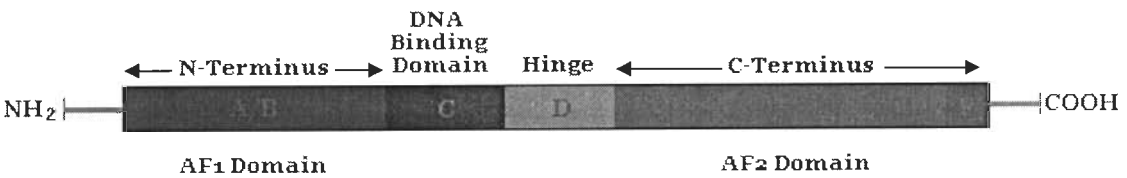


Fig 2.3 Schematic representation of the structure of steroid receptor.

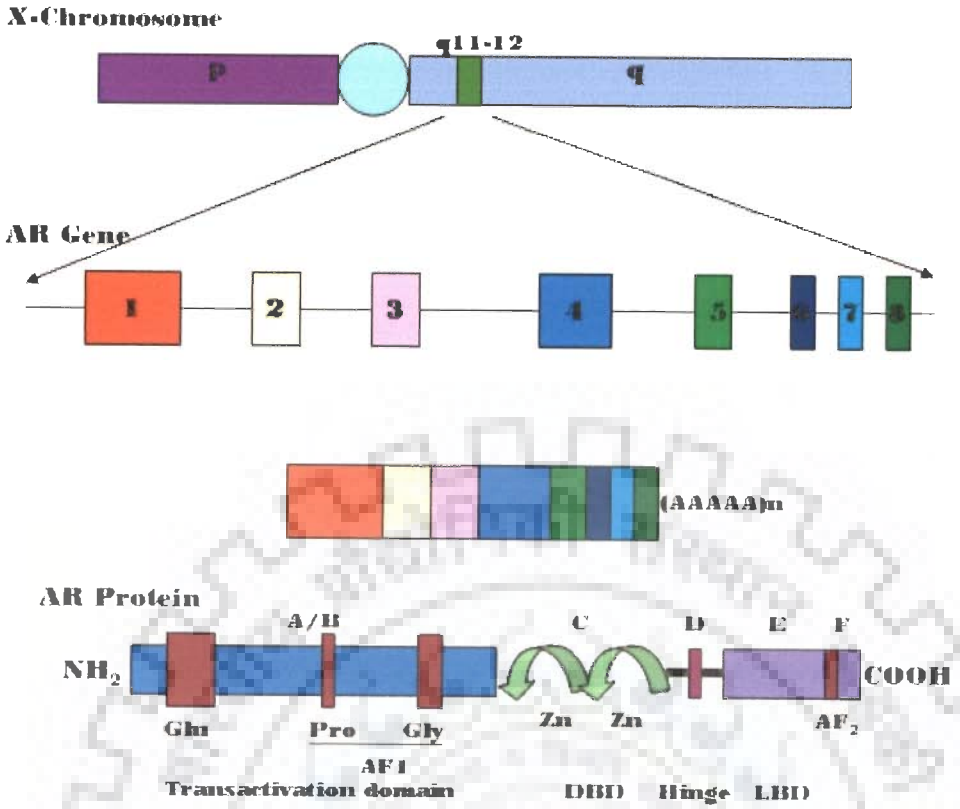


Fig 2.4 Schematic illustration of the location, exon structure and protein domain structure of the AR gene. (Top) The location of AR gene at the q11-12 of X chromosome. (Middle) The AR gene and its mRNA. The AR gene consists of 8 exons (boxes). (Bottom) The AR protein. Relative positions of glutamine (Gln), proline (Pro) and glycine (Gly) repeats within the N-terminal domain are shown by the indicated boxes. The transactivation function domains, AF-1 and AF-2 are located within the N-terminal domain and ligand-binding domain, respectively. Two zinc fingers in the DNA binding domain and a PEST sequence in hinge region are indicated.

2.3.2 Hormone response elements

Steroid receptor-ligand complex binds to specific DNA sequence in the promoter regions of target genes known as hormone response element (HRE). This complex bound to the *cis*-element (HRE) in the DNA recruits other DNA transcription factors and interacts either directly or indirectly with different co-factors, bringing about changes in the local chromatin structure, leading to the formation of a transcriptional active complex (Tsai & O'Malley, 1994; Mangelsdorf et al., 1995) (Fig

2.5). Steroid class III receptors normally homodimerize and recognize the HRE motif AGAACA hexamer palindrome separated by three bases while the remaining nuclear receptors (NRs) of the super family recognize AG(G/T)TCA in the same manner (Beato et al., 1995). However ER homodimer recognizes AGGTCA. Estrogen (ER), progesterone (PR), glucocorticoid (GR), mineralocorticoid (MR), and androgen (AR) homodimers recognize the palindromic hexameric hormone response element sequence, while thyroid hormone (TR), vitamin D (VDR), retinoic acid (RAR) *9-ci s* retinoic acid (RXR), PRAR receptors generally tend to form heterodimers more than homodimers, among themselves and bind to direct repeat response elements (Cooney et al., 1992). The steroid receptor dimer binding to the hexamer response element forms a symmetrical receptor structure that allows a head to head arrangement of the DNA-binding domains leading to target gene expression (Brosems et al., 2004).

Though steroids exhibit different physiological functions, there is a strong similarity between the DNA response element sequences recognized by their respective receptors. For example, the glucocorticoid response element (GRE) which is a semi palindromic repeat of 5'-TGTTCT-3' half-site separated by three base pair spacer is also recognized by AR, PR and MR apart from GR, thus giving it an alternate name, steroid response element (SRE) . Hence the selectivity of hormone action in cells containing more than one receptor form is questionable. Earlier in the evolution, estrogen receptor was separated from the other steroid receptors. This enabled the estrogen receptor to recognize a different palindromic repeat 5'-TGACCT-3' half site named as estrogen response element (ERE) (Zilliacus et al., 1995).

Lindzey et al, (1994) and Klinge, (2001) suggested the existence of minute sequence variations in the natural response elements that are actually recognized by these nuclear receptors in the cell which probably is important for the differential control of gene expression. It is further shown by Barbulescu et al. (2001) and Haendler et al. (2001) that the affinity of the steroid receptor for its response element is not always proportional to the transcriptional activity. This contradiction has to be further addressed. Moreover the magnitude of response from a single response element in the promoter is weak. Therefore in the regulatory regions of the target gene promoters multiple copies of these functional response elements, usually with slight sequence variations, exist in close vicinity, increasing the magnitude of the response (Lin et al., 2000; Klinge, 2001).

The DNA response elements, apart from binding steroid receptors to gene regulatory regions, also impart information onto them by acting as allosteric modulators (Lefstin & Yamamoto, 1998). Studies by them showed that the binding of the steroid-ligand dimer to the response element alters its conformation not only in the DBD but also in more distal regions, which affect the recruitment and interaction of the complex with cofactors and other regulators which help in target gene expression.

2.3.2.1 Selective binding of steroid response element (SRE)

As mentioned earlier, there is always a question regarding the specificity of response by GR, AR, PR and MR as they recognize a common class of steroid response elements. It was however shown that the binding affinity does not alone influence the specificity of steroid receptor action but also involves other important factors. Androgen receptor was recently shown to identify a different class of response

elements that are found to be present in the regulatory regions of genes coding for probasin, for the homeobox protein Pem, for sex-limited protein (Slp) and for the secretory component (Verrijdt et al., 2003). An important difference between selective AREs and SREs is the presence of a highly conserved, right half-site and a more degenerated left half-site (Barbulescu et al., 2001). The major role of specific sequences in the left half-site, in promoting AR selectivity was documented. Moreover, it is also found that the flanking sequences around the ARE also influences the specificity of AR binding (Haelens et al., 2003). All this makes it evident that the binding affinity alone does not affect the specificity but the information imparted by unique differences in the DNA sequences in and around ARE also influence the specificity of AR (Geserick et al., 2005).

Earlier studies suggested that ERE sequence is specific to the estrogen receptor and the receptor and ligand binding affected the receptor conformation and function. The transcriptional activity of ERα and ERβ bound to different EREs could not suggest a correlation between binding and transcriptional activities of these receptor sub groups. It had been suggested that these differences might be due to the differential recruitment of co-activators brought about by the DNA elements (Ramsey et al., 2004).

The function of GR is influenced by the nature of the DNA sequence bound. Two types of GREs are recognized – one is the classical GREs which are recognized by GR dimers and convey transcriptional activation and the second type is a group of negative GREs (nGREs) which mediate repression (Dostert & Heinzel, 2004) and possess one consensus half-site. Several mechanisms of GR binding to GREs and

nGREs have been described. One such mechanism is the allosteric conformational changes of GR bound to nGREs which can be seen in bovine prolactin promoter and mouse proliferin promoter (Dostert & Heinzel, 2004).

Miner & Yamamoto, (1992) have reported that composite GREs in the mouse proliferin promoter may convey stimulation or repression. This depends on the interaction of GR with different AP-1 complexes. Lysine residues, located in the GR zinc finger region, play an important role in the discrimination of the different GREs by the receptor (Starr et al., 1996).

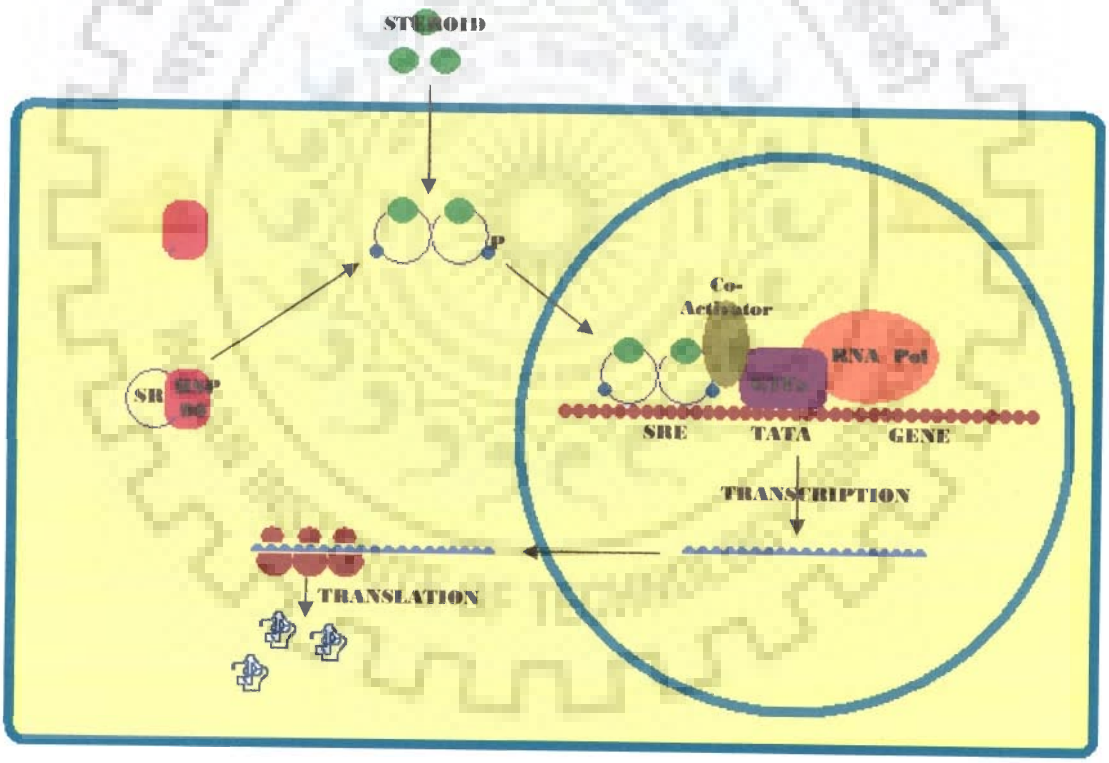


Fig 2.5 Schematic representation of the genomic action of a steroid in the target cell.

2.3.3 Non- genomic action of steroid hormone

Steroid can also act through the receptors present on the plasma membrane and this type of steroid mechanics is known as non-genomic action of steroids. Non genomic action of steroid involves the binding of the steroids to membrane receptors and activating them. This is followed by the up regulation of MAP kinases which leads to the response. While genomic action of steroids takes some time for its manifestation, the non genomic action is instantaneous (Falkenstein et al., 2000; Schmidt et al., 2000).

2.3.4 Interaction of steroid receptors with coregulators (coactivators and coregulators)

Nuclear receptors operate as molecular switches, alternating the transcriptional state of target genes as either repression or activation. Stimulation of the basal transcriptional machinery is incurred by a series of sequential events, including receptor phosphorylation, changes in conformation, and promoter association which in turn promote their interactions with an ever expanding list of co-regulatory proteins, which include dissociation of co-repressors (proteins that attenuate the functions of non-activated receptors) and recruitment of co-activators (proteins that augment the functions of activated receptors) (Wong et al., 1993; Smith & O'Malley, 2004; Lonard & O'Malley, 2005; Perissi & Rosenfeld, 2005). Participation in chromatin remodeling or interacting with general transcription machinery to affect the formation of the pre-initiation complex is the general method by which coregulator proteins modulate the transcription of NR target genes (Perissi & Rosenfeld, 2005).

Selective receptor modulators (SRMs), which are receptor ligands, exhibit

agonistic or antagonistic bio-character in a cell and tissue context-dependent manner. SRM-induced alterations occurring in the conformation of the ligand-binding domains of nuclear receptors, play an important role affecting their abilities to interact with other proteins, such as co-activators and co-repressors thereby suggesting that the relative balance of co-activator and co-repressor expression within a given target cell determines the relative agonist *versus* antagonist activity of SRMs (Smith & O'Malley, 2004).

2.3.4.1 Steroid receptor co-activators

Many studies have shown that the involvement of transcriptional regulators that do not have DNA-binding activity is essential for the steroid receptor target gene expression. Most of these co-regulator proteins are capable of directly interacting with the basal transcriptional machinery in a ligand dependent manner. Some of these proteins also exhibit enzymatic activities like those of histone acetyl transferase (HAT) or deacetylase (HDAC) that help in the modification of the nucleosomal proteins thus playing an important role in gene regulation.

Till date a number of nuclear receptor co-activators were reported and their importance in steroid gene transactivation was thoroughly studied. Some of the co-activators and the authors who reported them have been tabulated in Table 2.1.

Table 2.1 List of known steroid co-activators.

Ets	Schneikert et al., (1996)	FHL2	Müller et al., (2000)
TFIIF, TFIIH.	McEwan & Gustafsson, (1997). Lee et al., (2000)	HBO1	Sharma et al., (2000)
Retinoblastoma protein	Lu & Danielsen, (1998)	cyclin E, cyclin D1.	Yamamoto et al., (2000) Reutens et al., (2001)
CREB-binding	Fronsdal et al., (1998)	PDEF	Oettgen et al., (2000)

protein			
BAG-1L	Froesch et al., (1998)	BRCA1	Yeh et al., (2000)
ARA70/ELE1α	Zhou et al., (2002)	P-TEFb	Lee et al., (2001)
ARIP3	Moilanen et al., (1999)	caveolin-1	Lu et al., (2001)
p160s	Slagsvold et al., (2001)	ARA160, ARA54, ARA55/Hic5 , ARA24.	Hsiao & Chang, (1999) Kang et al., (1999) Fujimoto et al., (1999) Yang et al., (2000)

In the current review, a few co-activators which were extensively studied and shown to be important for steroid transactivation have been discussed below.

p160 Family

The p160 family proteins are the first cloned and best characterized amongst the wide range of coactivators (Lonard & O'Malley, 2005). These include three distinct but homologous members:

1. SRC-1/ nuclear receptor coactivator-1 (NCoA1) (Onate et al., 1995),

2. SRC-2/transcriptional intermediary factor 2 (TIF2)/glucocorticoid receptor interacting protein 1 (GRIP1)/nuclear receptor coactivator-2 (NCoA2) (Voegel et al., 1996).

3. SRC-3/amplified in breast cancer 1 (AIB1)/nuclear receptor co-activator 3 (NCoA3)/retinoic acid receptor-interacting protein 3 (RAC3)/acetyltransferase (ACTR)/thyroid hormone receptor-activated molecule 1 (TRAM1) or p300/CBP-interacting protein (p/CIP) and SRC-1 (NCoA-1) is the progenitor molecule for the SRC-1/p160 family of co-activators (Torchia et al., 1997).

Presence of multiple LXXLL signature motifs is a structural feature of p160 co-activators (Leo & Chen, 2000). It has been recently shown that the AF2 domain of the steroid receptor on ligand binding undergoes a conformational change that

accommodates the p160 family co-activators to bind in its hydrophobic cleft by interacting with the LXXLL motif (McKenna et al., 2002). CBP/p300 that has an intrinsic HAT activity and the CARM1 that shows arginine methyl transferase enzyme activity were found to be associated with these p160 co-activators (Chen et al., 1999). SRC1 is reported to show a weak intrinsic HAT activity. Apart form this, SRC1 also interacts with other transcriptional factors including AP1, NFkB, SRF and p53 (Na et al., 1998; Lee et al., 1999).

p/CAF

p/CAF interacts with CBP and p160 family members by its N-terminal amino acids. Ogryzko et al., (1998) suggested the possible interaction of p/CAF complex with RNA polymerase II core machinery. Though p/CAF alone is inert it is shown to acetylate histones when it is in complex with other associated proteins.

TRAP/DRIP

Thyroid receptor associated protein (TRAP)/vitamin D receptor interacting protein (DRIP) is a prominent group of co-activator that was shown to play an important role in directly contacting the basal transcriptional machinery acting as a mediator complex. After initiation, reinitiation/maintenance of transcription is carried out by TRAP220 and the TRAP/DRIP complex of proteins, which interact with RNA polymerase II. The ligand bound LBD domain AF2 core recruits the TRAP/DRIP through a single subunit (DRIP205/TRAP220/TRIP2) via the LXXLL motif (Rachez et al., 2000).

CBP/p300 (Co-integrator)

CBP/p300 interacts with the nuclear receptor AF2 domain in a ligand

dependent manner although this direct interaction in most nuclear receptors does not appear to be essential (Li et al., 2000). Apart from interacting with nuclear receptors directly, CBP is also capable of forming a ternary complex by interacting with SRC family members and nuclear receptors. The major function of CBP/p300 is to modify the chromatin structure via its intrinsic histone acetyl transferase (HAT) activity (Bannister & Kouzarides, 1996). Therefore it plays an important role in transcriptional initiation of the target gene during the steroid receptor transactivation. Apart from chromatin remodeling, CBP/p300 has also been reported to acetylate non-histone proteins including TF II Eβ, HMG1Y, p53, haematopoetic transcription factor GATA-1, erythroid Kruppel like factor and ACTR functionally modulating them in either negative or positive manner (Lee et al., 2001).

Other important co-activators

Some of the other important co-activators include SRA which selectively co-activates AF1 of steroid receptor; ARA which is an important co-activator interacting with AR and prostate cells, Trips that interact with RXR and show homology to yeast transcription activators, HMG1 that promotes the steroid receptor DNA binding and BRG1 that helps in chromatin remodeling by unwinding the DNA.

2.3.4.2 Functional role of co-activators

Transcription factors compete for binding to a limited pool of accessory factors necessary for gene expression (Wilson et al., 2003). Some co-activator proteins (RNA co-activator) generally do not bind to DNA, but interact indirectly through association with other DNA-binding proteins (*e.g.*, nuclear receptors) and once they are recruited to the promoter, they enhance transcriptional activity through a combination of

mechanisms which include efficient recruitment of basal transcription factors such as template activating factors and TATA-binding protein (Heemers et al., 2007). Moreover, nuclear receptor-interacting co-activators either possess or recruit other nuclear proteins that possess enzymatic activities crucial for efficient gene expression including the ATP-coupled chromatin-remodeling SWI-SNF complex, a number of acetyltransferase proteins (e.g., CBP/p300, pCAF, and p160s), methyltransferases (e.g., co-activator associated arginine (R) methyltransferase-1 (CARM1) and PRMT-1/2) and ubiquitin ligases (*e.g.*, E6-AP and Rsp5) (Chen et al., 2000).

The ligand-activated nuclear receptors bind to DNA and disrupt the local nucleosomal structure through interaction with the SWI/SNF chromatin remodeling machine and acetyltransferases. Then the TR-associated protein (TRAP)/vitamin D receptor-interacting protein (DRIP) complex which is recruited to target gene promoters makes direct contact with components of the basal transcription machinery to bring about transcriptional initiation. Subsequent downstream reactions in the transcription process, such as RNA processing (Monsalve et al., 2000; Auboeuf et al., 2002) and turnover of the receptor-co-activator complex (Lonard et al., 2000) are carried out by additional co-activator molecules (Fig 2.6). Though it has been suggested that receptor and co-regulator association in gene promoters is temporally regulated by primarily employing chromatin immunoprecipitation (ChIP) assays, what exactly controls the dynamic association of nuclear receptors and co-regulators with target genes, or whether these processes can be regulated in a cell-specific fashion has to be determined. But, it has been postulated that the ability of steroid receptors to activate transcription is a product of the ability of the receptor to interact with co-

activators and other proteins required for gene expression and the effect of various enzymatic activities on the formation, function, and disassembly of the receptor-co-activator complex.

2.3.4.4 Steroid receptor co-repressors

The two earliest discovered and best characterized co-repressors are, NCoR and SMRT (Fig 2.6). The current view on co-regulator action elucidates that in the absence of bound agonist, NRs bound to their response elements interact with a co-repressor. These co-repressors bind the nuclear receptors via the so-called co-repressor NR (CoRNR "corner") box (Perissi & Rosenfeld, 2005) which typically has a sequence L/V–X–X–I/V–I (where L is leucine, V is valine, I is isoleucine, and X is any amino acid) or alternatively LXXXI/LXXXI/L (Perissi et al.,1999) which interacts with the co-repressor docking surface on helices 3 through 5 of the receptor ligand-binding domain (LBD) (Privalsky, 2004). When these receptors are not bound to an agonist, they are held in the cytoplasm in a complex with heat shock protein 90 (Pratt et al., 2004).

While the receptor LBD has many common interaction sites for co-activators and co-repressors, helix 12 (also referred to as activation function 2 [AF-2]) of the NR is critical in determining which co-regulator docks: a co-repressor or co-activator. Helix 12 is in an extended conformation in unliganded NRs, providing access to the co-repressor binding site and upon agonist binding, helix 12/AF-2 is repositioned close to the LBD and this three-dimensional change in the NR, makes the three turn α-helix of the CoRNR box unfit for the co-repressor-binding area. Unlike the effect on co-repressors, this helix 12/AF-2 repositioning favors interaction with the two-turn α-

helix of a co-activator NR box, typically having a sequence of LXXLL and once the co-activators interact with the NR, helix 12 forms a charge clamp with helix 3 thus locking the co-activator into place on the agonist-bound receptor (Privalsky, 2004).

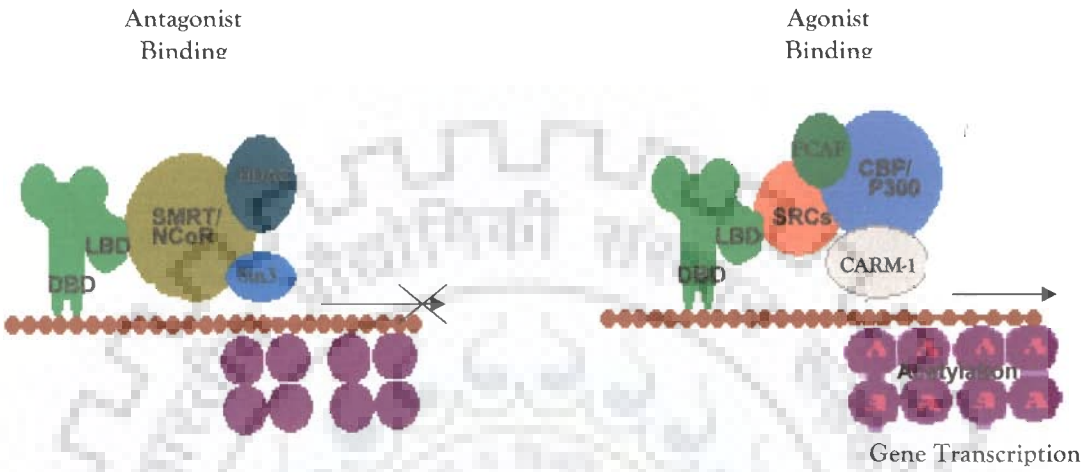


Fig 2.6 Schematic representation of the interaction of co-activators and co-repressors with steroid hormone receptor.

2.4. Endocrine disruptors

Endocrine system is an extremely sensitive system of our body. Any disturbances in this system involving hypersecretion or hyposecretion and activities of hormones can severely affect the physiological system or severely impair development of the organism. Endocrine disrupting chemicals (EDCs) are those chemicals which can cause adverse effects by obstructing the body's hormonal or chemical messengers. A number of descriptions have been proposed to define EDCs and during the Weybridge Conference, 1996, the European scientific and regulatory community has agreed on the following definition of a potential ED (Weybridge, UK, 1996)-

"An **endocrine disruptor** is an exogenous substance that causes adverse health effects in an intact organism, or its progeny, consequent to changes in endocrine function." or "A **potential endocrine disruptor** is a substance that possesses properties that might be expected to lead to endocrine disruption in an intact organism."

In May 1997, the U.S. Environmental Protection Agency (EPA) task force on endocrine disruption (EDSTAC) agreed on the following operational definition:

"An **endocrine disruptor** is an exogenous chemical substance or mixture that alters the functions(s) of the endocrine system and thereby causes adverse effects to an organism, its progeny, or (sub) population."

However the usage of the word "adverse" effect in the foresaid definition created ambiguity and thus in order to achieve consensus, the EDSTAC finally agreed to the following general description (EPA Final Report, 1998)

"The EDSTAC describes an **endocrine disruptor** as an exogenous chemical substance or mixture that alters the structure or function(s) of the endocrine system and causes adverse effects at the level of the organism, its progeny, the populations, or subpopulations of organisms, based on scientific principles, data, weight-of-evidence, and the precautionary principle."

2.4.1 Historical Perspective

Although the concept of endocrine disruption got prominence in the 1990s only, its history can be dated back up to 1930s when Sir Edward Charles Dodds, a British scientist, collected the scientific evidence on endocrine-disrupting effects, ultimately developing diethylstilbestrol (DES), a 'synthetic estrogen' eventually

banned in the U.S. (Krimsky, 2000). Afterwards in 1950, two scientists demonstrated that exposure of chickens to the pesticide DDT, a chemical very similar to DES, results in sexual underdevelopment and suppressed expression of secondary sexual characteristics (Burlington & Lindeman, 1950). They contended that there is a correlation between DDT and development of estrogenic effects in chicken and they finally hypothesized that pesticides may have adverse effects on the hormone system, however, this hypothesis remained scientifically dormant for decades. Again, the issue related to the adverse effects of chemicals on endocrine system received some pace in the late 1970s and early 1980s in conjunction with two initially isolated pathways: human health effects and wildlife biology. While John McLachlan showed the adverse effects of DES and DDT on human health (McLachlan et al., 1980), wildlife biologists commenced to accredit the harmful effect of chemicals on wildlife populations following Rachel Carson study in 1962 which was based on the harmful effects of pesticides on wildlife. The foresaid study showed that use of pesticides can be related with the following adverse effects in wildlife viz. widespread population declines of song birds, eggshell thinning in predatory birds (most notably the bald eagle), reproductive failure and declining populations of otters, dolphins, other sea mammals, alligators, sea gulls and other ecosystem consumers (Tiemann, 2008). In 1990s, Colborn was accredited with preaching the agenda of endocrine disruption in detail. He endeavored to amalgamate the science of wildlife ecology with the human health studies after being a part of the study of environmental degradation in the Great Lakes basin (Colborn et al., 1990). According to the references of Science Citation Index (ISI Web of Science), the word 'endocrine disruption (ED)' was first used by Colborn

in a work which depicted the idea of ecosystem-wide endocrine disruption due to environmental contamination (Colborn & Clemmens, 1992; Vogel et al., 2004). By the end of 1996, scientists were able to identify at least 51 chemicals which were suspected to be EDC including DES, DDT, PCBs, dioxins, and furans and this number increased up to 87 by the end of 2003 (Colborn et al., 1996). In United States, the endocrine disruption screening and testing advisory committee (EDSTAC) formed under the auspices of U.S. Environmental Protection Agency (EPA) is working exhaustively in this area. EDSTAC consists of the representatives from the industries, academic and research groups who help in devising the strategies for screening and studying the different EDCs having diverse chemical structures and belonging to different classes.

2.4.2 In vivo adverse effects of EDCs to Health

In the past few years an increase in the incidence of poor semen quality, testicular cancer, cryptorchidism and hypospadias has been noticed. Experimental and epidemiological studies suggest that these pathological conditions are a result of disruption in embryonic programming and gonadal development during foetal life. Statistical data on testicular dysgenesis syndrome in many industrial nations is alarming. But relatively few chemicals have so far been closely examined for their bioactivity in disrupting the hormonal balance. As per a recent summary, substantial variations in semen quality do exist in samples from different geographical locations (Sefe, 2002). Vinclozolin is known to be a potential antiandrogen and exposure to this chemical during testis differentiation alters programming of germ cells and Sertoli cells, leading to germ cell apoptosis and reduced sperm motility later in adult life

(Uzumcu et al., 2004). An inverse correlation between the concentration of PCB metabolites in blood, seminal plasma and in sperm motility and concentration has been found (Dallinga et al., 2002). A statistical correlation between poor semen quality and high levels of alachlor, diazinon, atrazine, metalachlor and 2,4-D has suggested that pesticide residues may be a factor for the differences in sperm quality between American population residing in rural and urban areas.

2.4.2.1 Testicular Cancer

Testicular cancer is often quoted as the most common type of cancer in young men. The obvious regional differences in incidence and association with birth cohort suggest a possible role of environmental factors in the development of testicular cancers (McKirenan et al., 1999; Nori et al, 2006; Takamiya et al., 2007). The main risk factor for testicular cancer is cryptorchidism, followed by hypospadiasis (Sharpe & Irvine, 2004). A study conducted on workers of the plastic industry exposed to polyvinyl chloride (PVC) demonstrated a significant increase in risk of seminoma (Ohlson & Hardeli, 2000). The mycotoxin, ochratoxin A, a naturally occurring contaminant of cereals, pork and other foods is known to be a xenotoxic carcinogen in animals (Schwartz, 2002). These evidences strongly suggest that occupational and dietary exposure can lead to cancer of reproductive tissue.

2.4.2.2 Congenital abnormalities (crytorchidism and hypospadias)

Cryptorchidism occurs when testis do not descend into scrotal sac, which is usually unilateral. Hypospadias is a developmental abnormality of the penis in which the urethral opening is not located at the tip of the glans penis but elsewhere along the shaft. Exposure assessment of selected organochlorine compounds has revealed that in

adipose tissue of boys undergoing correction for crytporchidism, there is a significant increase in heptachlorohepoxide and hexachlorobenzene residues when compared with adipose tissue from children undergoing surgery for other reasons (Hoise et al., 2000). It has been known that there is an increased transgenerational risk of hypospodiasis in sons of women exposed *in utero* to DES (Klip et al., 2002). However caution needs to be exercised since the registry data for both cryptorchidism and hypospodias could be highly unreliable due to different diagnostic approaches and there is need for prospective studies to make trustworthy conclusions. Nevertheless, proliferation of xenobiotic chemicals can produce potentially disastrous unintended consequences for the male gender development (Steinhardt, 2004).

2.4.2.3 Changes in Sex Ratio

A report from Turkey suggests that mothers who are exposed to hexachlorobenzene during their fertile period show a lower percentage of male births as compared to female ones (Jarell et al., 2002). A remarkable decrease in the birth of male children was noticed especially during the Yucheng disaster when fathers were exposed to PCBs at an age below nineteen. Sex ratios were determined in families who were accidentally exposed to Dioxin as a result of an industrial accident that occurred in Italy in 1976. Paradoxically, in another report, changes in sex ratio were not observed in offspring of parents who were occupationally exposed to relatively high dose of dioxin (Schnorr et al., 2001).

2.4.2.4 Effect on central nervous system

Central nervous system development is acutely sensitive to sex hormone levels, particularly in the perinatal period just prior to and after birth. The expression

of both ERα and ERβ has been shown distinctly in mammalian CNS, with ERβ showing a wider distribution and expression of several splice variants. Although not substantiated, there is suspicion that EDCs may pose a risk for the developmental neurotoxicity during this vulnerable period by interacting with these ERs. Few studies have addressed this issue, but epidemiological associations have linked perinatal exposure to PCBs, pesticides, and polychlorinated dibenzofurans with cognitive and behavioral deficits (Singleton & Khan, 2003). PCBs are known to activate estrogen receptors, alter thyroid hormone status and affect dopamine signaling together with related behavior in rodents. In addition, there are reports that EDCs such as bisphenolA transfer from the mother to the foetus and that the chemicals affect the developing brain, leading to behavior alterations such as impulsive or aggressive behavior (Adriani et al., 2003). Similarly, exposure to p-nitrotoulene a potent EDC has been shown to cause hyperactivity in the rat.

2.4.3 Modes of action of endocrine disruptor chemicals

2.4.3.1 By interfering with steroid hormone biosynthesis, storage, release and transport

A number of reports exist showing the pesticide and other chemicals interfering with the rate regulating steroid biosynthesis enzymes - 3β HSD, 17β HSD, P450scc and StAR protein (Kumar et al., 2008). For example, a wide range of chemicals like plasticizers, pesticides, fungicides, pharmaceuticals were shown to affect steroidogenesis and hypothalamic - pituitary-gonadal axis thereby altering the serum levels of testosterone, luteinizing hormone (LH) and follicle stimulating hormone (FSH). Exposure to the phthalates benzylbutyl (BBP), di(n)butyl (DBP), and

diethylhexyl (DEHP), fungicides like flutamide, procloraz, iprodione, ketoconazole, bisphnol A pesticides like fenarimol, dieldrin, dioxin decreases testicular testosterone production (Ankley et al., 2005; Seidlova-Wuttke et al., 2005; Blystone et al., 2007; Fowler et al., 2007; Adamsson et al., 2008; Howdeshell & Wilson, 2008; Perkins et al., 2008). It was shown *in vitro* that some EDCs like fenarimol, prochloraz and other imidazole fungicides inhibit estrogen biosynthesis by inhibiting the aromatase (CYP19) gene expression (Vinggaard et al., 2005). On the other hand, fenvalerate was shown to interrupt with the FSH stimulated calcium surge interrupting with the calcium homeostasis and its corresponding progesterone biosynthesis resulting in the blockage of ovulation in rats.

The other important aspect of steroid hormones is their bioavailability to the target tissues. Steroid hormones are generally found to be conjugated with sex hormone binding globulin (SHBG) and albumin in the blood. But the biologically available steroid constitutes only the free hormones. Therefore an alteration in the concentration of SHBG will have a major impact on the bioavailable steroid hormones. Estrogens are known to increase the synthesis of SHBG while the testosterone decreases its synthesis. Many pesticides which increase the UDP-glucuronosyl transferase enzyme in the liver were found to decrease the SHBG concentration there by altering the steroid biosynthesis.

2.4.3.2 Interference with hormone receptor and binding

A number of environmental chemicals may interfere with the natural hormones in the steroid receptor binding by mimicking the hormones structurally or by inhibiting receptor binding. However the affinity of these EDCs for the steroid

receptor is usually many times lower than the actual natural hormone (Roy & Pereira, 2005). These EDCs bind the receptor either activating the receptor (agonist) or binding without activating them (antagonist). For example, pyrethroid compounds (permethrin, cypermethrin, fenvalerate and deltamethrin) at high concentration compete with the estradiol in a competitive binding study with ER (Scippo et al., 2004). DDT and its metabolites show binding with the progesterone receptor (Viswanath et al., 2008).

2.4.3.3 By effecting xenobiotic and steroid hormone metabolism

Endocrine disruptors act on the endocrine system by modulating the steroid hormone metabolism by acting on the hormone receptors and further affecting their activity. These EDCs also show their effect by acting on the other related steroid and xenobiotic receptors. Two important regulators through which endocrine disruptive activity usually occurs are the human steroid and xenobiotic receptor/rodent pregnane X receptor (SXR/PXR) (Blumberg et al., 1998) and the androstane receptor (CAR) (Xie et al., 2000). These play a major role in the regulation of xenobiotic and steroid hormone metabolism. SXR/PXR and CAR respond to steroid hormones and xenobiotic ligands, thus mediating the induction of cytochrome P450 enymes (*e.g.* CYP3A, CYP2B, and CYP2C (Pascussi et al., 2003), conjugation enzymes (*e.g.* UGT1A1) (Xie et al., 2003), and transporters (*e.g.* P-glycoprotein, multidrug resistance- associated proteins, and organic anion transporter peptide 2) (Staudinger et al., 2003). These receptors are highly expressed in the liver and intestine and hence carry on all the above activity in these organs. These receptors play an important role in xenobiotic metabolism (e.g. CYP3A and CYP2B) and also regulate bile acid synthesis and cholesterol metabolism (Guo et al., 2003). Activation of transcription by

SXR is ligand dependent like any other nuclear receptor while CAR is always constitutively expressed whose activity is repressed by either unliganded SXR or steroids related to androstenol (Saini et al., 2005).

1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) and its structural analog methoxychlor show antiandrogenic activity by effecting the AR receptor transactivation causing sexual and reproductive abnormalities in male rats (Ousterhout et al.,1981; Gaido et al., 2000). However these compounds were also shown to activate the SXR/PXR and CAR receptors (Mikamo et al., 2003). DDT increased the expression of both these receptor significantly (Wyde et al., 2003). This shows the possibility of a new mechanism of action by these endocrine disruptors. SXR/PXR, unlike other nuclear receptors has broad ligand specificity. Large number of EDCs like bisphenolA activates this receptor. Most EDCs were reported to have a dual action by interfering with the steroid receptor as well as activating SXR/PXR and CAR (Schlumpf et al., 2004). These reports prove that EDCs can also show effect by not directly binding to the steroid receptors.

2.4.3.4 By interfering with the nuclear receptor co-activators

One of the major targets for EDCs is the steroid receptor transactivation. Steroid receptors bind to hormone response elements in the regulatory region of target genes, and then recruit a collection of co-activator proteins and the basal transcription machinery. Level of co-activator availability and the variable competition for them between the nuclear receptor and other transcription factors in different tissues are important factors that regulate the differences in the nuclear receptor transactivation.

It is observed that the changes in the receptor and co-activator expression level

affect the steroid receptor activity. Lonard et al. (2004) have shown that drug treatment increased the transactivational ability of ERα in the presence of xenobiotics by increasing the steady state levels of nuclear receptor co-activator levels. The EDC bisphenol A has shown an increase in the expression of co-activator TRAP220 and ERβ in mouse uterus cells while it only showed an increase in expression levels of ERβ alone in Ishikawa cells (Inoshita et al., 2003). This shows that the EDC can effect the target gene expression by altering the co-activators and that the effect is cell-type specific.

Another important type of EDC action was observed to be a result of the competition between the steroid receptors and the xenobiotic receptors for the available co-activators for their transcription. In this mode of action, the EDC may not interfere directly with the steroid receptor binding. Most of the xenobiotics act via the activation of xenobiotic receptor for their metabolism. However, as these SXR/PXR and CAR share some common co-activators with steroid receptors, an increase in the expression of these xenobiotic receptors during treatment with EDCs/xenobiotics would lead to a competition for these co-activators interfering with the steroid receptor transactivation. For example, in the cells treated with 1,4-bis[2- (3,5-dichloropyridyloxy)]benzene, CAR can interfere with ER mediated transcriptional activity without actually binding to the receptor or to its response element. This repression of ER was rescued when the co-activator GRIP1 is over-expressed in these treated cells (Min et al., 2002).

2.4.3.5 By effecting the proteasome mediated degradation of nuclear receptors

Nuclear receptor superfamily members are degraded through the ubiquitin-

proteasome pathway in a ligand-dependent manner; and receptor turnover by proteasome-targeted degradation prevents cells from over stimulation by endogenous hormones or other activating signals. This probably resets the transcriptional apparatus in preparation for a subsequent response (Dennis et al., 2001). The transcriptional activity of nuclear steroid receptors such as progesterone receptor is down-regulated by inhibition of the ubiquitin proteasome degradation pathway and AR (Lin et al., 2002). Proteasome-mediated degradation of ER in the presence of ER agonists, antagonists, selective ER modulators, demonstrates that transcriptional activity can be affected by modulating receptor stability (Wijayaratne & McDonnell, 2001). From this it can be hypothesized that EDCs can interfere with the proteasome-mediated degradation of receptors/co-regulatory proteins there by affecting the magnitude and duration of normal hormonal responses. For example, when the effects of bisphenol A and Estradiol treatment on ER mediated transcription were compared, it was observed that ERα and ERβ bound to Estradiol interacted directly with SUG1 (suppressor for Gal 1), a component of the proteasome, leading to the proteasome mediated degradation of these receptors and thus maintaining a high level of ER mediated transcription. On the other hand, bisphenolA treatment decreased the ERβ ability to interact with SUG1 although it triggered the transcriptional activity of ER. As a consequence of which the transcriptional activity decreased. Moreover ERβ degradation was much slower in the presence of bisphenolA than in the presence of estradiol or another estrogenic EDC, phthalic acid (Masuyama & Hiramatsu, 2004). Observations related to differential effects of bisphenolA treatment on ER levels (Inoshita et al., 2003) explained that inhibition of ERβ degradation increased its

protein levels, probably causing endocrine-disruption.

Proteasome degradation also maintains the protein levels of other important nuclear receptors like SXR/PXR. Phthalic acid decreases the proteasome degradation of SXR/PXR compares to the endogenous PXR ligand, progesterone, leading to an increase in its protein levels. Steroid hormone levels are increased by the induction of xenobiotic metabolic pathways (Lonning, 1989). However, even small changes in the levels of circulating sex steroids during critical periods of development are expected to have endocrine-disrupting effects. It has been reported by Lonard et al. (2004) that p160 family co-activators, such as GRIP1 and SRC-1, are also degraded via the proteasome.

2.4.3.6 Endocrine disrupters as hormone sensitizers

Short chain fatty acids like valproic acid and methoxyacetic acid (MAA) do not bind the steroid receptor or mimic the endogenous steroid hormones. These chemicals instead increase the steroid receptor activity by activating protein kinases or by inhibiting histone deacetylases enzyme activity (Jansen et al., 2004). Histone deacetylases lead to the decreased gene expression. Valproic acid acts as histone deacetylase inhibitor and also increases the expression of P21 cell cycle regulator (Zhu et al., 2004). Methoxyethanol or ethylene glycolmonomethyl ether which is a common constituent of paints, dyes and fuel additives popularly known as MAA increase the testosterone effect and ER expression (Tirado et al., 2004). Thus these chemicals act by increasing the functional sensitivity of their target steroid hormones there by enhancing the gene expression of the target genes *in vitro* and *in vivo* (Jansen et al., 2004). Font de Mora & Brown. (2000) reported the xenobiotic dependent

mitogen activated protein kinase (MAPK) activation leading to the increased phosphorylation of co-activators of the steroid receptors. Thus these hormone sensitizers cause endocrine disruption through a new nuclear receptor interaction pathway by increasing the sensitivity of the response of the steroids (Vaczek, 2005).

2.4.3.7 EDCs lead to reprogramming of DNA methlyation

DNA methylation is another important gene transcription regulation mechanism, especially in the primordial cells where DNA methylation and demethylation occurs in a sex-specific manner (Reik & Walter, 2001). EDCs interference with steroid receptors during gonadal differentiation could alter germ line development process. Methoxychlor, a fungicide has been shown to affect the sperm viability by interfering with the DNA methylation. It has been shown that the rat embryonic exposure to vinclozolin or methoxychlor during sex development reduced sperm development and fertility in adult testis (Staudinger et al., 2003). These EDCs exposure led to irregularities in genome wide DNA methylation pattern in the male germ line leading to the sexual impairments even in the F4 generation of the exposed rats progeny although they are not subjected to the exposure of these chemicals in their later generations (Anway et al., 2005). Thus the epigenetic effects caused by these EDCs unravel a novel mechanism for EDC disruption of gene expression. The different targets of the endocrine disruptors were depicted schematically in the Fig 2.7 as shown below.

2.4.3.8 Endocrine Cross Talk and Endocrine Disruptors

As described earlier, there are increasing mechanisms reported of EDCs action without directly interfering the receptor binding. EDCs were found to cause steroid

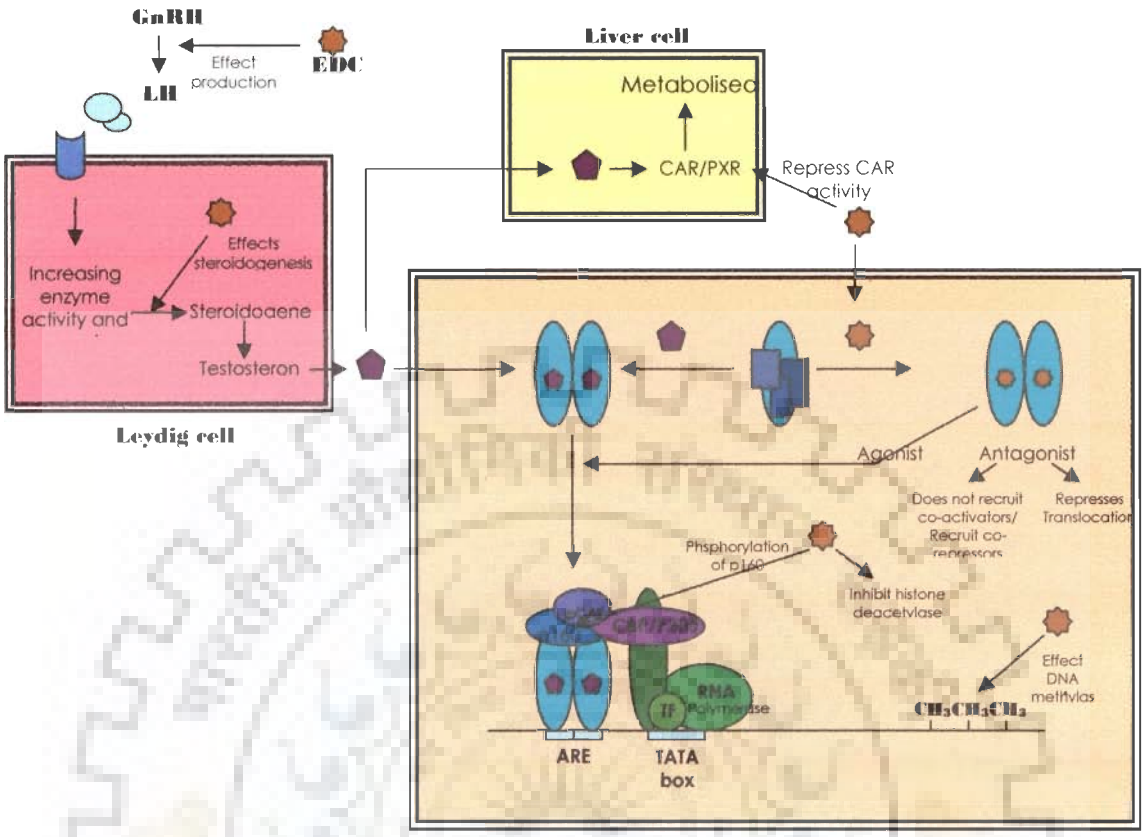


Fig 2.7 Schematic representation of various targets of endocrine disruption chemicals.

endocrine disruption by affecting a different endocrine pathway because of the cross talk between the two pathways. For example, an EDC that affects insulin levels leads to altered biologically available steroid hormone by interfering with the SHBG biosynthesis. In another such condition, DDT and some phthalates were found to exhibit both antiandrogenic and estrogenic effect. Antiandogens are likely to increase the endogenous estrogen levels by blocking he negative feed back loops. This blockage leads to increased LH surges resulting in elevated testosterone levels which finally get converted into estrogens by the increase in aromatase activity. On the other hand, tamoxifen was shown to act as antagonist in one tissue and an agonist in

another. This chemical was studied to affect the tissue specific co-activators. Some co-activators are probably shared by different members of the steroid receptor super family; therefore there exist a competition for these co-activators leading to altered availability of them by the steroid receptors in different tissues leading to differences in their responses in the corresponding tissues.

2.4.4 Endocrine Disruptors: Different Sources and Categories (types) and biological relevance

Our present lifestyle involves usage of a number of synthetic chemicals in various forms like insecticides, pesticides, drugs, detergents, cosmetics, sanitary cleansers, and also the industrial wastes which pollute the air and water. Exposure to these chemicals and their by products may result in manifesting of clinically relevant disturbances both in humans and wildlife. Roy et al. (2004) have shown that one prominent disturbance caused by these chemicals is their contrary effect on the endocrine system, which is also known as endocrine disruption. Exposure of EDC may lead to the production of a number of sex related disorders (Guillette, 2006; Massart et al., 2006). A possible link has been suggested between exposure to EDC and production of a number of diseases like reduced fecundity, abnormal fetal development, delayed onset of puberty, cryptorchidism, abnormal lactation, testicular dysfunction and even various types of cancers (Roy et al., 2005; Buck et al., 2006; Guillette, 2006; Maffini et al., 2006). A list of various pesticides and their mode of action, affecting the endocrine system were grouped in Table 2.2.

Table 2.2. List of few chemicals which have been earlier shown to be potent EDC.

2,4-D	Synergistic androgenic effects when combined with testosterone.	Kim et al. (2005)

Aldrin	Antagonises the action of androgens by binding competitively to their receptors and inhibiting the genetic transcription they induce.	Lemaire et al. (2004)
Atrazine	Androgen inhibitor with a weak oestrogenic effect. Disrupts the hypothalamic control of lutenising hormone and prolactin levels. Induces aromatase activity, increasing oestrogen production. Damages the adrenal glands and impairs steroid hormone metabolism.	Thibaut & Porte (2004)
Carbendazim	Increases estrogen production by increasing aromatase activity.	Morinaga et al. (2004)
Carbofuran	Acute doses increase levels of progesterone, cortisol and oestradiol whilst decreasing testosterone levels.	Goad et al. (2004)
Chlordane	Antagonises the action of androgens via binding competitively to their receptors and inhibiting the genetic transcription they induce.	Lemaire et al. (2004)
Cyproconazole	Inhibits the enzyme aromatase, decreasing the production of oestrogens and increasing the available androgens.	Trosken et al. (2004)
DDT and metabolites	Mimics the action of oestrogen, antagonises the action of androgens via binding competitively to their receptors and inhibiting the genetic transcription they induce. Promotes the proliferation of androgen-sensitive cells. Mimics the actions of oestrogens indirectly by stimulating the production of their receptors.	Lemaire et al. (2004)
Dicofol	Inhibits androgen synthesis, increases the synthesis of oestrogens. Also binds to the oestrogen receptor.	Okubo et al. (2004)
Dieldrin	Antagonises the action of androgens via binding competitively to their receptors and inhibiting the genetic transcription they induce. Mimics the actions of oestrogens indirectly by stimulating the production of their receptors.	Bulayeva. (2004)
Endosulfan	Antagonises the action of androgens via binding competitively to their receptors and inhibiting the genetic transcription they induce. Mimics the actions of oestrogens indirectly by stimulating the production of their receptors. Weak aromatase inhibitor.	Lemaire et al. (2004)
Endrin	Antagonises the action of androgens via binding competitively to their receptors and inhibiting the genetic transcription they induce.	Lemaire et al. (2004)
Heptachlor	Binds to cellular oestrogen and androgen receptors.	Fang et al. (2003)

Mancozeb	Inhibits the production of thyroid hormones.	Fang et al. (2003)
Methoxychlor	Strong oestrogen mimic. Also antagonises the action of androgens via binding competitively to their receptors and inhibiting the genetic transcription they induce. Interacts with the pregnane X cellular receptor, interfering with the manufacture of enzymes responsible for steroid hormone metabolism.	Eriko et al. (2003)
Prochloraz	Antagonises the cellular androgen and oestrogen receptors, agonises the Ah receptor and inhibits aromatase activity, diminishes foetal steroidogenesis	Vinggaard et al. (2006)
Vinclozolin	Potent androgen-receptor antagonist. Competitively inhibits the binding of androgen to its receptor and inhibits androgen-inducing gene expression. Alters androgen-dependant ventral prostate gene expression. Interacts with the pregnane X cellular receptor, interfering with the manufacture of enzymes responsible for steroid hormone metabolism.	Eriko et al. (2003), Fang et al. (2003)

2.5 Development of assay systems for screening of EDC

Incorporation of the issue of endocrine disruption into the Food Quality Protection Act (FQPA) and amendments made to the Safe Water Drinking Act (SWDA) in 1996 made it mandatory for the U.S. Environmental Protection Agency (EPA) to develop and implement a screening program to determine whether hormonal activity existed in environmental chemicals. Consequently the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) was established which recommended the setting up of a program with tiered approach to evaluate potential endocrine activity of chemicals (EDSTAC, 1998). Organization of Economic Cooperation and Development (OECD) developed the conceptual framework having several levels of screening and testing, for the testing and assessment of endocrine disrupting chemicals (OECD 2003). EDSTAC had proposed a two tiered approach

which started with – Tier 1 screening : identifying substances that exhibit hormonal activity using a variety of *in vitro* and *in vivo* bioassay systems and Tier 2 testing : then determining which of these can evoke detrimental effects in test animals (Witorsch, 2002). In the approach proposed by the US-EPA, tier I screening battery detects the hormonal activity of the compounds and tier II characterizes and quantifies those effects (Eertmans et al., 2003). Environmental chemicals have exhibited marked species differences in tier endocrine disruptive activities therefore posing the need for multi-tiered approaches. Several *in vitro* and *in vivo* bioassays available to screen EDCs used today are based on different principles. A few of the assays currently used to screen EDC's are described below.

2.5.1 In vitro Tests

2.5.1.1 Receptor Binding Assay

This assay reports whether the concerned chemical interacts directly with the steroid receptors and determines the affinity of that chemical for the receptors. The chemical has to compete with radio-labeled ligands (like estradiol, androgen) for binding a particular receptor isolated from the nuclear or cellular extract of the target tissue or cells (Wong et al., 1995). *In vitro* binding assays using recombinant receptor proteins synthesized in bacterial systems, for estrogen (Scippo et al., 2004), androgen (Freyberger & Ahr, 2004; Scippo et al., 2004; Yamasaki et al., 2004) and progesterone (Scippo et al., 2004; Viswanath et al., 2008) have been optimized.

These assays cannot discriminate between agonistic and antagonistic actions of particular substances, which is a major drawback. They are suitable only for the detection of hormone-receptor mediated effects but cannot detect pro-steroids

(eg. Pro-estrogen) due to the absence of metabolism in the cell free system (Baker, 2001).

2.5.1.2 Cell Proliferation Assay

Estrogen or estrogen like chemicals causing cell proliferation of the human breast cancer cell line (MCF-7) can be best identified using the E-Screen assay (Lippman & Huff, 1976; Soto et al., 1995). A MCF-7 based proliferation assay, which is one of the most simplest and sensitive assays available for estrogenic compounds, was developed and optimized by Desaulniers et al. (1998) after thoroughly considering all parameters. All the critical parameters that should be taken into account for performing the E-screen assay have been described in a detailed protocol by Rasmussen & Neilsen. 2002. A study by Körner et al. (2004), reported the screening of androgenic compounds using the proliferation of human mammary carcinoma cell line stably transfected with human AR. The disadvantages in using this test are that- a) batch to batch variation of cells results in change of cell proliferation rates, b) it is difficult to perform on environmental samples containing constituents that are toxic to cells and c) it has been shown in some studies that MCF-7 cells proliferate in response to a number of non-estrogenic substances like EGF, progesterone, DHT, insulin-like growth factors, lithium chloride and ethanol thus generating false data (Baker, 2001).

2.5.1.3 Reporter Gene Assays

In this assay, the level of expression of the reporter genes in response to the induction by some ligands or ligand molecules is measured. The mammalian cell lines (MCF7, COS1, CHO, etc.) or yeast strains such as *Saccharomyces cerevisiae* are

transfected with a reporter plasmid and receptor plasmid (in case the cell line does not have the endogenous receptor), for being used in this assay. The receptor plasmid consists of hormone response element (HRE) coupled to the reporter gene, such as luciferase (mostly in the case of mammalian cells) and β-galactosidase (Metzger et al., 1998; Hoogenboom et al., 2001; Roy et al., 2004). The principle behind this assay is that exposure of the cells to a hormonally active compound, will result in the binding of this compound to its receptor and the ligand receptor complex dimerizes and binds to the HRE resulting in the transcription and expression of the reporter genes. β-galactosidase metabolizes the substrate generating a red colour that can be estimated using spectrophotometer (Sohini & Sumpter, 1998), and where luciferase is the reporter, the substrate is luciferin and this generates a flash/glow of light, which can be determined using a luminometer (Roy et al., 2004) (Fig 2.8).

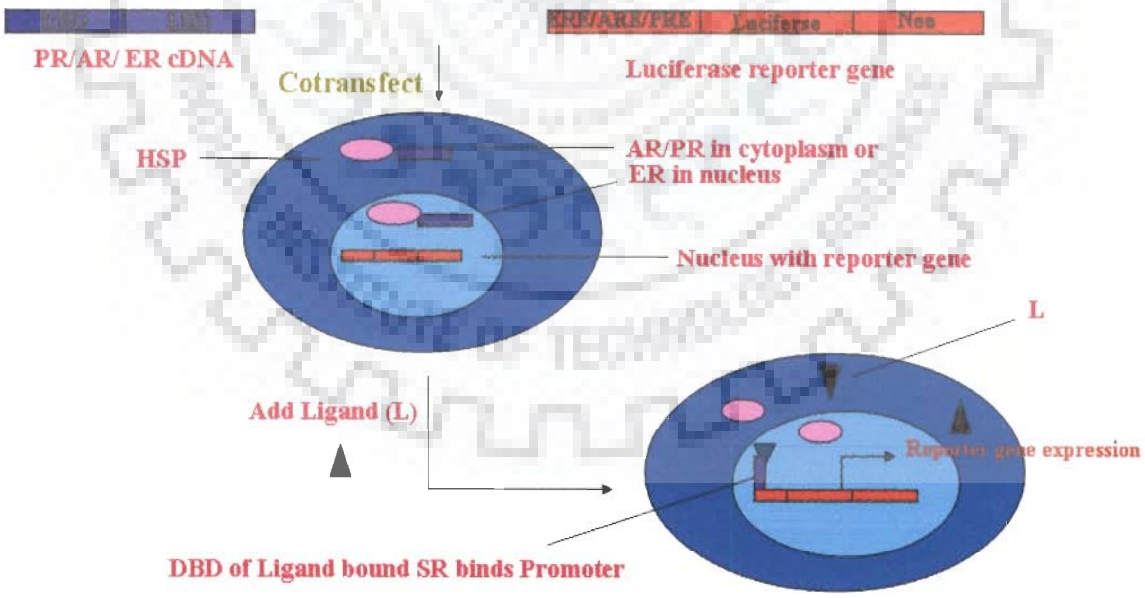


Fig 2.8 Scheme of reporter based transactivation assay

Unlike cell proliferation assay, reporter gene assays avoid batch-to-batch variations as was seen in the transient expression of reporter and receptor genes in cells (Vinngard et al., 1999). Moving a step ahead towards the development of a high-throughput screening system, Bovee et al. (2004) reported the use of green fluorescent protein as the reporter gene expressed in yeasts. This assay reduces time and labour to a great extent by by-passing the cell lysis step done in other reporter based assays.

2.5.2 In vivo Tests

2.5.2.1 (Anti-) Estrogenic Assays

The rodent uterotrophic assay which is being done since 1930 to screen estrogenic chemicals involves the *in vivo* binding of the chemicals to the estrogen receptor. The principle of this assay is, the exogenous estrogen or estrogen like chemical can cause hypertrophy of the uterus in immature rats or ovarectomized rats. The rats are treated for 3 days with the chemicals and then they are sacrificed to collect the uterus, which is physiologically characterized. Owen & Ashby. (2002) evaluated this test by screening some chemicals for their estrogenicity and concluded that it is reliable and can detect estrogenic chemicals. Vaginal cornification assay is another assay used extensively for the identification of estrogenic chemicals where histological changes in vaginal epithelium are detected in response to different chemicals (Martin & Claringbold, 1960). These tests have to be properly validated and standardized by the EPA (Gray et al., 2002) and then they can be used to screen compounds with estrogen and thyroid hormone like activities.

2.5.2.2 (Anti-) Androgenic Assays

Hershberger et al. (1953) demonstrated the best assay to detect androgenic

properties of different chemicals. They analyzed castrated rats, the response of the ventral prostate, seminal vesicles and coagulating glands to exposure of several chemicals, mostly androgenic and some even estrogenic or progesterogenic. The capacity of exogenously administered chemicals to restore the weight of the above mentioned organs of castrated male rats was determined and validated by Dorfman. (1962). A detailed study was performed by Yamasaki et al. (2004), using Hershberger test and binding assay to identify thirty different chemicals for their androgenic activities. Another assay, based on the same principle as the rodent pubertal female assay used for estrogenic chemicals, has been developed that utilizes immature male rats for identifying and screening androgenic and thyroid properties of chemicals. It examines abnormalities in the development of male sex hormones and secondary sex characteristics (Gray et al., 2002).

2.5.2.3 (Anti-) Progesterogenic Assay

Very few chemicals with progesterone like activities have been identified. Tabata et al. (2002) utilized rabbit endometrial transformation test for identifying the *in vivo* activity of progesterogenic chemicals. In this assay, immature female rabbits are administered these chemicals for 5 consecutive days and then they are sacrificed and their uterine sections are studied histologically for their extent of transformation in response to the chemicals.

2.5.2.4 In vivo Assays using Non-Mammalian Vertebrates

Fish is a popular and commonly used model amongst non-mammalian vertebrates used for identifying EDCs. A fish reproductive assay is used to test agents for estrogenic and androgenic effects, in which adult reproductively active fishes are

exposed to the chemicals for 21 days and finally the abnormalities associated with survival, reproductive behavior, secondary sex characteristics and fecundity (number of spawns, number of eggs per spawn, fertility and development of offspring) are examined. Hutchinson et al. (2000) have proposed that this model be included in the higher tier screening program. A second test using fish model is vitellogenin (vtg) protein regulation and its plasma clearance rates. Vtg is a protein expressed mostly in female fish and though the male fish posses this gene they do not express it in a level equal or greater than that expressed in females under normal conditions. Therefore it has become an excellent marker for estrogenic chemicals in aquatic environment. Hemmer et al. (2002) reported that investigation of vtg regulation and plasma clearance in male sheepshead minnows showed that liver vtg levels return to the base-line in estradiol exposed fish, but remain elevated in nonylphenol-treated fish.

2.6 Concluding remarks

Environmental toxicology studies have revealed that EDC could be of real threat to human health and wild life in the coming future. Although concerns have been raised among scientists and public officials, many countries have yet to take serious action to handle the environmental problem. One impediment to achieve proper management of this major problem is the general belief that environmental endocrine disruptors (agonists and antagonists) are less potent than their natural endogenous counter parts and therefore do not have the capacity to cause health-threatening effects. However, it has been shown that when these EDCs act through the same receptor pathway as endogenous hormones, they cause a magnified response. For example, xenoestrogens, which have been deemed of low potency relative to

estradiol are able to shift the dose response curve for estradiol in a reverse order. Moreover, endogenous hormones and their environmental mimics show non-monotonic dose-response curves resulting in different effects at low and high doses. Considering these facts, it seems that the endocrine disruptors are not the class of chemicals that can be ignored and therefore proper risk management strategies for potential adverse effects on human health need to be established.

CHAPTER 3

MATERIALS & METHODOLOGY

CHAPTER 3 (METHODOLOGY)

3.1 Plasmid vectors used in the study

3.1.1 pMALc2X vector used in cloning of hPR(LBD)

pMALc2X is an *E.coli* expression plasmid used in the protein expression and purification (Fig 3.1). It is a high copy number plasmid containing the pMB1 from pBR322 and M13 origin of replications. The multiple cloning site (MCS) is positioned to allow translational fusion of the *E.coli* maltose binding protein (MBP, encoded by the *malE* gene) to the N-terminus of the cloned target protein. Cloning of the target gene at the MCS disrupts expression of *lacZα*, allowing for insert screening by α-complementation. MBP's affinity for amylose allows easy purification of the fusion protein by affinity chromatography with amylose resin. The MBP tag can be cleaved using Factor Xa protease. Transcription of the fusion gene is controlled by the tightly inducible "tac" promoter (P_{tac}). Basal expression from P_{tac} promoter is minimized by lac repressor protein binding, encoded by $lacI^q$ gene. Ampicillin resistant gene is present as a selection marker. The *malE* gene on this vector is deleted for the signal sequence, so, the fusion protein produced remains in the cytoplasm.

3.1.2 pET28c vector used for cloning hAR(DBD & LBD)

The pET28c plasmid is a translational vector containing the highly efficient ribosome binding site from the phase T7 major capsid protein (T7 promoter) (Fig 3.2). "c" suffix denotes the reading frame relative to the BamHI cloning site in the MCS, the vector express from the ATC triplet of the BamHI recognition sequence. pET28c contains a sequence for six histidine residue tag which remains fused to the C-terminal of the expressed protein. The tag can be cleaved using thrombin protease. This tag allows

the affinity purification of the fused protein on Ni-NTA agarose resin. The vector also includes a kanamycin resistant gene (*kan*^{r}) and F1 origin of replication site for synthesis of single stranded DNA. The promoter driving the fusion protein is IPTG inducible.

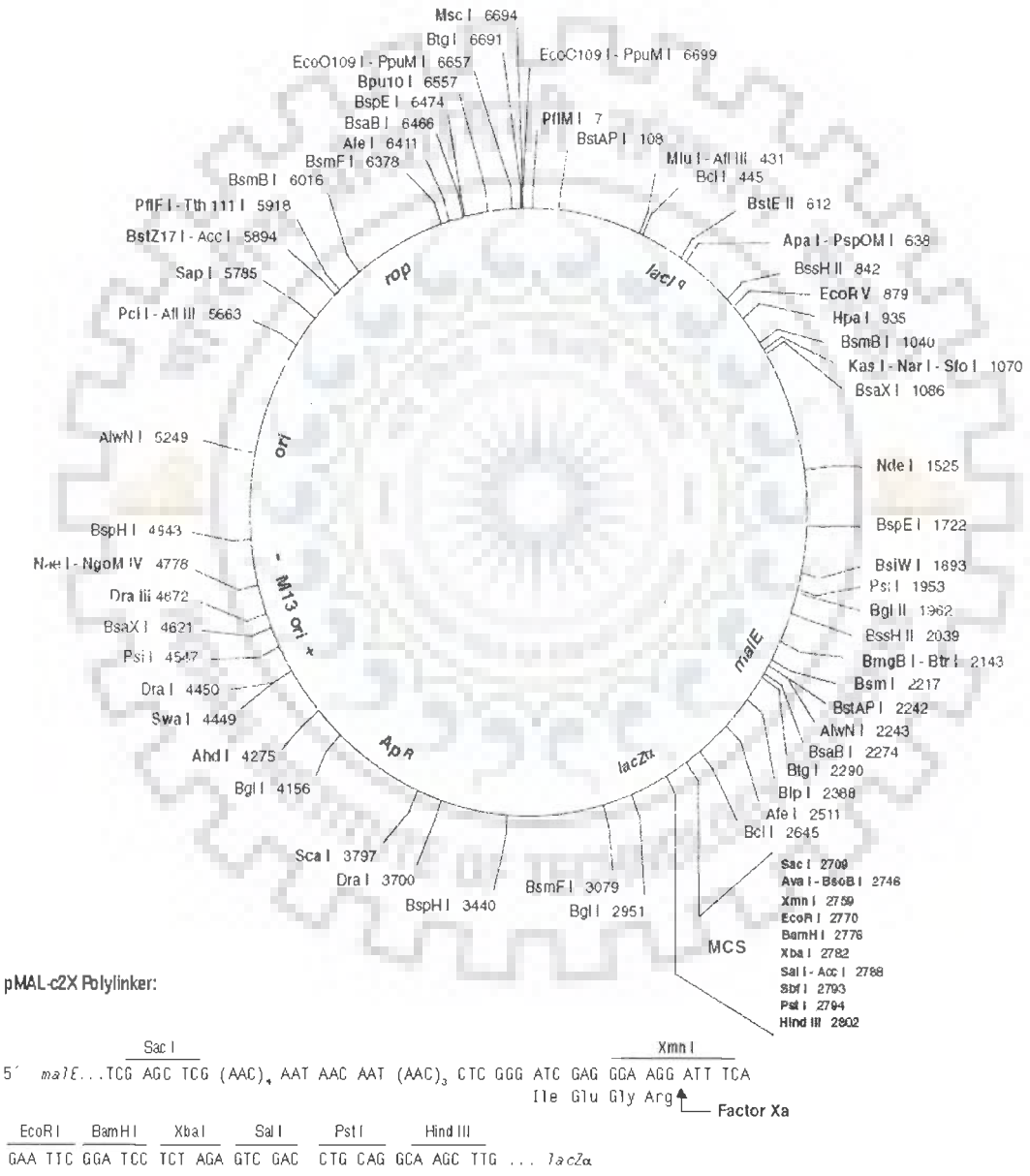


Fig 3.1 Graphical representation of pMALc2X vector and its MCS.

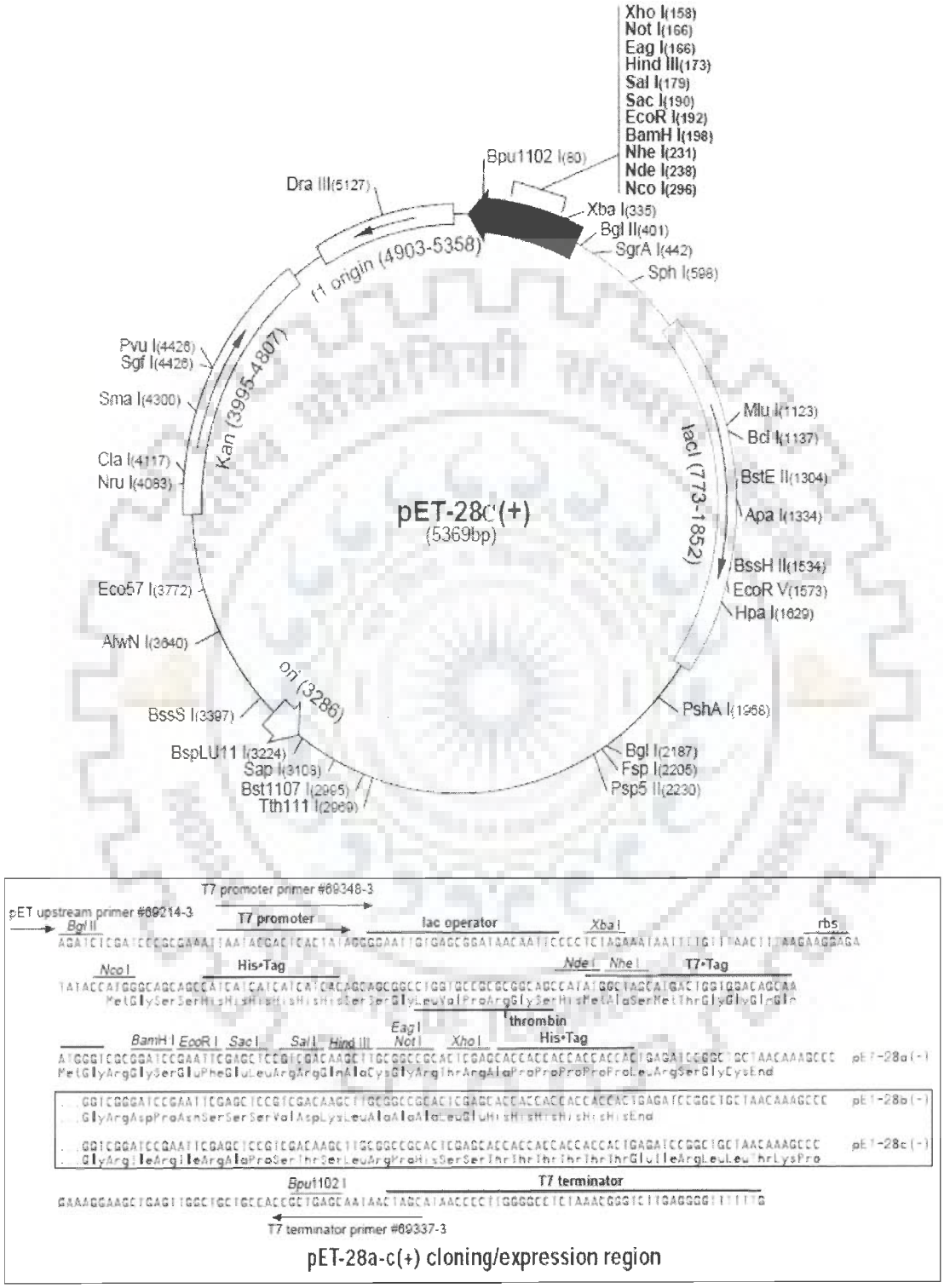


Fig 3.2 Graphical representation of pET28c vector and its MCS.

3.1.3 pcDNA3.1 vector

pcDNA3.1(+) and (-) are derived from pcDNA3 and designated for high level stable and non-replicative transient expression in mammalian hosts. The vector contains - human cytomegalovirus immediate-early (CMV) promoter for high level expression in a wide range of the mammalian cells; multiple cloning sites in the forward and reverse orientation to facilitate cloning in either directions; neomycin resistant gene for selection of stable cell lines; episomal replication in cell lines that are latently infected with SV40 or that express the SV40 large T antigen (e.g. COS-1, COS-7). It also includes pUC origin of replication and ampicillin resistant gene for harvesting the vector in bacteria.

pCMV-CBP vector used in the present study has full length functional CREB binding protein (CBP) cloned under the CMV promoter in pcDNA3.1 vector.

3.1.4 pSG5 vector

The pSG5 is useful for both *in vitro* and *in vivo* expression. Expression *in vivo* is achieved via transient expression in a variety of cell lines (highest level of expression is obtained following transfection of a cell line expressing the T antigen). The f1 origin allows rescue of ssDNA for use in mutagenesis and sequencing. The SV40 early promoter and polyadenylation signal promotes expression *in vivo*, and the T7 bacteriophage promoter facilitates *in vitro* transcription of cloned inserts. The β-globin intron II allows splicing of expressed transcripts. To ligate the gene of interest into the pSG5 vector, unique restriction sites EcoR I, BamH I, and Bgl II (downstream from the promoter) are present.

The pSG5-hAR and pSG5-hPR vectors used in this study were a kind donation from Professor Ilpo Huhtaniemi & Professor Jan Brosens, Imperial College, London, UK.

The vectors have full length human androgen receptor and human progesterone receptor cloned in between EcoRI and BglII sites respectively in the pSG5 vector described above.

3.1.5 Other plasmids used in the study

pGL3-PRE-TK-Luc (referred in the thesis as PRE-Luc for convenience) (kindly provided by Professor Ilpo Huhtaniemi of Imperial College London, UK) consists of multiple copies of progesterone response elements cloned in the upstream of minimal thymidine kinase (TK) promoter driving the luciferase reporter gene in pGL3 vector. pGL3-Probasin-TK-Luc (referred in the thesis as pProbasin-Luc) (also kindly provided by Professor Ilpo Huhtaniemi of Imperial College London, UK) consists of androgen receptor specific probasin *cis*-element cloned in the upstream of the minimal tk promoter driving the luciferase reporter gene in pGL3 vector.

For determining the cAMP responses using the trans-reporter system, the pCRE-Luc plasmid (Stratagene, UK) was used. In this vector, the expression of the *Photinus pyralis* (firefly) luciferase gene in the reporter plasmid is controlled by a synthetic promoter that contains six direct repeats of the cAMP response element (CRE) (Fig 3.3). When a reporter plasmid is transfected into mammalian cells and induced externally by a ligand or adenylate cyclase activator (forskolin) or PKC activator (12-*O*-tetradecanoylphorbol- 13-acetate) the activation of endogenous protein kinases results in the binding of respective transcription factors to the corresponding enhancer elements which in turn stimulate reporter gene expression.

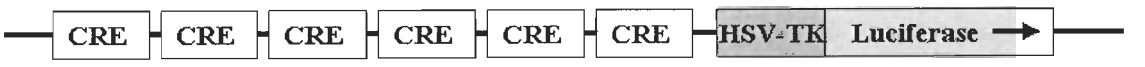


Fig 3.3 Schematic representation of pCRE-Luc plasmid.

The signal transduction pathway was determined using pathway profiling luciferase system (BD Biosiences Clontech, UK). This system contains different luciferase reporter vectors that contain a specific *cis*-acting DNA sequence (enhancer element) upstream of the luciferase gene and one construct (pTAL) without any enhancer element upstream of luciferase reporter gene used as negative control. The key *cis*-acting elements tested in the study were: activator protein 1 (AP1) (pAP1-Luc), cAMP response element (CRE) (pCRE-Luc) and nuclear factor of kB (pNFkB) (pNFkB-Luc) (Fig 3.4). All these specific *cis*-acting DNA binding sequences were located upstream of the TATA-like promoter (pTAL) region from the herpes simplex virus thymidine kinase (HSV-TK) promoter. The vector pTAL was used as null vector, which did not have any *cis*-acting elements in its promoter region and was a negative control in the assay. The increase in the expression of any of these secondary molecules leads to the increase in the luciferase induction thus making them quantifiable.

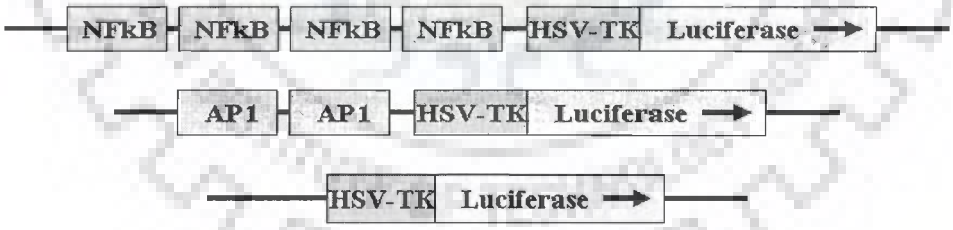


Fig 3.4 Schematic representation of pAP1-Luc, pNFkB-Luc and pTAL.

3.2 Cell lines used in the study

All the cell lines were purchased from National Centre for Cell Sciences, Pune, India. All the cell culture media were purchased from GIBCO (GIBCO, BRL, Inchinnan,

UK) while the antibiotics puromycin and G418 were obtained from Sigma, St. Louis, MO, USA and Promega, Southampton, UK respectively. Superfect transfection reagent was purchased from QIAGEN, Valencia, CA.

NIH3T3 and CHO cells were grown in DMEM medium with or without phenol red supplemented with 10% heat-inactivated fetal calf serum (FCS) (GIBCO, BRL, Inchinnan, UK), penicillin (10^5 U/l) and streptomycin (100 mg/ml) (GIBCO, BRL, Inchinnan, UK) in a humidified 5% CO_2 incubator at 37°C. All the treatments of the cells were performed with charcoal-stripped FCS to reduce the contaminating steroids from the serum.

T47D human breast cancer cells were maintained in Iscove's Modified Dulbecco's Medium supplemented with 10% fetal bovine serum (FBS), BME and MEM amino acids, L-glutamine, sodium pyruvate, and penicillin (10^5 U/l), streptomycin (100 mg/ml) and porcine insulin (10^{-8} M) (Sigma, St. Louis, MO, USA). The culture flasks were maintained in a humidified atmosphere of 5% CO_2 incubator at 37°C.

LNCaP cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, penicillin (10^5 U/l), streptomycin (100 mg/ml). Media was changed every 3 days and stock cultures were passaged once a week by plating out trypsinized cell suspensions. Cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 incubator.

3.3 Transformation of bacterial cells

All the procedures were strictly performed under sterile conditions. DH5α bacterial cells were grown at 37°C, 250 rpm to an OD_{600} of 0.8. The culture was then allowed to chill at 4°C in a pre-chilled falcon tube. These cells were pelleted at 5000 rpm

for 10 min at 4°C and re-suspended in 35 ml of pre-chilled 80 mM $CaCl_2$ and 20 mM $MgCl_2$ solution. The cells were re-pelleted and suspended in 2 ml of 0.1 M $CaCl_2$ to obtain the competent cells. An aliquot of these cells were added with 5 ng of plasmid DNA, mixed well and incubated at 4°C for 30 min. Proper negative and positive controls with no plasmid and known plasmids respectively were included. The cells were subjected to heat shock at 42°C for 90 sec and immediately transferred to ice. LB broth was added to each tube and incubated at 37°C for 45 min. The transformed cells were finally spread plated on antibiotic containing plates corresponding to the antibiotic resistant gene in the plasmid. The plates were incubated overnight at 37°C.

3.4 Plasmid Isolation

The transformed bacteria was grown overnight at 37°C, 250 rpm in LB broth containing an antibiotic. A small aliquot, 1.5 ml culture was pelleted by centrifugation at 5000 rpm for 10 min and the bacterial pellet was resuspended in 100 µl of 50 mM glucose, 25 mM TrisCl (pH 8.0), 10 mM EDTA (pH 8.0) solution. To the suspended cells, 200 µl of freshly prepared 0.2 N NaOH, 1% SDS solution was added and mixed by inverting the tube gently and then incubated for 5 min at room temperature. After the incubation, 150 µl of ice-cold 5 M potassium acetate (pH 4.7) solution was added and the tubes were incubated on ice for 10 min. The solution was then centrifuged at 15,000 rpm for 15 min, at 4 °C and the supernatant was precipitated for the plasmid DNA by adding two volumes of ethanol. The DNA was pelleted at 14,000 rpm for 20 min at 4°C. Finally, the pellet was washed with 70% (v/v) ethanol, re-pelleted, air dried and suspended in nuclease free water. The quality of the plasmid DNA was confirmed by running the DNA on the agarose gel.

3.5 Quantification of DNA using spectrophotometer

2 µl of the DNA/RNA sample was diluted in 1 ml of nuclease free water and its OD was determined at 260 nm and 280 nm against nuclease free water as blank. The ratio of the OD_{260}/OD_{280} was calculated to check the purity of the nucleic acid. The quantity of DNA and RNA can be calculated using the standard value-

For DNA: 1 OD_{260nm} = 50 µg/ml

For RNA: 1OD_{260nm} = 33 µg/ml

3.6 Agarose gel electrophoresis

For a 0.8% gel, 0.4 g of agarose was added to 50 ml 1X TAE Buffer (24.2 g Tris base, 5.71 ml glacial acetic acid, 2 ml of 0.5 M EDTA (pH 8.0) for 100 ml). The mixture was heated in a microwave oven till the agarose was completely dissolved. Two microliter of ethidium bromide (10 mg/ml stock) was added after the solution became lukewarm. This solution was then poured into the gel casting tray fitted with appropriate comb and allowed to solidify. This gel was used for the submerged electrophoresis for separating the DNA samples using 1X TAE as the running buffer. DNA samples (isolated plasmids) were mixed with 6x gel loading dye (10 mM TrisCl pH 7.6, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol, 60 mM EDTA pH 8.0) and loaded into the wells. Gel was run at 7-8 V/cm for 1 h till the dye front reached the last one-third of the gel. The agarose gel was observed under U.V/visible transilluminator.

3.7 RNA isolation from tissues and cell lines

3.7.1 Sample preparation

One milliliter of tri reagent (Sigma, St. Louis, MO, USA) was added to the cells of each well in the 6 well plate. The cell lysate was passed several times through a pipette

to form a homogenous lysate and was allowed to stand for 5 min at room temperature. The lysate was then added with 200 µl of chloroform, shaken vigorously and allowed to stand for 15 min at room temperature. This mixture was then centrifuged at 12,000 rpm for 15 min at 4°C to obtain three phases: a red organic phase, an interphase and a colorless upper aqueous phase. In case of the tissue, it was pulverized in liquid nitrogen using a mortar and pestle. The homogenized tissue was transferred into an eppendorf containing tri reagent and the same procedure was followed as described above.

3.7.2 RNA isolation

The aqueous phase was transferred to a fresh tube and precipitated with 0.7 volume isopropanol for 1 h. After the incubation, it was centrifuged at 12,000 rpm for 15 min at 4°C. The pellet was washed by adding 1ml of 75% ethanol to the tube and re-centrifuging at 12,000 rpm for 5 min at 4°C. The obtained RNA pellet was air-dried at room temperature and dissolved in 30 µl of nuclease free water. The isolated RNA was run on a formaldehyde agarose (denatured) gel and observed under an UV illuminator. Finally, the RNA was quantified and stored in -80°C refrigerator.

3.7.3 RNA gel electrophoresis (formaldedyde gel electrophoresis)

For casting a 1.5% agarose formaldehyde (2.2 M) gel, 1.5 g of agarose was melted in 72 ml of nuclease free water, allowed to cool to 55°C and added with 10ml of 10x MOPS buffer (0.2M MOPS, 20mM sodium acetate, 10mM EDTA) and 18ml of deionised formaldehyde. The gel was allowed to set and later transferred to the horizontal electrophoretic unit. The sample for the electrophoresis was prepared by adding the following components in the same order in an eppendorf tube:

20 µl RNA (upto 20µg)

2 µl 10X MOPS electrophoretic buffer

4 µl Formaldehyde

10 µl Formamide

1 µl Ethidiumbromide (200µg/ml)

The above mentioned sample was boiled at 55°C for 1 h and then cooled in ice. To this sample, 2 µl of 10X formaldehyde gel loading buffer (50% glycerol, diluted in DEPC water, 10mM EDTA, 0.25% w/v bromophenol blue) was added. The RNA was electrophorised with MOPS buffer at a voltage of 4-5 V/cm until the bromophenol blue had migrated approximately $2/3^{rd}$ of the gel length and then visualized under the UV illuminator and documented.

3.7.4 Semi quantitative Reverse Transcriptase PCR (RT-PCR)

All the enzymes and dNTPs were purchased from Genei, Bangalore, India. Total RNA extracted from the control and test samples were quantified and equal amounts from these samples were then reverse transcribed. The reaction was carried out in two steps: cDNA synthesis and PCR amplification.

3.7.4.1 First strand cDNA synthesis (reverse transcription)

As the first step, total RNA was reverse transcribed to form cDNA by adding approximately 100 ng of RNA sample from all the groups in individual 0.2 ml tubes and sterile water was added to bring the volume to 9 µl. To this, 1 µl of $Oligo(dT)_{18}$ primer was added and the vial was placed at 65°C for 10 min and then at room temperature for another 2 min (to remove any secondary structures). The following reagents were added sequentially–

1 µl RNase inhibitor (10 U/µl)

1 µl DTT (0.1 M)

4 µl RT buffer (5x)

2 µl dNTP mix (30 mM)

0.5 µl M-MuLV Reverse transcriptase (50 U/µl)

1 µl sterile water

The solutions were mixed well and incubated at 37°C for 1 h, followed by incubation at 95°C to denature RNA-cDNA hybrids. The samples were then spun briefly and quickly placed on ice.

3.7.4.2 PCR amplification

PCR amplification for a 25 µl reaction volume with the desired number of cycles was performed by preparing a PCR cocktail consisting of following components:

14.2 µl sterile water

2.5 µl 10x PCR buffer

1 µl 30 mM dNTP mix

1 µl Forward primer (100 ng/µl)

1 µl Reverse primer (100 ng/µl)

0.3 µl Taq polymerase (3 U/µl)

4 µl DNA (obtained above)

Once the PCR cocktail was ready, PCR was performed in the thermocycler machine (PTC-200 thermalcycler, MJ Research, USA) using the following program.

	Temperature	Time
Step1 (Denaturation)	94°C	60sec
Step2 (Denaturation)	94°C	30sec

Step3	(Annealing)	**°C	30sec
Step4	(Extension)	72°C	60sec
Step5	Goto Step2 for 30-35cycles		
Step6	(Final extension)	72°C	2min
Step7	End		
Step8	(Product storage)	4°C	1hr

** annealing temperature depends on the primer length and base composition.

The products were stored at -80°C refrigerator and run in an agarose gel as described earlier. All the primers used were purchased from Sigma genosys, Bangalore, India (Table 3.1).

Table 3.1 Primers used for the semi-quantitative RT-PCR in the present study

Gene Primer	Sequence	Product size	Gene bank accession number
P450SCC-F	CgCTCAgTgCTggTCAAAA	688	J05156
P450SCC-R	TCTggTAgACggCgTCgAT		
P450C17-F	GACCAAGGGAAAGGCGT	302	M22204
P450C17-R	GCATCCACGATACCCTC		
3β-HSD-F	CCgCAAgTATCATgACAgA	547	M38178
3β-HSD-R	CCgCAAgTATCATgACAgA		
17β-HSD-F	TTCTgCAAggCTTTACCAgg	653	AF035156
17β-HSD-R	ACAAACTCATCggCggTCTT		
AR-F	TTACgAAgTgggCATgATgA	570	M20133
AR-R	ATCTTgTCCAggACTCggTg		
GAPDH-F	AgACAgCCgCATCTTCTTgT	207	NM017008
GAPDH-R	CTTgCCgTgggTAgAgTCAT		

F, Forward primer. R, Reverse primer.

3.7.5 Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis (SDS PAGE)

For the preparation of 5 ml, 12% resolving polyacrylamide gel, 2ml of 30% acrylamide mix (29% w/v acrylamide and 1% N,N'-methylene-bis-acrylamide) was mixed with 1.3 ml of 1.5 M Tris (pH 8.8), 50 µl of each of 10% SDS (w/v) and 10% ammonium persulfate (w/v) and the volume was made up with de-ionised water. 20 µl TEMED (Sigma, USA) was added to the solution which was then rapidly poured into the gel casting tray. Once the resolving gel was set, 5% stacking gel (containing 170 µl acrylamide bisacrylamide mix (29:1), 130 µl 1.0 M Tris (pH 6.8), 10 µl each of 10% SDS and 10% ammonium persulfate, 1 µl TEMED per 1 ml of solution) was over layered on it. The samples were prepared by adding the sample with 1xSDS gel loading buffer (50 mM TrisCl (pH 6.8), 100 mM DTT, 2% SDS, 0.1% Bromophenol blue, 10% glycerol) in a 1:1 proportion and heating at 100°C for 3-5 min to denature the proteins. The samples were then loaded into the wells of the acrylamide gel and separated using 1xTris-glycine running buffer (25 mM Tris, 250 mM Glycine (pH 8.3), 0.1% SDS) at a voltage of 8 V/cm until the sample dye front had moved to the end of stacking gel and there after the voltage was increased to 15 V/cm. After the electrophoresis was completed, the gels were fixed and stained with Coommassie brilliant blue. For this, the gels were initially stained in the staining solution (0.25 g of commassie in 100 ml of methanol: acetic acid: water solution in the ratio of 5:1:4 respectively), followed by repeated destaining in the destaining solution (methanol:water:acetic acid in the ratio of 5:4:1 respectively).

3.7.6 Western Blot analysis

3.7.6.1 Tissue Preparation

The tissue from control and various treatment groups were homogenized in phosphate buffer saline containing 20% v/v glycerol and 1 mM EDTA and centrifuged at 10000xg for 30 min at 4°C. In case of cultured cells, the cells were scraped and transferred into an eppendorf tube and washed twice with PBS. The cells were then lysed by adding lysis buffer (160 mM Tris, pH6.9, 200 mM DTT, 4% SDS, 20% glycerol, 0.004% bromophenol blue). The protein was stored in -80°C refrigerator. In the case of bacteria, the cells were pelleted by centrifuging at 10,000 rpm for 5 min, re-suspended in lysis buffer and subjected to freezing and thawing for 5 to 6 times to lyse the cells. The lysate was centrifuged at 12,000 rpm for 30 min to obtain the soluble fraction of the protein lysate. Once the protein was obtained, it was quantified using Bradford's reagent using commercially available kit (Genei, Bangalore, India). An equal quantity of protein from sample and control groups was run in 12% polyacrylamide gel electrophoresis (Laemmli et al., 1970; Sambrook &Rusell, 2001) as described earlier.

3.7.6.2 Membrane transfer

After the electrophoresis run was completed, the gel was equilibrated in transfer buffer (3.033 gm of Tris base, 14.42 gm of glycine, 150 ml of methanol in 1 l, the volume made up with water) for 30 min. Simultaneously, PVDF membrane (Millipore corporation, USA) was immersed in 100% methanol for 2-3 seconds, followed by equilibration in transfer buffer for 20 min. The transfer cassette was arranged appropriately with the gel and the membrane and allowed to blot at 100 volts for 120 min.

3.7.6.3 Ponaceau Staining

The membrane was removed and stained in ponaceau stain (0.05 gm of ponaceau,

0.1 ml of glacial acetic acid in 10 ml, the volume made up with water) for 5 min at RT followed by destaining in water for 10 min to check the transfer of proteins. Membrane was completely destained by rinsing the membrane for 3-4 times with distilled water.

3.7.6.4 Immunoblotting of membrane

The membrane was initially incubated in 10 ml of blocking buffer (10% w/v skimmed milk in TBS) for 1 h. After incubation, the blocking buffer was replaced with 5 ml of primary antibody diluted in the ratio of 1:1000 in blocking buffer, and incubated for 1 h at room temperature. The membrane was then washed four times in 200 ml of TTBS (0.1% Tween 20 in TBS) for 10-15 min each. After the final wash, 5 ml of secondary antibody conjugated with ALP (Alkaline Phosphatase) (Genei, India) diluted 1:1000 in blocking buffer was added and further incubated for 1 h. This was followed by four times wash again with 200 ml of TTBS for 10-15 min with a final wash with 50 ml of TBS for 15 min. Finally, the membrane was placed in 5 ml of the substrate solution (BCIP/NBT) (Genei, India) for 30 min and then washed in distilled water to visualize the developed bands.

The antibodies used in the present study were against – human androgen receptor (A kind donation by Dr. R. K. Thyagi, Jawaharlal University, New Delhi, India), human progesterone receptor (Santa Cruz biotechnology, CA, USA), StAR (Kindly donated by Professor D. M. Stocco, Texas Technical University, U.S.A), CBP and NFkB (Santa Cruz, CA, USA) (Kindly donated by Dr. Arun Bandopadhyay, IICB, Calcutta, India).

3.8 Transfection of DNA into mammaliam cell lines

The day before transfection, $8x10^5$ cells per 60mm dish were seeded in complete growth media. The cells were incubated at 37°C in a 5% CO_2 incubator until they grew to

40-80% confluency on the day of transfections. Then, 2.5 µg of DNA was dissolved in TE buffer pH 7.0 (minimum DNA concentration of 0.1 µg/µl) and diluted with cell growth medium without serum or antibiotic to a total volume of 150 µl (in case of co-transfection with two plasmids in the case of transactivation, the receptor containing plasmid DNA and the reporter plasmid DNA were taken in the ratio of 1:4 concentrations respectively). The solution was mixed and spun down for a few seconds. 15 µl of polyfect reagent (Superfect transfection reagent, Qiagen, CA, USA) was added to the DNA solution and mixed well by pipetting up and down. The samples were then incubated for 5 to 10 min at room temperature to allow the complex formation. During the incubation, the cell medium was aspirated from the petriplate and the cells were washed twice with PBS. To the plate, 3 ml of medium was added and allowed to stand. After the incubation of the transfection mix, 1 ml of complete growth medium was added to it and gently mixed. The whole transfection mix was then added to the petriplate. The plate was gently swirled to ensure uniform distribution of the complexes. The cells were incubated with the complexes at 37°C and 5% CO_2.

3.9 MTT assay for cell viability

3.9.1 Plating out cells

Sub confluent layer of cells were trypsinized and collected in DMEM medium containing serum. Cells were counted and seeded at a density of 2.5-50x10^3 cells/ml in a 96 well microtiter plate. 200 µl of the suspension was added to the central 8 columns of the 96 well plate, starting with column 3 and ending with column 10. Then 200 µl of only medium was added to columns 1, 2, 11 and 12. Column 1 was used as blank. The plate was incubated in incubator at 37°C, 5% CO_2.

3.9.2 Test compound addition

Serial dilutions of the test compounds were made in sterile water to prepare required concentrations. Media was replaced with fresh media and the test compounds were added with each concentration in duplicate to the wells of the microtitre plate. The plate was then incubated in incubator at 37°C, 5% CO_2. At the end of the exposure period, the media containing test compounds was replaced with 200 µl of fresh media. The cells were allowed to grow for 2-3 population doubling times..

3.9.3 Estimation of surviving cell numbers

The plates were fed with fresh media at the end of growth period and 50 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5mg/ml) was added to the wells from column 3 to 10. The plates were wrapped in aluminum foil and incubated for 4 h. At the end of the incubation, media was removed and the MTT formazan crystals formed around the viable cells were dissolved in 200 µl of DMSO. 25 µl of Sorenson's glycine buffer (0.1 M glycine, 0.1 M NaCl adjusted to pH 10.5 with 1 M NaOH) was added to each well and the absorbance was recorded at 570 nm immediately in an ELISA plate reader (Oasys, Austria) as the product is unstable. On the basis of comparison to the control, depending on absorbance, percentage viability/well was calculated for every concentration and plotted on a bar graph.

3.10 Cloning of hPR(LBD) in pMALc2x bacterial expression vector

pMAL2x- hPR(LBD) was constructed by sub-cloning the ligand binding domain of the hPR downstream maltose binding protein (MBD) tag in pMalc2X vector (a gift from Dr. Pradeep Chakraborty, Institute of Microbial Technology, Chandigarh, India). The ligand binding domain was amplified from a plasmid pSG5-hPR containing full

length human progesterone receptor (a kind gift from Professor Jan Brosens, Imperial College, London). The following procedure was followed to achieve the goal-

The primers were designed based on the sequence obtained from Genbank, NCBI (accession number: NM_000926). The nucleotide sequence from 3484-4256 codes for the hPR-ligand binding domain (256 amino acids). To enable the directional cloning into the MCS of the pMALc2x vector, two restriction enzyme sites, EcoRI and BamHI, were included into the 5' overhangs of the primers designed. All the guidelines suggested by New England Biolabs catalogue for high efficient digestion by these restriction enzymes were carefully followed.

The primer sequence was as follows-

Primer	Sequence, 5'-3'	Tm
hPRS	GGAATTCCCAGGTCAAGACATACAGTTG	70.5
hPRAs	CGGGATCCCGTCACTTTTTATGAAAGAG	72.5

The PCR was performed using XT5-polymerase (Genei, Bangalore, India) as described earlier with an annealing temperature of 55°C for the first five cycles followed by an annealing temperature of 65°C for the rest of the cycles. The amplified product was purified using the PCR product purification kit from Millipore Corporation. The amplified product and the vector pMalc2X were completely digested with EcoRI/BamHI restriction enzymes. The digested DNA was checked on the agarose gel. To decrease the background of the self ligated vector colonies, the linearised vector was eluted from the agarose gel (Montage Life Sciences Kit, Millipore Corporation, USA) and treated with calf intestinal alkaline phosphatase. Ligation reaction was then set with the vector and insert DNA with the ratios of 1:5 and 1:10 incubated overnight at 16°C. The ligated

product was transformed into DH5α and selected on ampicillin containing LB agar plates. Proper controls were included with all the reaction setups. All the restriction enzymes, ligase, calf intestinal alkaline phosphatase were purchased from New England Biolabs, Ipswich, MA.

Incase of pMALc2x vector, blue white screening was performed for initial screening of positive colonies. For this, 90mm ampicillin containing LB agar medium plate was spread with 40 µl of 2% X-Gal and 7 µl of 0.8 M IPTG and the colonies replica plated on it. Further confirmation of the positive clones was performed by the restriction digestion of the plasmid obtained from the white colonies (Fig 3.5) with EcoRI and BamHI. The recombinant plasmid was sequenced and finally transformed into TB1, *E.coli* bacterial strain recommended for the expression of pMALc2x cloned proteins.

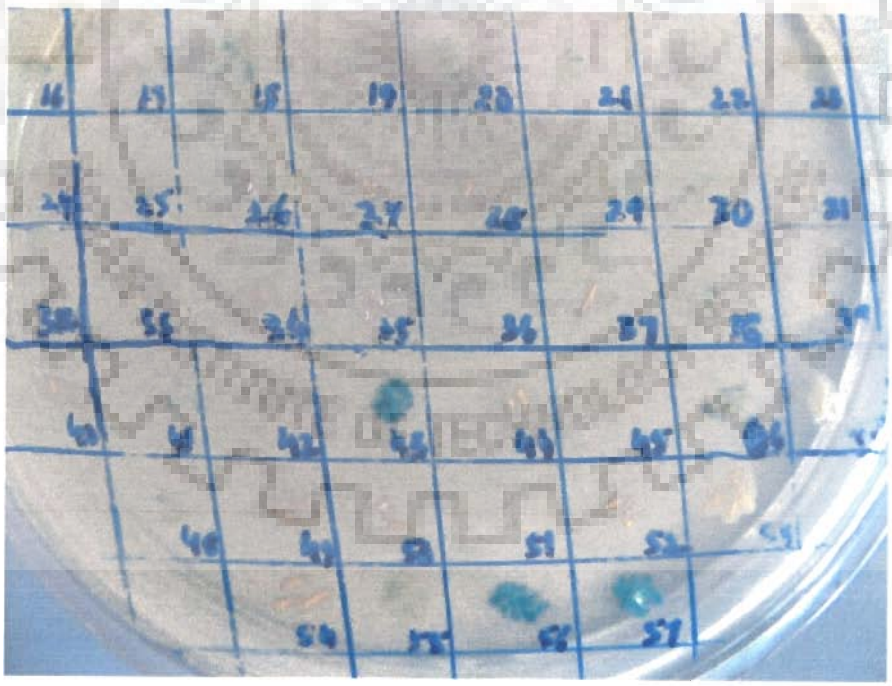


Fig 3.5 Blue white screening of the transformed colonies with ligation product of pMALc2Xvector and hPR(LBD) on Ampicillin, X-Gal and IPTG containing LB agar.

3.11 Purification of hPR(LBD) recombinant proteins

E.coli TB1 carrying plasmid MBD–hPR (LBD) was grown in LB broth containing 0.2% (w/v) glucose and 100 µg/ml ampicillin at 37 °C until the absorbance of 0.6 was achieved at 600 nm. Isopropyl-1-thio-β-D-galactoside (IPTG) at a final concentration of 0.1 mM was added and the culture was induced for 10 h at 25 °C. Cells were harvested by centrifugation at 5000 rpm at 4°C for 10 min. The cell pellet was re-suspended in 20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA, 1mM phenylmethylsulfonyl fluoride (PMSF) and disrupted by freezing and thawing in liquid nitrogen and 37°C respectively. The whole cell lysate was centrifuged at 40,000 rpm for 10 min at 4°C to obtain the clear soluble fraction of the proteins. The concentration of the total soluble fraction was estimated using Bradford estimation kit (Genei, Bangalore, India).

For purification of the recombinant hPR protein, amylose resin (New England Biolabs, Ipswich, MA) was equilibrated with the column buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1mM EDTA). The sample (5-10 mg protein/ml resin) was loaded onto the column and eluted with 10 mM maltose containing column buffer. The flow rate was 1 ml/min. The eluted protein was dialysed against 20 mM Tris-Cl, pH 7.2, 20 mM NaCl buffer. The dialysed protein was further loaded on a DEAE- Sepharose column which was equilibrated with the same buffer. The column is washed with 20 mM Tris-Cl, pH 7.2, 200 mM NaCl (as optimized by step gradient elution to remove the non-specific proteins bound to the column) and later the protein was eluted with 20 mM Tris-Cl, pH 7.2, 350 mM NaCl. The eluate was then concentrated and sample prepared for SDS-PAGE.

3.12 Cloning of hAR(DBD & LBD) in pET28c bacterial expression vector

pET28c- hAR(DBD & LBD) was constructed by sub-cloning the DNA binding domain and ligand binding domain of the hAR downstream the 6XHis tag in pET28c vector (a gift from Dr. Pradeep Chakraborty, Institute of Microbial Technology, Chandigarh, India). The following procedure was followed to achieve the goal-

RNA was isolated from LNCaP cells using Tri reagent (Sigma) as described earlier. The Androgen receptor DBD & LBD was amplified by RT-PCR. Androgen receptor DBD & LBD was amplified using sequence specific primers containing the restriction sites for BamHI and XhoI in their overhangs (underlined). The primers were designed based on the sequence available from Genbank, NCBI (accession no. NM_000044).

The primer sequence was as follows

Primer	Sequence, 5'-3`	Tm
hARS	CGGGATCCGAAAGACCTGCCTGATC	76.4
hARAs	CCGCTCGAGCTGGGTGTGGAAATAG	75.4

The RT-PCR was performed using XT5-polymerase (Genei, Bangalore, India) as described earlier with an annealing temperature of 58°C for the first five cycles followed by an annealing temperature of 68°C for the rest of the cycles. The amplified AR(DBD & LBD) was then cloned into pET28c. A similar procedure described earlier in case of hPR(LBD) cloning was followed. The positive colonies were screened and confirmed by double digestion with the respective cloning enzymes (BamHI and XhoI). After the positive clone was reconfirmed by sequencing, the recombinant plasmid was retransformed into BL21, specific protein expression strain for pET28c vector.

3.13 Purification of hAR(DBD & LBD) recombinant protein

The cloned AR-LBD gene fragment was cloned in the MCS of the pET28c vector so that the expressed protein was tagged with 6xHis on its N-terminal end. This protein was therefore purified on Nickel- NTA column beads (Invitrogen-life technologies, CA, USA) that have the affinity for His tag. The culture was grown at 37 °C initially to an O.D of 0.8 and then the protein expression was induced with 0.5 mM IPTG and further incubated at 25°C for 3 h. At the end of the incubation, the cell pellet was obtained by centrifuging the bacterial broth at 10,000 rpm for 10 min. The cell pellet was re-suspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) and lysed by sonication. The cell lysate was centrifuged at 40,000 rpm for 10 min at 4 °C to obtain the soluble fraction which was precipitated with 60% ammonium sulphate to concentrate the AR protein fraction in the protein sample. The protein was then subjected to dialysis against the lysis buffer several times to remove all the excess salts. The protein was then mixed with lysis buffer equilibrated Ni-NTA beads and allowed to mix gently on a rocker for an hour. The beads were loaded into a column and washed extensively with 25 volumes of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 150 mM imidazole, pH 8.0). The protein was finally eluted with 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0. The protein eluate was collected and subjected to SDS-PAGE and immunoblot analysis using hAR antibodies.

3.14 Ligand binding assay

The saturation, ligand binding analysis and dissociation constant (K_D) was determined as per the method described earlier (Pillon et al., 2005). Briefly, the 1 mg/ml recombinant protein hPR(LBD) or hAR(DBD & LBD) was incubated with a range of

[^{3}H]Progesterone (57 Ci/mmol specific activity) (BARC, Mumbai, India) or [^{3}H]R1881 (60 Ci/mmol) (Amersham Pharmacia Biotech, Roosendaal, Netherlands) concentrations, respectively in a reaction mixture (100 µl) containing 50 mM KH_2PO_4 pH (7.4), 10% glycerol, 0.1% α-thioglycerol, 25 µg/ml leupeptin, 1mM EDTA in the presence or absence of about 100-fold excess of unlabeled progesterone and testosterone respectively. The reaction mixture was then incubated at 4°C for 24 h. At the end of the incubation, 100 µl of dextran-coated charcoal solution consisting of 0.5% Norit A (Sigma, USA) and 0.05% dextran-70 (Pharmacia Fine Chemicals) was added to the incubation mixture and further incubated for 10 min at 4°C to remove the unbound free ligand. The mixture was then centrifuged at 1800 rpm for 5 min. The radioactivity of 100 µl of the supernatant was measured in 2 ml of Aquasol-2 (Packard) using a liquid scintillation counter (Beckman-Coulter, Roissy, France). The K_D was calculated as the free concentration of radioligand at half-maximal specific binding by linear Scatchard transformation using Graph-Pad Prism statistics software.

For screening the chemicals by competitive receptor binding assay, relative binding affinity (RBA), the recombinant protein was incubated with radio-labelled steroid and increasing concentrations of test chemicals. The unbound labeled steroid was removed and the radioactivity of the bound steroid was determined. Experiments were performed in triplicates and repeated twice. For each chemical, the concentration required to inhibit specific progesterone/testosterone binding by 50% (IC_{50}) was determined.

3.15 Sample collection and preparation

Water effluents were sampled from the outlets of five different waste water treatment plants. These samples (1 litre) were extracted immediately after collection by

using solid phase extraction. Water samples were filtered through 0.1μm glass fibre filters (Type GMF5, Rankem, Mumbai, India), acidified with concentrated sulfuric acid to pH 2 into two 1 l samples. One litre of this sample was then extracted using reverse phase C18 solid phase extraction columns (RP-C18 SPE, Rankem, Mumbai, India) and dissolved in 500 μl of dimethylsulfoxide (concentration factor of 2000). Further, a 1:100 dilution in medium for the sample extracted with C18 solid phase extraction columns resulted in the highest test concentration of 2ml equivalent/well. This was further serially diluted to 1, 0.5, and 0.25 ml equivalent/well to determine the concentration dependent effects.

Most of the test chemicals and pesticides screened were purchased from Rankem, Mumbai, India while the other chemicals were a kind gift from Ilpo Huhtaniemi, Imperial College, London, UK. All the test chemicals were dissolved in ethanol to prepare 100 mM stock solution. The stock solution concentration was selected based on the solubility of the different compounds used in the assay.

3.16 Stable transfection

Stable transfections were performed by the Lipofection method according to the manufacturer's recommendation (Superfect transfection reagent; QIAGEN, Valencia, CA). After 48 h, the cells were trypsinized and fresh cells were plated on a 150mm diameter plate and selected in medium containing 1 g/l of neomycin (G418) (Promega, Southampton, UK). The medium was changed two times a week. After about 2-3 weeks, some antibiotic resistant clones appeared in the plates. They were picked using cloning rings and re-plated in 24-well culture plates. Confluent cells were split into triplicate in 24-well plates. When these cells were confluent, one plate was treated with 10 nM of the

respective steroid, and the incubation continued for 24 h. Thereafter, the cells in the treated plate and in one untreated plate were assayed for luciferase activity using the luminescence kit (Promega, Madison, USA). The clones showing highest activity in the presence of its respective steroid with a low background were selected from the remaining untreated plate. The assay was finally optimized for the number of cells/well of a 96 well plate and the time of incubation with the test compound that gave the maximum luciferase response.

3.17 Screening and Luciferase assay

During the assay, about 20,000 cells per well were plated on 96-well plate in 200 µl of DMEM medium without phenol red and with 10% FCS. The next day, the cells were washed with PBS and the medium was changed to 200 µl fresh DMEM with 10% charcoal-stripped FCS. After about 3 h, the test compounds were added to the cells. The concentrations of the stock solutions of all the test compounds were 100 mM in ethanol. They were then further diluted in the medium, resulting in the final concentration of ethanol in the incubations to be 0.1%. The cells were incubated with the compounds for another 24 h. Luciferase activity was measured using the luciferase reporter assay system kit (Promega, Madison, USA) according the manufacturer's instructions in a multiplate reader (BMG Labtech, Offenburg, Germany). In case of screening for antagonistic activity, the cells were added with test chemicals along with either 0.4 nM progesterone or 0.3 nM testosterone (determined IC_{50} values for the corresponding cell lines) for their respective assays and the decrease in luciferase activity was determined.

3.18 *In vivo* screening of EDCs

3.18.1 Animal maintenance

The *in vivo* study was performed using adult male albino rats, *Rattus norvegicus,* of age group around 6-7 weeks. Animals were purchased from the animal house facility of All India Institute of Medical Sciences (New Delhi, India) and were in healthy condition at the time of purchasing. They were housed in a well-ventilated animal house with a temperature maintained at 22-23°C, humidity 50-55% and light cycle of 14 h light: 10 h dark. The animals were fed with a balanced animal feed (Ashirwad Animal Feed Industries, Punjab, India) and had access to hygienic drinking water *ad libitum*. The animals were acclimatized to the animal house condition for 10 days prior to the experiments. All the procedures were approved by the Institutional Animal Ethics Committee and confirmed to the UFAW Handbook on the Care and Management of Laboratory Animals.

3.18.2 Screening of test samples for (anti)androgenicity by Hershberger assay.

Rats when forty-two-days old were castrated by removing testis and epididymis and were allowed to recover for next 10 days. The animals were then grouped (n = 8) as follows:

For determining androgenicity in WWTP (inlet and outlet) efluents

Group I : treated with 10% alcohol (control).

Group II : treated with 50 ml equivalent of inlet (IIa) and outlet (IIb) water samples.

Group III : treated with 100 ml equivalent of inlet (IIIa) and outlet (IIIb) water samples.

Group IV: treated with 150 ml equivalent of inlet (IVa) and outlet (IVb) water samples.

Group V : treated with 200 ml equivalent of inlet (Va) and outlet (Va) water samples.

For determining the anti-androgencity of chlorpyrifos and piperophos

Group I : treated with 10% alcohol (-ve control)

Group II : subcutaneous injections of testosterone propionate (0.4 mg/kg/day).

Rats in groups III to VI received 0.4 mg/kg testosterone propionate along with the test chemicals during the treatment.

Group III : treated with 5 mg/kg/day of chlorpyrifos.

Group IV : treated with 10 mg/kg/day of chlorpyrifos.

Group V : treated with 15 mg/kg/day of chlorpyrifos.

Group VI : treated with 5 mg/kg/day of piperophos.

Group VII: treated with 10 mg/kg/day of piperophos.

Group VIII: treated with 15 mg/kg/day of piperophos.

Vehicle and extracted water samples were administered via gavages to 51 day old castrated rats for 20 consecutive days. The dose used in the experiment was optimized earlier and was below the LD_{50} dose. After approximately 24 h of final treatment, androgen-dependent accessory sex glands or organs namely, ventral prostrate, seminal vesicles, glans penis, vas defferentia, Cowper's gland were carefully removed and weighed.

3.18.3 Effects of test samples in intact rats

Intact rats were grouped (n = 8) as follows and treatment was initiated when rats were 51 days old:

Group I : treated with only alcohol (10%) as vehicle

Group II : treated with 50 ml equivalent of inlet (IIa) and outlet (IIb) water samples

Group III : treated with 100 ml equivalent of inlet (IIIa) and outlet (IIIb) water samples

Group IV : treated with 150 ml equivalent of inlet (IVa) and outlet (IVb) water samples

Group V : treated with 200 ml equivalent of inlet (Va) and outlet (Va) water samples.

Group VI : treated with 10 mg/kg/day of chlorpyrifos.

Group VII: treated with 10 mg/kg/day of piperophos.

Vehicle and extracted water samples were administered via gavages to 51 day old castrated rats for 20 consecutive days. After 24 h of final dose, the rats were sacrificed; testis and blood from each of control and treated groups were collected and immediately placed in liquid nitrogen for further analysis. RNA was isolated from the testes of the control and treated groups and used for the analysis of expression profiles of key steroidogenic enzymes (3β-HSD, 17β-HSD, P450scc, P450C17) and AR by semi quantitative RT-PCR as described earlier. The isolated protein was used for the analysis of expression profile of StAR protein by immunoblot and for the estimation of the activity of the 3β -HSD and 17β-HSD enzyme *in vitro*. The blood serum was used for the estimation of LH, FSH and testosterone hormones.

3.18.4 Estimation of steroidogenic enzyme activity in vitro

3β-HSD and 17β-HSD are the two crucial enzymes in the steroid biosynthesis pathway and their activities have been found to be effected by some EDCs. These two enzymes were assayed according to the method described earlier (Talalay, 1962; Sarkar et al., 1991; Shukla et al., 2001; Krazeisen et al., 2001). Briefly, the testes and ovaries removed from different treated and control groups of rats were homogenized in 20% spectroscopic grade glycerol containing 5 mM potassium permanganate and 1 mM EDTA followed by centrifugation at 10,000xg for 10 min at 4°C. For 3β-HSD activity, 1 ml aliquot of the supernatant was mixed with 100 μM sodium pyrophosphate buffer (pH 8.9), 0.9 ml double distilled water and 30 μg DHEA making up the incubation mixture to a volume of 3 ml. Enzyme activity was measured at 25°C after the addition of 0.5 μM of NAD^+ to the mixture against a blank (without NAD^+). For the determination of 17β-HSD

(type III activity), 1 ml aliquot of the above centrifuged supernatant was mixed with 400 μM sodium pyrophosphate buffer (pH 10.2), 25mg bovine serum albumin and 0.3 μM testosterone bringing the total reaction volume to 3 ml. Enzyme activity was measured after the addition of 1.1 μM NADH to the mixture against a blank without NADP. By this experiment, the activity of reverse reaction catalysed by 17β-HSD (conversion of testosterone to androstenedione) was determined. The forward reaction (conversion of androstenedione to testosterone) was performed under similar conditions using 50 mM phosphate buffer, 30 nM androstenedione and 7 mM NADPH. One unit of enzyme is equivalent to a change in the absorbance of 0.001units/min at 340 nm.

3.18.5 Estimation of blood serum Testosterone, LH and FSH using ELISA

For the determination of serum hormones, blood was collected by cardiac puncture from the intact rats on completion of the treatment and allowed to clot at 4°C overnight. The serum was then aspirated, centrifuged at 2000Xg for 10 min and the clear supernatant was used for hormone assays. The assays were performed using the commercial enzyme immunoassay kits as per manufacturer's instruction (Omega Diagnostics, UK and Transasia Biomedical, Mumbai, India). The hormones that were assayed included testosterone, luteinizing hormone (LH) and follicle stimulating hormone (FSH). The interassay and intrassay co-efficient of variations for all the assays were below 12% and 6% respectively and the assays were highly specific for each hormone analyzed.

3.18.6 GC-MS analysis of the WWTP effluent

One liter of collected effluent water sample was extracted with DCM in same ratio as prepared for gavaging to the rats, concentrated to 1 ml and then evaporated to dryness in a 1.5 ml vial. Further extract was derivatized in same vial by addition of N,O

bis(trimethylsilyl) trifluoroacetamide (BSTFA) as a silylation agent. The derivatization was performed by reconstituting the dried elute sample with 110 µl of acetone:hexane (1:1, v/v) and 20 µl of BSTFA. After that, the vials were capped and placed in a water bath at 65°C for 30 min to ensure complete derivatization of the compounds of interest. The derivatization leads to the silylation of all the target compounds except hexachlorobenzene. After completion of derivatization, 2 µl of the reaction mixture was injected (in split less mode) into the GC-MS system. GC-MS analysis was performed using the protocols described earlier (Liu et al., 2004; Leusch et al., 2006) with some modifications. EI-MS analysis was performed on a Perkin-Elmer Clarus 500 gas chromatograph coupled with a mass spectrometer and an autosampler. An Elute -1 Crossbond® (5% Diphenyl – 95% Polysiloxane) column of 30 m x 0.25 mm i.d. x 0.25 µm film thickness was used. The GC column temperature ranged from 75°C (initial equilibrium time for 2 min) to 155°C at a temperature increase of 10°C/min, 155 - 260°C at a temperature increase of 15°C/min and 260°C -300°C at a temperature increase of 12°C/min.. The mass spectrometer was operated in the full acquisition electronic impact mode (70 eV). The presence of the compounds was confirmed by matching the retention time of the standards with that of corresponding peaks in the chromatogram of sample and further by analyzing mass spectra of the matching peaks.

3.18.7 Determination of serum alkaline phosphatase, Acid phosphatase, SGPT and SGOT

3.18.7.1 Serum oxaloacetate and pyruvate transaminase activity (SGPT and SGOT)

SGOT and SGPT activity were determined by the method described by Reitman & Frankel. Each substrate (0.5 ml) (2 mM α-ketoglutarate and either 200 mM α-L-

alanine or L-aspartate) was incubated for 5 min at 37 °C in a water bath. Serum (0.1 ml) was then added and the volume was adjusted to 1.0 ml with 0.1 M, pH 7.4-phosphate buffer. The reaction mixture was incubated for exactly 30 and 60 min at GPT and GOT, respectively. Then to the reaction mixture, 0.5 ml of 1 mM DNPH was added and left for another 30 min at room temperature. Finally, the color was developed by addition of 5.0 ml of NaOH (0.4N) and product read at 505 nm.

3.18.7.2 Serum alkaline phosphatase and acid phosphatase

Alkaline Phosphatase was estimated based on Bessey et al. (1946) in which the rate of formation of the yellow colour of p-nitrophenol (p-NP) produced by hydrolysis of p-nitrophenylphosphate (p-NPP) in alkaline solution is measured spectrophotometrically at 405 nm and 37°C. In the assay, 0.3 M 2-Amino -2 Methylpropane -1,3 Diol/0.002 M $MgCl_2$ Buffer pH 10.25, Substrate [0.4 M p-Nitrophenyl Phosphate] and the sample tissue were mixed and incubated at 37°C and the increase in absorbance was monitored for ± 5 min.

For acid phosphatase estimation, the method used here was based on the biochemical estimation by Hudson et al. (1947). Acid phosphate estimation assay utilizes para-nitrophenyl phosphate (pNPP) as the chromogenic substrate for the enzyme. In the first step, acid phosphatase dephosphorylates NPP. In the second step, the phenolic OH-group is deprotonated under alkaline conditions resulting in the p-nitrophenolate that yields an intense yellow colour which can be measured at 405-414 nm. L-tartarate acts as an inhibitor of non-tartarate resistant acid phosphatases.

3.18.8 Rabbit endometrial transformation assay

Immature female rabbits, weighing 800–1100 g, were used, three animals per

group. All rabbits were primed with estrogen for 6 days, and then administered with 200 ml equivalent of WWTP extract orally everyday for 5 consecutive days to determine the progestagenic activity. On the other hand 5 and 10 mg/kg/day chlorpyrifos and piperophos were administer orally in combination with progesterone (0.1 mg/kg per day, administered subcutaneously) to determine antiprogestative activity. Rabbits administered with only 10% ethanol or 0.1 mg/kg/day progesterone subcutaneously served as negative and positive vehicle controls, respectively. All rabbits were sacrificed on the day following the final administration of test compound or progesterone. The uteri were excised, weighed, and fixed in buffered formalin, and 2- to 3-mm sections were cut with a razor blade. Transversal slices from each of the right and left uterine horns were prepared, stained with haematoxylin and eosin, and examined histologically. The extent of endometrial transformation was recorded as a progestational effect in accordance with the method previously described (McPhail, 1934).

3.19 Effect of test EDCs on primary Leydig cell culture

3.19.1 Isolation of Leydig cells from testes

Leydig cells were isolated according to the method as described earlier (Parthasarathy & Balasubramanian, 2008). Briefly, the testes collected from freshly sacrificed rats were decapsulated and digested in collagenase (type I) (0.25 mg/ml) containing DMEM-F12 medium at 37 °C for 15 min in a shaking water bath. On completion of the incubation, the tubes were gently shaken, and then 10 ml of DMEM-F-12 medium without collagenase was added and allowed to stand for 15 min. The supernatant was aspirated and transferred to a sterile tube and the procedure was repeated again. The crude Leydig cell preparation obtained was further purified on discontinuous

percoll gradients. The purity of Leydig cells was assessed by immunocytochemical staining of 3β-HSD and further viability was determined by trypan blue dye exclusion method. The purity was 85%-90% and viability was 90%. Purified Leydig cells were plated in culture plates containing DMEM-F12 medium with 2% FBS. After 24 h, cells were washed twice with FBS-free medium and starved for an hour at 34 °C. After starvation, the medium was replaced with fresh medium containing different concentrations of test compounds (0.1, 1, 10 and 100 µM) and incubated for 24 h at 34°C.

Leydig cells were treated with 10 µM of chlorpyrifos and piperophos, which showed a significant decrease in the testosterone production by these cells. The treated cells were used for the isolation of RNA and protein for the analysis of expression profiles of key steroidogenic enzymes (3β-HSD, 17β-HSD, P450scc) and StAR protein using semi-quantitative RT-PCR and immunoblot respectively.

3.19.2 Immunocytochemistry of isolated Leydig cells

For immunocytochemical staining of primary Leydig cells, protocol described by Shiraishi & Ascoli, (2007) was followed with some modifications standardized in the laboratory. Leydig cells were initially fixed in the wells of the 24 well plate with 200 µl of fixative incubated for 30 min. After the incubation, the cells were allowed to dry at 37°C for 20 min followed by RT for another 1-2 h. To these cells, 80 µL of formamide was added and further incubated at RT for 10 min. The plate was then transferred to a water bath at 75° C for 10 min, followed by another incubation at 4° C for 5 min. Formamide was then replaced with 200 µl of blocking buffer (3% (w/v) skimmed milk) and allowed to stand at 37° C for 1 h. After the blocking, the cells were added with 100 µl of 3β-HSD primary antibody diluted in the ratio of 1: 2000 in blocking buffer and

incubated for 30 min at RT. The plate was then washed three times with 500 µl PBS and finally incubated with 100 µl of fluorescein conjugated secondary antibody (Genei, Bangalore, India) diluted in blocking buffer (1:2000) for one hour at RT. The plate was again washed three times with distilled water and observed under fluorescence microscope (Leica, USA).

3.19.3 Estimation of testosterone production by Leydig cells primary culture medium

To analyze testosterone producing efficacy, Leydig cells were exposed to test compounds with and without LH (100 ng/ml) for 24 h at 34°C, then the culture media were collected for testosterone assay. The assays were performed using the commercial enzyme immunoassay kits as per manufacturer's instructions (Omega Diagnostics, UK). Each experiment was performed in quadruplicates to avoid statistical errors. Cells treated with 1% ethanol were used as vehicle control, throughout the study.

3.20 Indirect estimation of cAMP in CHO-K1 cell lines

LH receptor is a transmembrane G-protein coupled receptor that acts through the production of cAMP. Naturally, the estimation of cAMP level in a LH/hCG target cell could demonstrate the status of LH/hCG interactions with LH receptor in that cell. Here we estimated the cAMP by an indirect method using the luciferase driven cAMP response element according to the method described earlier (Jia et al., 1993). Briefly, CHO-K1 cells were transiently transfected with hLH receptor and CRE-luc using lipofection reagent (Superfect transfection reagent, QIAGEN, Valencia, CA) according to the manufacturer's instructions. Twenty four hours after transfection the cells were treated with hCG or other test chemicals chemicals and then assayed for reporter gene expression (luciferase measurement). Luciferase activity was determined using the

luminescence kit (Promega, Madison, USA).

3.21 Estimation of cAMP in the treated cells by ELISA

Non-radioactive, competitive immunoassay purchased from Sigma, St. Louis, MO, USA was used for the quantitation of total cAMP in Leydig cell cultures as per manufacturer's instructions. Buffer contains 0.1 N HCl that aids in the lysis of cells, inhibits endogenous phosphodiesterases and stabilizes the cyclic nucleotides. This kit uses a polyclonal antibody to cAMP to competitively bind cAMP or cAMP which has been covalently linked to an alkaline phosphatase molecule. The assay was performed in a 96 well plate coated with anti-rabbit IgG antibody. The coloured end product, produced by the addition of substrate to the wells, was read at 405 nm on a ELISA plate reader. The intensity of the color was inversely proportional to the concentration of cAMP present in the well.

3.22 Co-immunoprecipitation

Whole cell extracts were prepared in extraction buffer containing 20 mM TrisCl (pH 7.8), 140 mM NaCl, 1 mM EDTA, 0.5% sodium deoxycholate, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 5 mM DTT and protease inhibitor cocktail. The extracts were incubated with human anti-androgen receptor antibody for 1 h at 4°C, followed by 1 h incubation with 15 ml of protein A-Sepharose (Sigma-Aldrich, CA, USA). After five washes with 0.5 ml of the above extraction buffer, the pellets were re-suspended in electrophoresis sample buffer, boiled for 5 min, and analyzed on a 7.5% polyacrylamide gel. Proteins were transferred to an Immobilon-P membrane and immunoblotted with rabbit anti-CBP antibody (Santa Cruz biotechnology, CA, USA) (Kindly donated by Dr. Arun Bandopadhyay) .

3.23 Statistical analysis

For the calculation of progesterone and testosterone equivalents (expressed in ng/l) in the waste water treatment plant effluent, progesterone and testosterone standard curve was plotted with log (effect ratio) against log of concentration. Effect ratio was calculated according to:

$$\text{Effect ratio} = A / (B - A)$$

Where A is the corrected luminescence for background and B is the limit luminescence in excess of progesterone/testosterone, usually the average of 3 to 5 wells at the highest concentrations of the steroid. EC_{50} was calculated by linear regression in log–log plots of the quotient versus concentration. At EC_{50} this quotient is unity. WWTP effluent tests were evaluated similarly and their progesterone/testosterone equivalents were extrapolated from the progesterone/testosterone standard curve (Svenson et al., 2003).

The values shown in the results were mean ± SD from three independent experiments each performed in quadruplicates for each treatment. Data was analyzed by one-way analysis of variance (ANOVA), followed by Duncan's multiple comparisons test or Student's t-test when appropriate. The level of significance was set at $p < 0.05$. For agonists, the treatments were compared to vehicles treated control group, while for antagonists; treatments were compared to the positive control groups.

RESULTS & DISCUSSION

CHAPTER-4

4.1 Introduction

There is an alarming increase in the number of Endocrine disrupting chemicals (EDCs) that were being shown to interfere with the endocrine system affecting the health of humans, wildlife and their progeny. Earlier studies indicate that environmental chemicals, which are able to inhibit PR and AR binding may have an important impact on abnormalities associated with the developing reproductive system (Vonier et al., 1996; Pickford & Morris, 1999). This poses a need to identify these environmental compounds. Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) and OECD suggested a tiered approach to evaluate potential endocrine disrupting activity of these chemicals. The receptor binding assay represents an important component as part of the US EPA tier 1 screening battery and of level 2 of the conceptual frame work.

Numerous reports exist on testing the estrogenic activities of these chemicals which have been shown to exert their effects through interaction with estrogen receptor using competitive binding assays (Bolger et al., 1998; Scippo et al., 2004; Pillon et al., 2005; Laws et al., 2006). However, much less is known about the competitive binding of these EDCs with androgen receptor (Gaido et al., 1997; Freyberger & Ahr, 2004; Wilson et al., 2007; Hartig et al., 2008) and especially the progesterone receptor (Scippo et al., 2004, Viswanath et al., 2008).

In the present chapter of thesis, the androgen and progesterone receptors' ligand binding domain (LBD) that interact with the corresponding ligands were cloned in bacterial expression vectors and their recombinant proteins were purified. These proteins were then utilized for the development of competitive binding assays for screening EDCs

as a part of the tier1 screening. The assays were initially optimized with known compounds and then followed by screening of some unknown chemicals which were not reported earlier. Finally, the water samples collected from the waste water treatment plants (WWTP) from Northern part of India were evaluated for their androgenic and progestagenic activities.

4.2 Production of progesterone receptor ligand binding domain (LBD) and screening of progestagenic EDCs

4.2.1 Cloning of progesterone LBD in pMalc2x plasmid

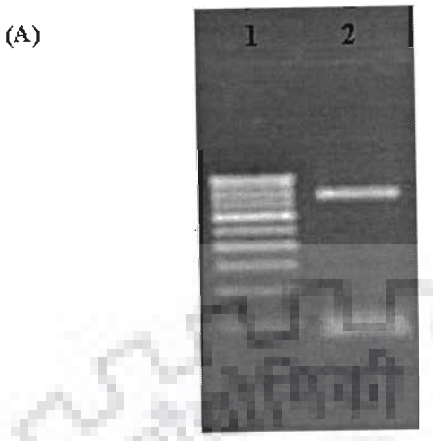
The ligand binding domain of progesterone receptor was cloned into pMalc2x bacterial expression vector. pSG5 containing full length hPR (a kind gift from Professor Jan Brosens, Imperial College, London) was used as the template for amplifying the hPR-LBD. The primers for the LBD amplification were designed from the hPR sequence available on Genbank (NM_000926). Restriction sites for EcoRI and BamHI were included into the primers for cloning-

Primer1– 5'-G**GAATTC**CCAGGTCAAGACATACAGTTG-3'

Primer2– 5'-CG**GGATCC**CGTCACTTTTTATGAAAGAG-3'

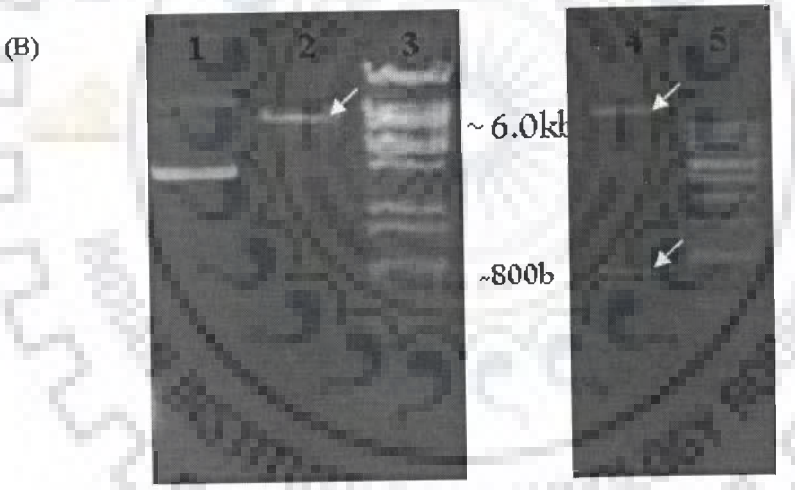
An amplified product of ~800bp was obtained by PCR using XT-5 polymerase (Fig 4.1A). The amplified product and the pMalc2x vector were digested using the corresponding restriction enzymes and the digested products were ligated to obtain hPR-LBD-pMalc2x clone. The colonies were initially screened for positive clones using X-Gal plate. White colonies from the X-Gal plate were picked and used for confirmation of the clone by double digestion of the plasmid DNA with EcoRI and BamHI (Fig 4.1B). Plasmid was prepared from the positive clone, verified by sequencing and retransformed

into TB1 *E.coli* strain, expression strain for pMalc2x, for the protein expression and purification.



Lane 1 : 100 bp ladder

Lane 2 : PCR amplified product of hPR ligand binding domain (~800bp)



Lane 1 : Undigested pMAL-C2X plasmid

Lane 2 : Double digested pMAL-c2X plasmid with EcoR1 and BamH1

Lane 3 : λ DNA digested with Bgl21 marker

Lane 4 : Double digestion of hPRLBD-pMAL-c2X plasmid with EcoR1 and BamH1 (For confirmation of the clone)

Lane 5 : 500bp Ladder

Fig 4.1 Cloning of Human progesterone receptor ligand binding domain (LBD) in bacterial expression vector pMAL-c2X.

4.2.2 Expression and purification of the MBD-hPR(LBD) receptor

The ligand binding domain (LBD) of human progesterone receptor was ligated to the C-terminal end of the maltose binding protein tag in pMALc2X vector to obtain a fusion protein. The fusion protein product was ~68 kD comprising about ~43 kD MBD tag and the 256 amino acid hPR- LBD. This expression vector with cloned hPR- LBD was transformed into TB1 strain of *E.coli* to express the desired protein. The expressed proteins were separated into soluble and insoluble fractions and subjected to SDS-PAGE and western blot analysis. This demonstrated that at 37°C of incubation temperature, only small amount of the expressed MBD-hPR(LBD) fusion protein was produced in soluble fraction while the bulk of it was in the form of inclusion bodies. However, production of soluble MBD-hPR(LBD) was greatly increased by decreasing the induction temperature from 37 to 25 °C.

An efficient, two step purification procedure consisting of amylose resin followed by ion exchange using DEAE-sepharose as described in Methodology, resulted in 35 to 45 fold purification of MBD-hPR (LBD) to near homogeneity as seen in SDS-PAGE (Fig 4.2) and immunoblot analysis (Fig 4.3).

Once the recombinant protein was obtained, the next step was to develop a receptor binding assay. For initial experiments, fixed amount of receptor protein was incubated with increasing concentration of protein (as described in materials and methods). Saturation plot of progesterone binding to the recombinant hPR-LBD protein was shown in Fig. 4.4. The data was extrapolated to build a Scatchard plot using Graphpad Prism stastical software to determine the dissociation constant (K_D) for progesterone binding. The purified hPR-LBD protein showed a K_D value of 8.9 nM.

This value is within the range of values reported by others for this receptor LBD protein (Scippo et al., 2004).

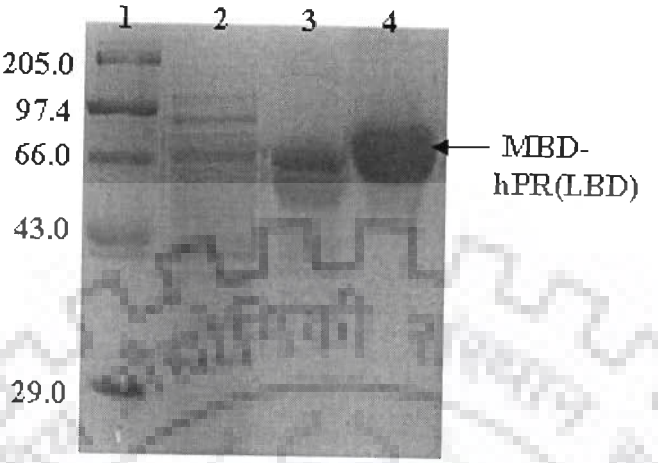


Fig. 4.2 Purification of recombinant MBD-hPR(LBD) expressed from pMalc2X-hPR(LBD). SDS–PAGE gel analysis of the various steps of the purification of MBD-hPR(LBD). Lane 1, Molecular weight standards; Lane 2, Soluble fraction of cell extract; Lane 3, 10 mM maltose elute from amylose column; Lane 4, 350 mM elute from DEAE-Sepharose column.

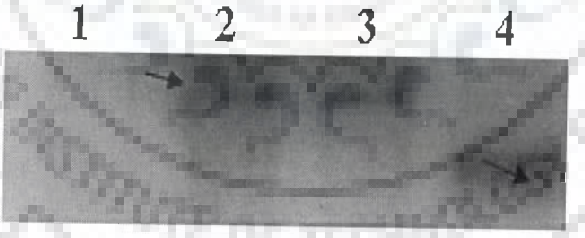


Fig. 4.3 Western blot analysis of hPR(LBD). Lane 1, Untransfected cell line, NIH3T3 (negative control). Lane 2, Full length progesterone receptor obtained from T47D cell line (positive control); Lanes 3, hPR expressed in the stable cell line(NIH3T3-hPR-Luc) (described in chapter 5) and Lane4, MBD-PR(LBD) recombinant protein over-expressed in bacteria; Arrows indicate the progesterone receptor bands.

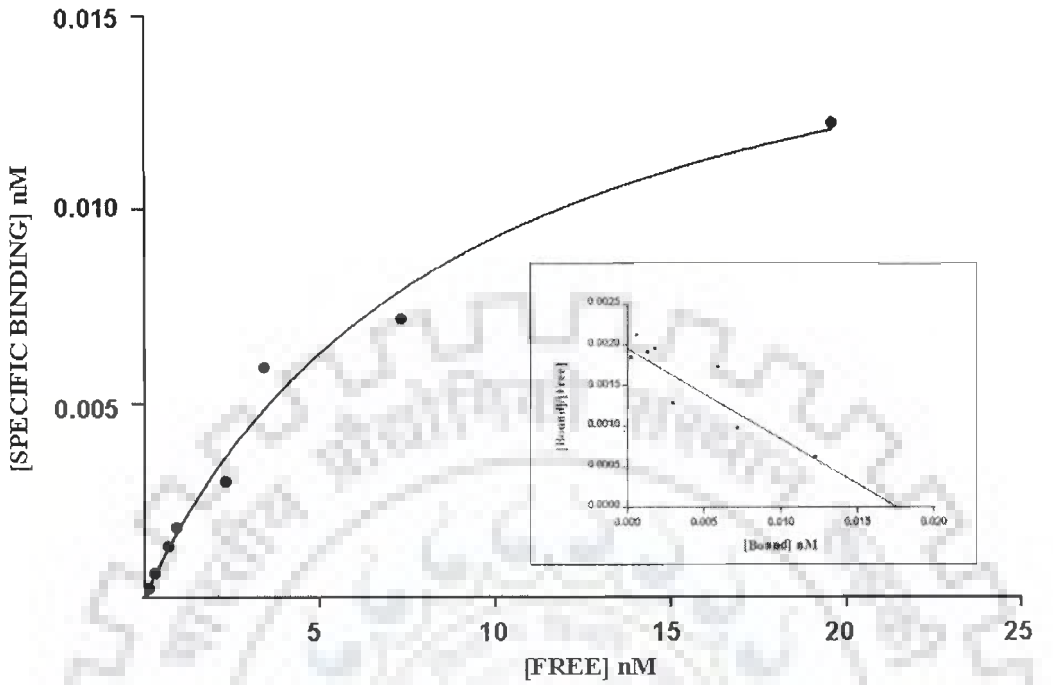


Fig. 4.4 Progesterone binding to ligand binding domain (hPR-LBD) of recombinant human progesterone receptor. Specific binding was calculated as the difference in binding between samples containing radioactively labeled progesterone and samples containing radioactively labeled progesterone and a 100-fold molar excess of unlabeled progesterone. Inset shows the Scatchard plot of the data presented in main panel. Equilibrium dissociation constant (K_D) of the receptor is determined to be 8.9±0.1 nM.

4.2.3 Competitive Binding analysis of some (anti)progestative chemicals using recombinant hPR-LBD protein

The recombinant progesterone receptor protein was incubated with [^{3}H]Progesteone (57 Ci/mmol specific activity) in the presence and absence of 1000-fold molar excess of other non specific steroids and synthetic chemicals. As shown in Table 4.1, most of the compounds tested did bind to the progesterone receptor. Trichlorophenol, piperophos and DDT metabolites p,p'-DDT, o,p'-DDT, p,p'-DDE showed a strong affinity with their IC50 values of 34 , 42 , 8.5 , 21 and 12 μM, respectively. Vinclozolin, dialifos and BHA did not bind to the receptor. The other test chemicals and the WWTP

effluents bound to the receptor with apparently lower affinity, and their IC50 values ranged from 50-240 μM.

4.3 Screening of androgenic EDCs

4.3.1 Cloning of human Androgen receptor (DBD & LBD) in pET28c bacterial vector

RNA was isolated from LNCaP cells using TRI reagent (Fig 4.5A). The Androgen receptor DBD & LBD was amplified by reverse transcription PCR. For this, cDNA of the total RNA was synthesized from which, androgen receptor DBD & LBD was amplified using sequence specific primers containing the restriction sites for BamHI and XhoI in their overhangs (Fig 4.5B). The primers for the amplification of the hAR(DBD & LBD) was designed based on the sequence available on Genbank, NCBI (accession no. NM_000044).

Primer1 –5'- CG**GGATCC**GAAAGACCTGCCTGATC-3'

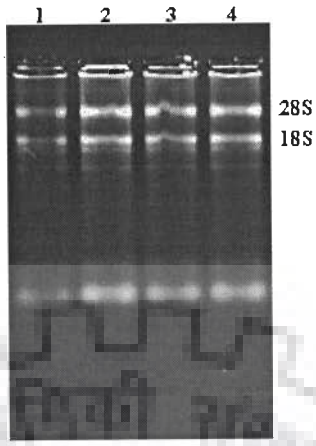
Primer2 – 5'- CCG**CTCGAG**CTGGGTGTGGAAATAG-3'

The amplified AR (DBD & LBD) was then cloned into pET28c vector. For this the amplified product and pET28c vector were both double digested with the respective enzymes and ligated to obtain pET28c-AR(DBD & LBD) clone. The clone was confirmed by double digestion with the respective cloning enzymes (BamHI and XhoI) used (Fig 4.5C). Plasmid miniprep was prepared from this clone, sequence verified and retransformed into BL21, specific protein expression strain for pET28c vector.

4.3.2 Purification of pET28c-AR(DBD & LBD) and androgen ligand binding assay

The amplified AR(DBD & LBD) gene fragment was cloned in the MCS of the pET28c vector in a way that the expressed protein was tagged with 6xHis on its N and C-terminal ends. This protein was therefore purified on Nickel - NTA column beads that

(A)

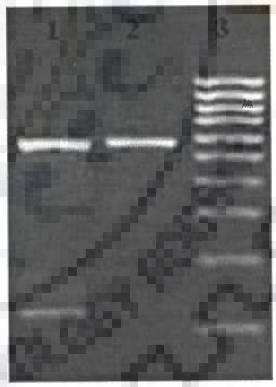


Lane 1, 2, 3, 4 : Total RNA from LNCAP cells using Guanidinium thiocyanate – chloroform extraction.

(B)

Lane1 : 1Kb ladder
Lane2 : PCR amplification of AR (DBD+LBD)

(C)



Lane1 : Double digestion of hAR(LBD)-pET28c clone with Bam HI and Xho I
Lane2 : Double digested pET28c vector
Lane3 : 1Kb DNA ladder

Fig 4.5 Cloning of Human androgen receptor ligand binding domain (LBD) in bacterial expression vector pET28c.

have the affinity for His tag. The culture was grown at 37 °C to an O.D of 0.8 and then the protein expression was optimized to be at 25°C for 3 h with 0.5 mM IPTG induction. About 40-50% of the AR expressed protein was in soluble fraction while the rest had been into the insoluble fraction of the total protein. The soluble protein was precipitated by 60% ammonium sulphate to concentrate the recombinant protein in the fraction as well as to remove some fraction of the nonspecific proteins. The protein was then purified on a Ni-NTA column as described in methodology. However we could only partially purify the hAR(DBD & LBD) protein (Fig 4.6B). The purified protein was confirmed by the immunoblot with specific AR antibodies (Fig 4.6A)

For the competitive binding assay, the recombinant protein was purified, it was incubated with increasing concentration of [H^3]R1881 with and without 100 fold excess of unlabelled testosterone. The K_D was calculated as the free concentration of radioligand at half-maximal specific binding by fitting data to the Hill equation and by linear Scatchard transformation. Testosterone binding by the recombinant hAR(DBD & LBD) protein is shown in Fig. 4.7. Determination of the dissociation constant (K_D) for testosterone binding was analyzed by Graphpad Prism statistical software which showed a K_D value of 0.9±0.2 nM. This value is within the range of values reported by others for full length androgen receptor (Hartig et al. 2007). The receptor specificity for R1881 and the test chemicals IC50 values were listed in the Table 4.1.

Most of the tested chemicals screened by competitive ligand binding assay were able to inhibit the binding of [^{3}H]R1881 to the hAR(DBD & LBD) protein indicating a relatively high binding affinity of these chemicals with their IC_{50} values in the range of 0.25 to 350 μM (Table 4.1).

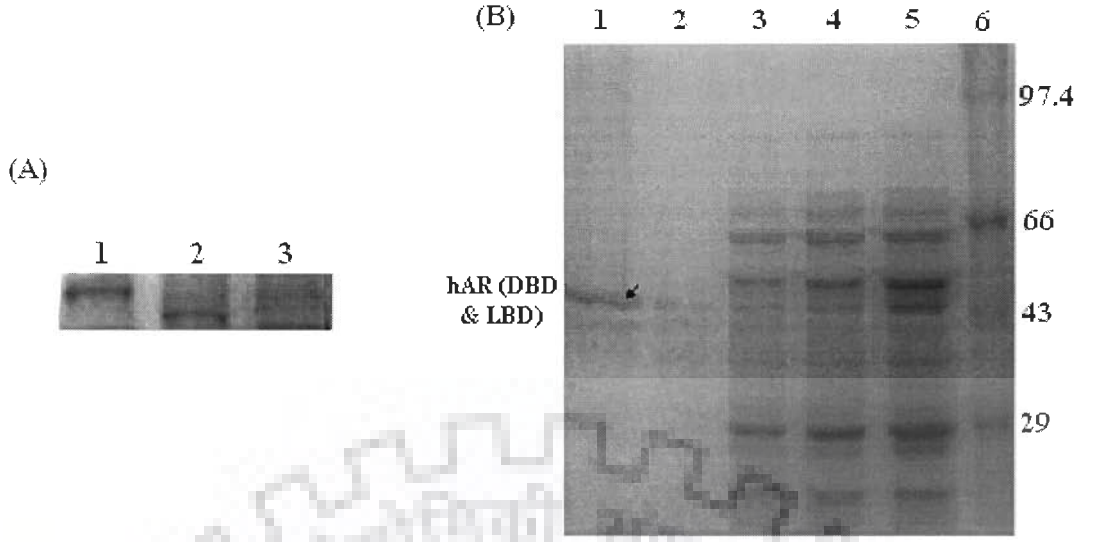


Fig 4.6 (A) Western blot analysis for AR(DBD & LBD). Lane 1, Full length hAR obtained from testes (positive control). Lane 2, hAR(DBD & LBD) expressed in BL21. Lane 3, untransformed BL21 bacterial lysate (negative control). (B) Expression and purification of hAR (DBD&LBD) expressed in BL21 on a Ni-NTA agarose resin. Lane 1, Elution wash containing the receptor protein. Lane 2, Final wash elute. Lane 3, Column wash fraction. Lane 4, Flow through. Lane 5, Soluble fraction of the bacterial lysate precipitate with 60% ammonium persulphate. Lane 6, Medium size protein ladder.

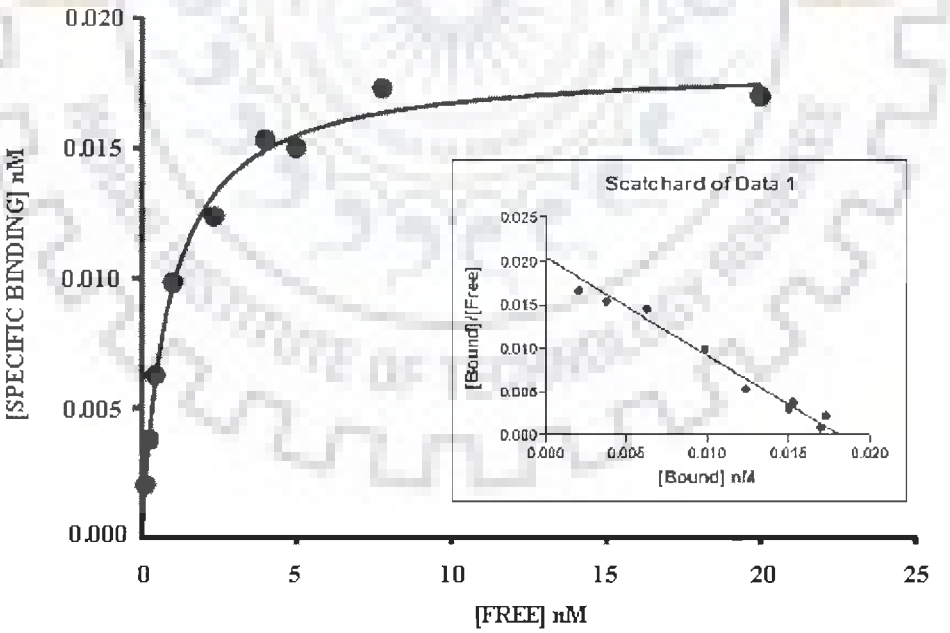


Fig. 4.7 Testosterone binding to hAR(DBD & LBD) recombinant receptor protein. Inset shows the Scatchard plot of the data presented in main panel. Equilibrium dissociation constant (K_D) of the receptor is determined to be 0.9±0.2 nM.

Table 4.1 Screening of some chemicals for their competitive binding to recombinant progesterone and androgen receptors.

Chemicals	Androgen IC50 (μM)	Progesterone IC50 (μM)
R1881	0.06	NB
17β-Estradiol	NB	NB
Progesterone	NB	0.09
p,p'-DDT	21	8.5
o,p'-DDE	33	21
p,p'-DDE	199	12
BHA	165	NB
Vinclozolin	230	NB
Methoxychlor	185	160
Dieldrin	NB	275
Bisphenol A	101	180
Chlorpyrifos	29	56
Piperophos	13	42
Dialifos	367	NB
Trichlorophenol	NB	34
3,3',4,4',5-Pentachlorobiphenyl	NB	NB
Heptachlor	22	292
3-Methyl hexane	NB	NB
Pentyl phenol	NB	NB
Nialate	155	220
Anthracene	186	NB
2,3,5-Trichlorobenzoic acid	NB	58
Caffeine	NB	NB
Bifenox	45	NB
Dichloryos	32	NB
Spiroxamine	NB	NB
Fenitrothion	64	NB
Malathion	NB	140
Oxasulfuron	NB	NB
Diphenamid	188	NB
Methiocarb	38	NB
Triclosan	64	NB
Benazolin	NB	NB
Extracted sewage treatment plant effluents	225	340

4.4 Discussion

Steroid receptor binding assay is an important step for screening of the EDCs. The value of receptor binding assays both as a mechanistic tool to characterize receptor

mediated endocrine activity and as an important screening assay for endocrine active compounds, is well recognized (Soto et al., 2006). These assays are also important in elucidating the mode of action of a particular EDC, i.e., to confirm the chemical's physical interaction with the receptor. In the present study, *in vitro* binding analysis of various (anti)progestagenic and (anti)androgenic chemicals with their respective human recombinant receptor protein was studied.

The use of recombinant receptors for binding assays offers a number of advantages which include an inexhaustible source of material, homogeneity of the binding proteins and stability of the binding properties compared to receptors prepared from animal tissues (Scippo et al,. 2004). In the present thesis, the recombinant proteins, hPR(LBD) and hAR(DBD & LBD) expressed and purified from the bacteria exhibited remarkable affinity with progesterone and testosterone showing a dissociation constant of 8.9 nM and 0.82 nM, respectively. These binding affinities were in good comparison to the earlier reports utilizing progesterone LBD and full length androgen receptor (Scippo et al., 2004; Hartig et al., 2007). These receptor proteins also exhibited high specificity to their respective steroid ligands as seen in Table 4.1.

In the first part of the study, some well established EDCs for both AR and PR were screened using our assays. In this regard, DDT and its metabolites exhibited very strong binding with both hPR(LBD) and hAR(DBD & LBD). Similarly, a commendable binding was also shown by methoxychlor and bisphenolA with both the receptor proteins while BHA and vinclozolin have bound with only androgen receptor. Their IC_{50} values were at par with earlier reports with marginal variations (Kelce et al., 1995; Lambright et al., 2000; Scippo et al., 2004). This variation could be attributed to several factors like

handling of receptor proteins and differences in methodologies applied for the binding assay (Freyberger and Ahr, 2004). Once the assays were validated with the known EDCs, a wide range of chemicals were tested for their androgen and progesterone receptor binding abilities. Most of the chemicals selected for screening in this thesis were not earlier reported to be screened by any of the *in vitro* assays (especially in terms of progesterone receptor). Triclosan, fenitrothion, bifenox, dichloryos and heptachlor showed strong binding with androgen receptor while diphenamid, anthracene, dialifos showed weak binding with their IC_{50} ranging from 150 to 350 μM. Malathion and 2,3,5-trichlorobenzoic acid showed binding specifically only with progesterone receptor with their IC_{50} vaues of 140 and 58 μM respectively. On the other hand, nialate, heptachlor, piperophos and chlorpyrifos exhibited binding with both hAR(DBD & LBD) and hPR(LBD) proteins. All the chemicals discussed here in this chapter were also screened using the cell based reporter bioassay (chapter 6) to further validate these binding results.

In the next part of the study, WWTP effluent was tested for the hAR(DBD & LBD) and hPR(LBD) binding. There were almost no reports available on the endocrine disruptors in WWTP effluents from Indian subcontinent except by Senthilkumar et al. (1999) which demonstrated the presence of potential EDCs in the river Ganges, one of the major rivers in India and Chatterjee et al. (2008) who showed the progestagenic activity of WWTP effluent using yeast cell based reporter bioassay. Here in the thesis, the screened WWTP effluent showed a significant progestagenic activity with an IC_{50} value of 340 μM. This effluent also competed with the labeled [^{3}H]R1881 showing the presence of androgenic EDCs. To the best of our knowledge, this is the first ever report on the presence of the progesterogenic compounds in the effluents of WWTPs using

receptor binding assays. The possible EDCs in these effluents were analyzed using GC-MS, the details of which are discussed in chapter 7.

In conclusion, we cloned the hAR and hPR ligand binding domain in bacterial expression vectors and purified the protein from the bacterial source. The functionality of the receptor was assessed by saturation binding assay which conclusively proved that the recombinant LBDs has strong affinity for their corresponding steroids. This receptor protein was finally used for competitive binding assays for screening various chemicals and WWTP effluents for their endocrine disruption activity. The developed *in vitro* bioassay thus could be used as the tier1 assay system.

CHAPTER- 5

5.1 Introduction

(Anti)estrogenic endocrine disruptors were well studied over the decades by different multi-tier bioassays including the hormone binding assays, transactivation bioassays and *in vivo* models. However androgen and especially progesterone like endocrine disruptors were not so extensively studied. Although binding assays give an idea of the receptor's ability to bind to the ligand molecules, it neither recognizes them to be an agonist or antagonist nor do they help to determine the other possible mechanisms by which the EDCs can block the steroid receptor function (Tabb & Blumberg, 2006). Thus, *in vitro* reporter gene assay systems have been developed that imitate the mechanism of receptor action via the steroid receptor pathway and are thus suitable to assess the potential of a variety of compounds. The assay is developed based on the principle reviewed in chapter 2 (2.5.1.3).

Transient transfections do not reflect physiological conditions because the target DNA sequences are over expressed and maintain their responsiveness only for a limited time to a single passage of cells. Moreover, there is a high inter assay and intra assay variation. Therefore cell lines stably transfected with steroid receptor and steroid responsive reporter gene proved to be more convenient. There were some stable cell lines reported earlier for screening (anti)androgenic EDCs (Furmann et al., 1992; Schrader & Cooke, 2000; Korner et al., 2004; Roy et al., 2004, 2006; Sonneveld et al., 2005) utilizing a wide range of cell lines like CHO-K1, COS1, COS7, CV1, MDA-kb-2, PC3 and human U2-OS. Blankvoort et al. (2001) developed a stably expressing AR reporter gene cell line utilizing the endogenous AR of these cells. Roy et al. (2006) and Chen et al. (2006) have

reported a sensitive HEK293 cells stably expressing AR and reporter gene to measure androgen activity in human serum samples. Raivio et al. (2001) in their *in vitro* bioassay, cotransfected the COS 1 cells with AR-interacting protein 3 (ARIP3) to increase the sensitivity of the assay. Each of these reported assays have some advantages as well as some limitations.

On the other hand, there were not many reports on the progesterone transactivation assays for screening EDCs. One of the first mammalian cell based PR transactivation assay was reported by Koltz et al. (1997) where they analyzed the role of DDT in transactivating PR using T47D cell line which was followed by another bioassay by Schoonen et al. (1998) using CHO cells. Using a similar approach based on endogenous PR in T47D cell line, Willemsen et al. (2004) later developed a stable cell line for screening EDCs. Recently vinclozolin has been demonstrated to possess anti-progestative activity using a HeLa cell line stably transfected with hPR and response element driving the expression of luciferase gene (Molina-Molina et al., 2006). Our group also reported the presence of progestagenic endocrine disruptor chemicals in waste water treatment plant effluents using transactivation assay (Viswanath et al., 2008).

In the present chapter, we report the development of two stable cell lines, NIH3T3 expressing the human progesterone receptor (pSG5-hPR-B) and pPRE-Luc and NIH3T3 stably expressing the human androgen receptor (pSG5-hAR) and pProbasin-Luc. These cell lines were characterized for their EC_{50} and optimized for various parameters that would ensure high sensitivity and efficiency for high throughput screening of EDCs in a 96-well plate format.

5.2 Screening of (anti)progestagenic EDCs

5.2.1 Development of stable cell line (NIH3T3-hPR-Luc) with PR transactivation assay

Both pSG5-hPR and pGL3-PRE-Luc plasmids does not have any mammalian cell specific antibiotic resistant genes for selection during the stable cell line development. So, these plasmids were co-transfected along with pcDNA3.1 empty vector into NIH3T3 cells. Neomycin resistant gene in pcDNA3.1 was used as a selection marker during the stable cell line development screening (Fig 5.1). As shown in Fig 5.1B & C, with time, there was a gradual increase in the size of the clones indicating their expansion. Since these clones may or may not be positive for progesterone response, about 100 neomycin resistant colonies were randomly picked and analyzed for progesterone responsiveness. Of these, 13 clones showed positive response in the presence of 10 nM progesterone with varing degrees of luciferase induction (Fig. 5.2) The colony #8 (Fig 5.2) which produced the best response in 10 nM progesterone treatment was chosen and later subjected to test its dose dependency for increasing concentrations of progesterone and other assays. This clone was indicated as NIH3T3-hPR-Luc cell line in this thesis. The data was then plotted graphically to obtain a standard response curve. The expression of the progesterone receptor by the best clone (clone#8) was confirmed both by RT-PCR analysis and immunoblot with the progesterone antibody (Fig. 5.3).

5.2.2 Dose dependent induction of luciferase by progesterone

Analyses of progesterone-response capacity of the transfecetd NIH3T3 cells were carried out by the transactivation assay with increasing concentrations of progesterone in 96 well plates (Fig. 5.4A&B). The luciferase expression was induced by progesterone in a dose dependent manner with the saturation of reporter gene induction at 50 nM resulting in about 26 fold induction at this concentration. The developed assay showed a

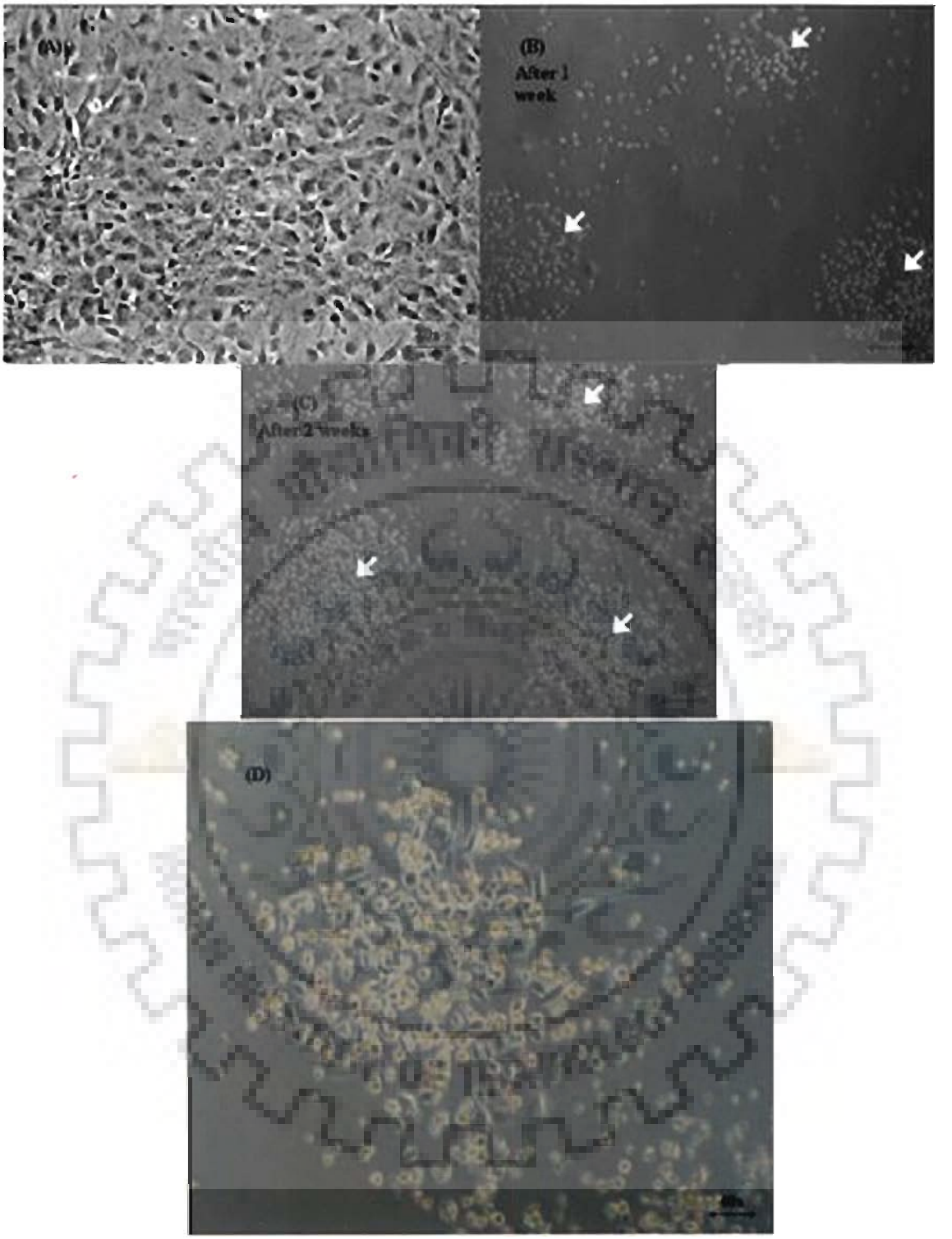


Fig. 5.1 Stable cell line development by antibiotic selection (neomycin) of transfected clones (A) Untransfected confluent NIH3T3 cells. (B) Representative small colonies of the stably transfected NIH3T3-hPR-Luc cells appearing after one week selection with antibiotics. (C) Increase in the size of colonies as shown in (B) after 2 weeks. (D) Closer view of a stably transfected growing colony at the end of 2nd week.

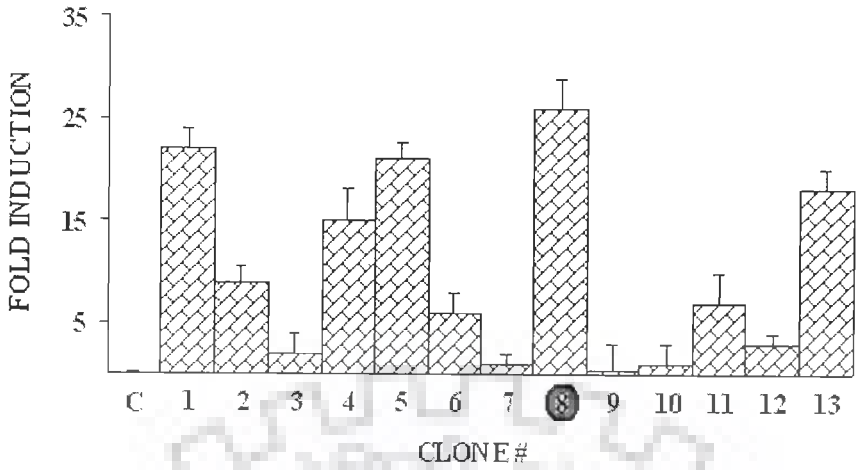


Fig 5.2 Luciferase activity of the 13 neomycin resistant colonies transfected with pSG5-hPR, pGL3-PRE-Luc and pcDNA3.1 in response to 10 nM progesterone. C, represents the untransfected NIH3T3 cells (vehicle control).

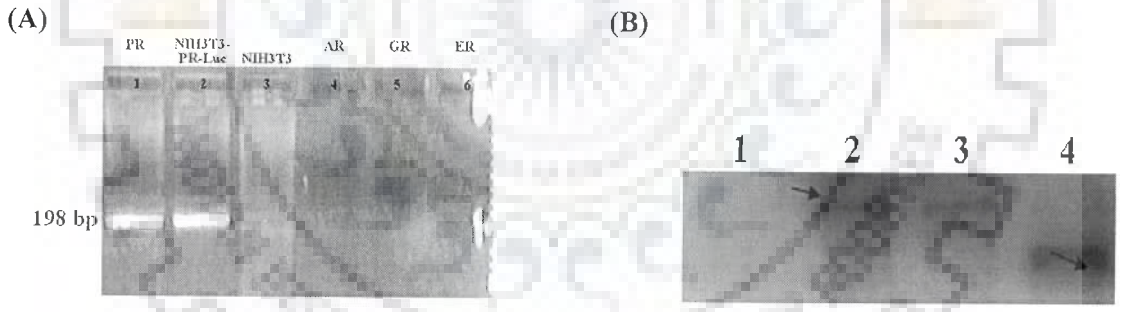


Fig 5.3 (A) RT-PCR analysis of NIH3T3-hPR-Luc. Lane 1, PR expressed in T47D (positive control); Lane 2, PR expressed in NIH3T3-hPR-Luc; Lane 3, Untransfected NIH3T3 cells (negative control); Lane 4, 5, 6, RT-PCR analysis for the expression of AR, GR and ER respectively in the NIH3T3-PR-Luc cell line. (B) Western blot analysis for PR in stable cell line NIH3T3-hPR-Luc. Lane 1, Untransfected cell line, NIH3T3 (negative control). Lane 2, Full length progesterone receptor obtained from T47D cell line (positive control); Lanes 3, hPR expressed in the stable cell line(NIH3T3-hPR-Luc) and Lane4, MBD-PR(LBD) recombinant protein over-expressed in bacteria; Arrows indicate the progesterone receptor bands.

half maximal effect (EC_{50}) at 4 nM for progesterone, which is consistent with the activity of reporter based bioassay developed for screening progestagenic EDCs in other mammalian and yeast cells (Death et al., 2004; Willemsen et al., 2004; Molina-Molina et al., 2006). The dose dependent response progressed in a linear fashion (Fig 5.4B).

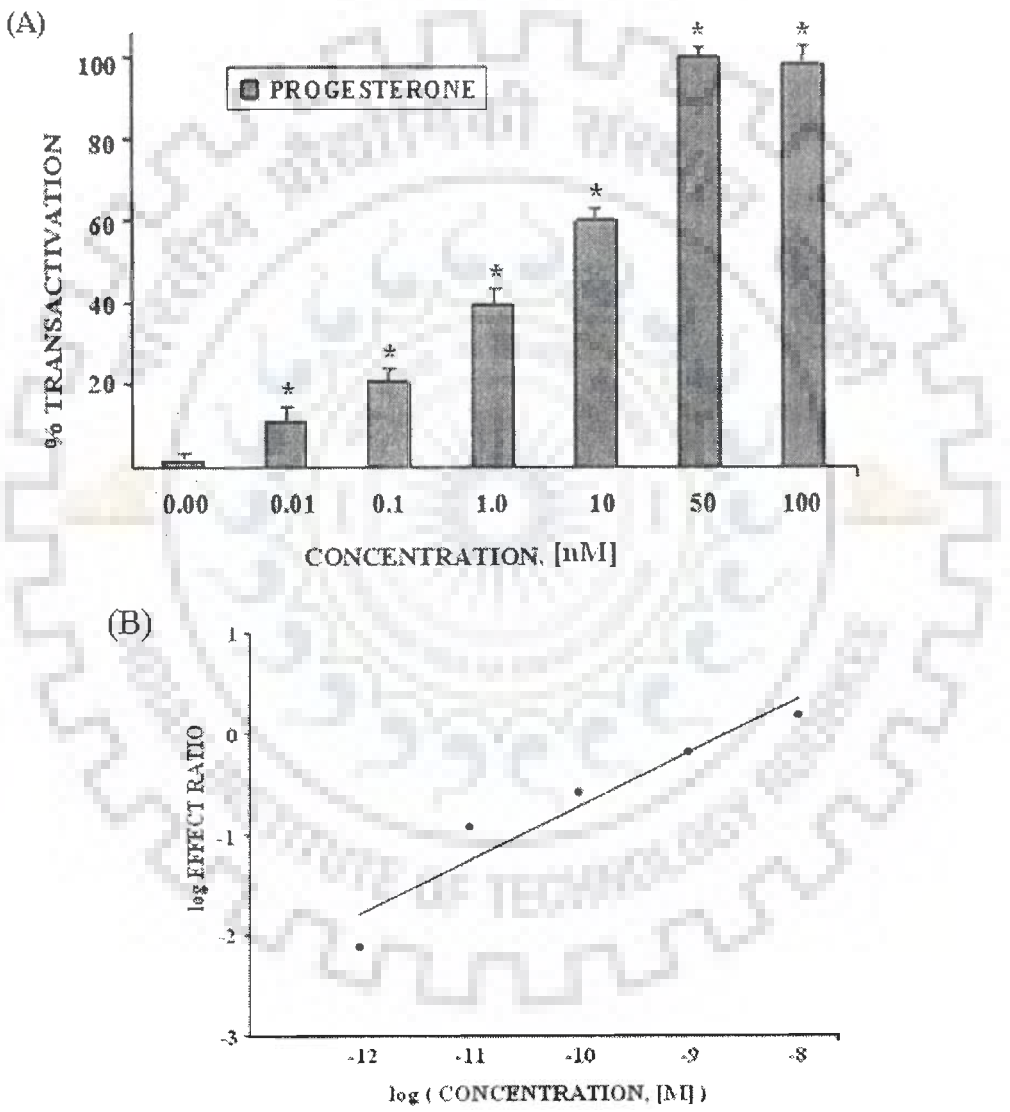


Fig 5.4 (A) Dose-dependent luciferase activity induction by increasing concentrations of progesterone in stably transfected NIH3T3-hPR-Luc cells. Luciferase activities were expressed as percentage of that obtained with 100 nM progesterone which was given the value of 100%. (B) Double log plot dose curve of progesterone. The values represent the mean ± S.D. of four independent experiments each performed in quadruplicates.
* Significant luciferase activity as compared to vehicle treated cells ($p<0.05$).

The assay was adapted for 96 well format with an aim to develop high throughput assay system. The cell number was optimized to be 20,000 cells/well above which the response did not increase significantly. Time kinetics study with the progesterone showed that significant ($p<0.05$) detectable response with progesterone was found to be at 5 h incubation after which there was progressive increase in the response upto 35 h (Fig. 5.5). This further confirmed that the assay can be used in the detection of less stable progesterogenic compounds as well.

5.2.3 Specificity of the hPR transfected cells to stimulation by other steroids

The induction of transactivation of PR in the recombinant cells was tested for its specificity to steroidal hormones. The NIH3T3-hPR-Luc cells were incubated with increasing concentrations of estradiol, testosterone, dexamethasone and measured for their luciferase activity. As shown in Fig 5.6, none of the non-progestin steroids could activate the PR induced transactivation till 100 nM concentration and above confirming the specificity of the developed assay.

5.2.4 The effect of known potent anti-progestin RU486 on NIH3T3-hPR-Luc cell line

The effect of RU486, a potent anti-progestin compound, was tested in the NIH3T3-hPR-Luc cell line. For this, the anti-progestin activity was checked by treating the cells with increasing concentrations of RU486 in the absence and presence of half-maximally stimulating concentration of progesterone. As shown in Fig. 5.7, RU486 dose dependently inhibited the progesterone induced transactivation with an effective IC_{50} value of around 0.1 μM. However, RU486 alone did not demonstrate any agonistic activity, confirming further that RU486 is a full antagonist for PR atleast in this cell line (Fig. 5.7).

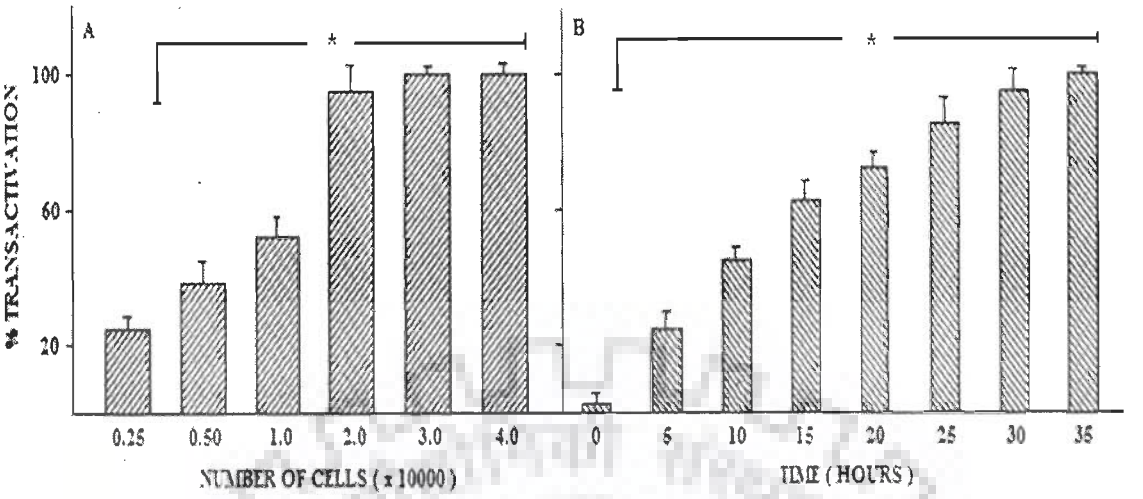


Fig. 5.5 Optimization of the time and number of cells for luciferase induction in the stable cell clone on stimulation with progesterone. A) The cells were stimulated with 4nM progesterone for 24 h. The results were expressed as percentage of luciferase activity, measured with 30,000 cells (100%). B) Time course of luciferase induction in NIH3T3-PR-Luc cells by progesterone. The cells were incubated with 4nM progesterone for 0–35 h. The results are expressed as percentage of luciferase activity, taken the mean activity at 30 h as 100%. The results represent the mean ± S.D. of three similar experiments each performed in quadruplicate.
* Significant luciferase activity as compared to control ($p<0.05$).

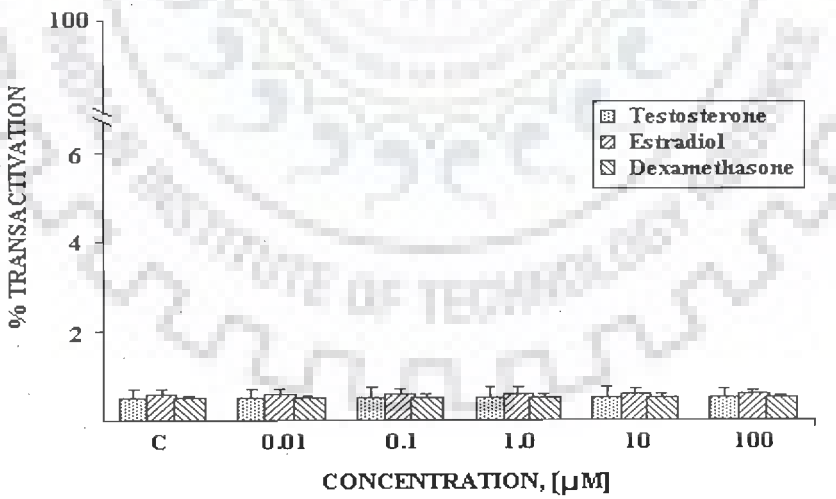


Fig 5.6 Determination of ligand specificity in NIH3T3-hPR-Luc cells in response to increasing concentrations of non-progestagenic steroids (0.01-100 μM). Luciferase activities were expressed as percentage of that obtained with 100 nM progesterone which was given the value of 100%. The values represent the mean ± S.D. of three similar experiments each performed in quadruplicates. No significant luciferase activity as compared to the control ($p<0.05$)

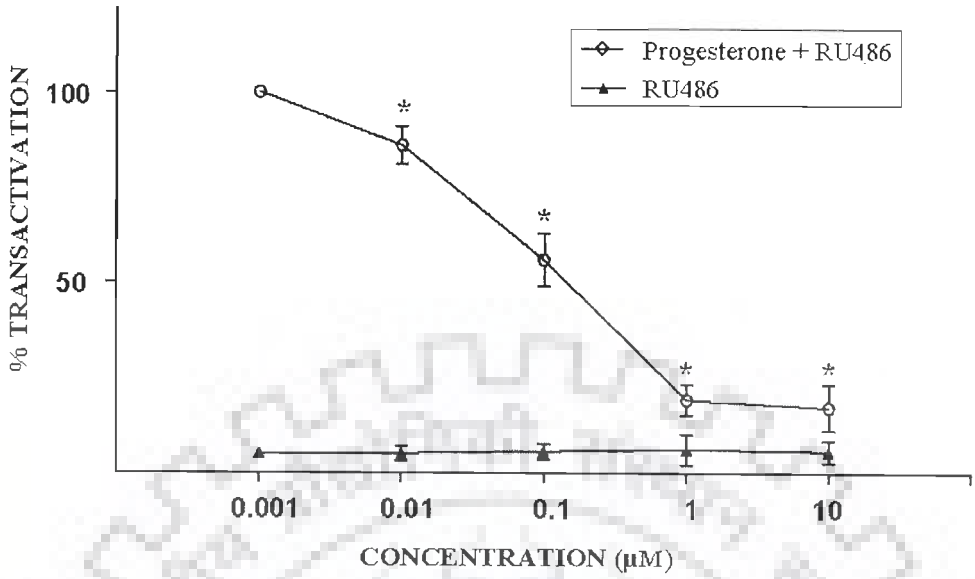


Fig 5.7 Demonstration of antagonistic activity of known anti-progestin RU486 in NIH3T3-hPR-Luc cells. Cells were either incubated with increasing concentration of anti-progestin alone or in the presence of 4 nM progesterone. The mean transactivation obtained with 4 nM progesterone alone was given a value of 100%. The values are the mean ± S.D. of three similar experiments performed in quadruplicates.
* Significant inhibition of the progesterone induced luciferase activity as compard to vehicle treated cells ($p<0.05$).

5.3 For screening androgenic EDCs

5.3.1 Development of cell based Androgen Reporter assay

NIH3T3 cells were co-transfected with pSG5-hAR-puro and pProbasin-Neo-Luc plasmids that contained puromycin and neomycin resistant genes for selection, respectively. About 10 neomycin resistant clones were obtained after selection by incubating in the presence of 1 g/l of neomycin (G418). Some of the clones were found to exhibit good transactivation response in the presence of testosterone (Fig 5.8). One of the clones (clone#5) was marginally more active than the others showing about 24 fold inductions in the presence of 10 nM testosterone which increased linearly when treated with increasing concentrations of testosterone (Fig. 5.9); it was therefore selected for

further studies and was indicated as NIH3T3-hAR-Luc cell line in this entire thesis. The expression levels of AR were analysed until passage 18, and they remained stable, even after freezing. Thereafter, the cells showed gradual reduction in AR activity. The expression of AR by the clone was indicated by both RT-PCR amplification and western blot of the receptor against the untransfected NIH3T3 cells as shown in the Fig 5.10A&B, respectively. The finding indicated stable AR expression in the clone, while no AR was expressed in untransfected NIH3T3 cells. The EC_{50} of the clone to testosterone is 2.9 nM.

Fig 5.8 Luciferase activity of the 10 neomycin resistant colonies transfected with pSG5-hAR, pGL3-probasin-Luc and pcDNA3.1 in response to 10 nM testosterone. C, represents the untransfected NIH3T3 cells (vehicle control).

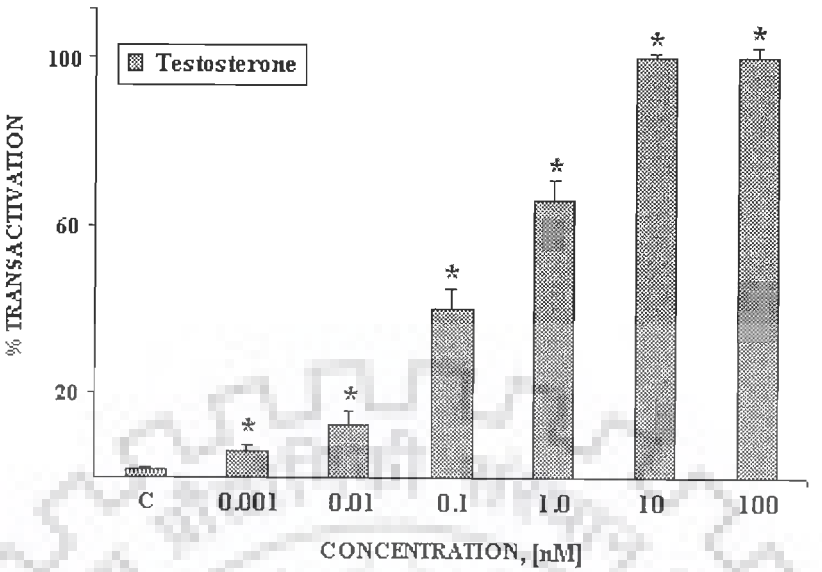


Fig 5.9 The most active clone (clone #5) was selected and incubated with increasing concentrations of testosterone. NIH3T3-hAR-Luc cells showed dose dependent luciferase activity showing the maximum induction at 10nM testosterone concentration. The values represent the mean ± S.D. of four independent experiments each performed in quadruplicates ($p<0.05$).
* Significant luciferase activity as compared to vehicle control ($p<0.05$).

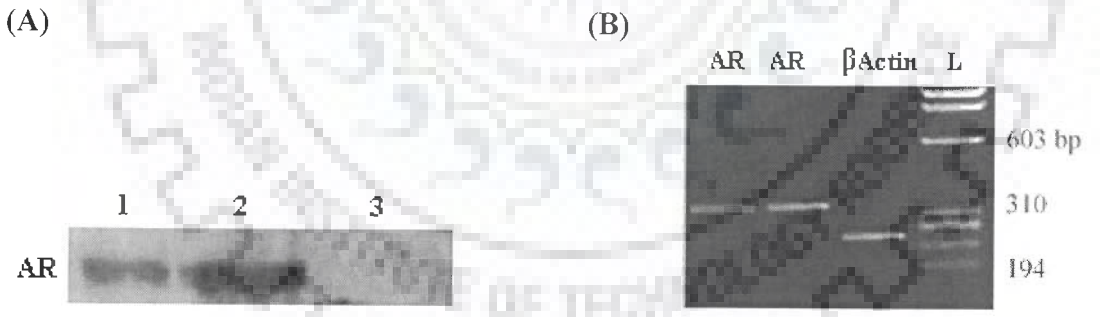


Fig 5.10 (A)Western blot analysis for AR in stable cell line NIH3T3-AR-Luc. Lane 1, AR protein from LNCaP cells (positive control); Lane 2, AR protein expressed in NIH3T3-hAR-Luc cells; Lane 3, Protein from untransfected NIH3T3 cells (negative control) (B) RT-PCR analysis of NIH3T3-AR-Luc cells. Lane 1, AR expression in LNCap cells (positive control); Lane 2, AR expression in NIH3T3-hAR-Luc cells; Lane 3, β-actin expression in NIH3T3-hAR-Luc cells; Lane 4, DNA ladder.

The cell number was optimized to be 25,000 cells per well of the 96-well plate. It can be seen that the luciferase activity was clearly detectable at 5 h and increased in a progressive fashion about five-fold when the incubation was continued up to 30 h (Fig 5.11). These findings demonstrated that the cell line had the potential to be used for measuring androgenic activities of compounds that were stable even for short duration.

To examine the specificity of the NIH3T3-AR-Luc cell line, the cells were treated with estradiol, progesterone and dexamethasone. All these steroids showed <3% transactivation (Fig 5.12) at a concentration of 100 nM which was insignificant, at a concentration 1000 fold higher than the 100% transactivation obtained with testosterone ($p<0.05$).

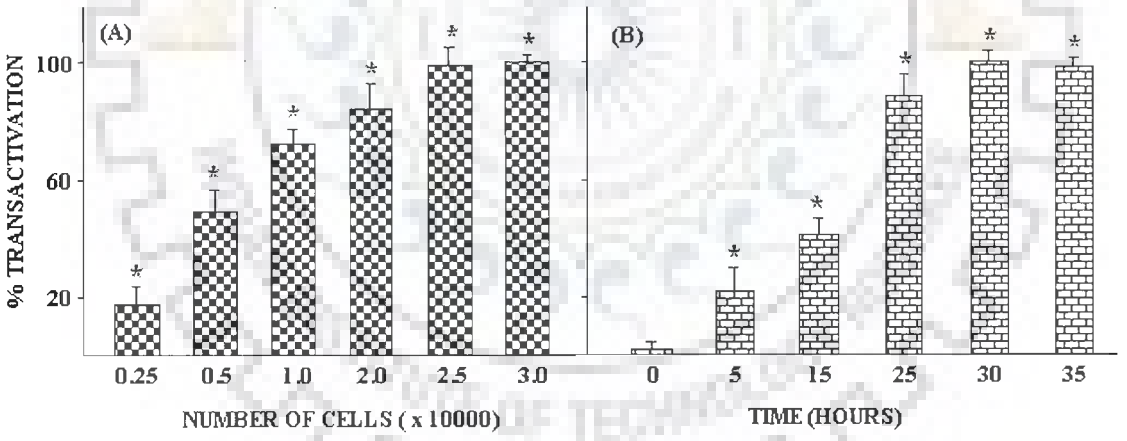


Fig 5.11 Optimization of the time and number of cells for luciferase activity in NIH3T3-hAR-Luc cells on stimulation with testosterone. A) The cells were stimulated with 3 nM testosterone for 24 h. The results were expressed as percentage of luciferase activity, measured with 30000 cells (100%). B) Time course of luciferase induction in NIH3T3-AR-Luc cells by testosterone. The cells were incubated with 3 nM testosterone for 0–35 h. The results are expressed as percentage of luciferase activity, taken the mean activity at 30 h as 100%. The results represent the mean ± S.D. of three similar experiments each performed in quadruplicate.
* Significant luciferase activity as compared control ($p<0.05$).

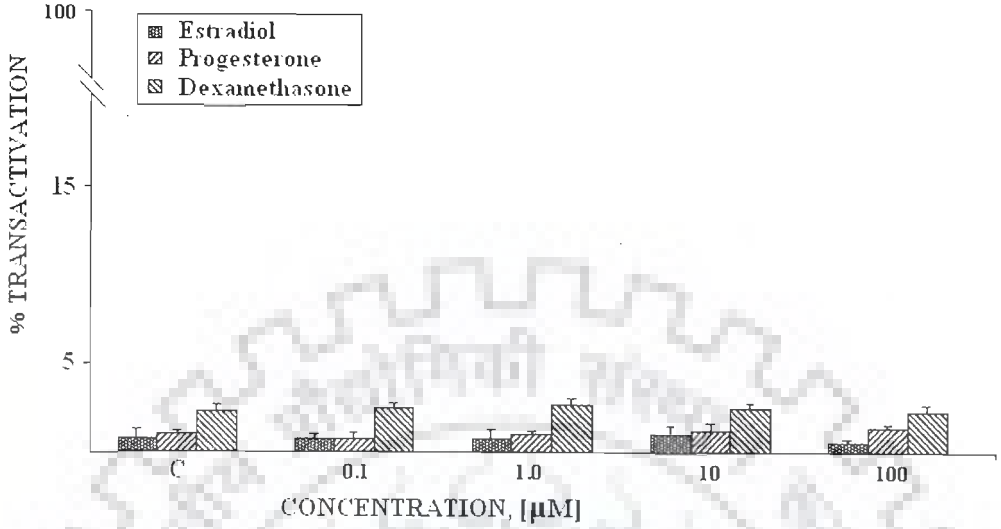


Fig 5.12 Determination of ligand specificity in NIH3T3-hAR-Luc cells in response to increasing concentrations of non-androgenic steroids (0.1-100 μM). Luciferase activities were expressed as percentage of that obtained with 10 nM testosterone which was given the value of 100%. The values represent the mean ± S.D. of three similar experiments each performed in quadruplicates. No significant transactivation observed ($p<0.05$).

5.3.2 The effect of anti-androgens and androgens on luciferase activity of NIH3T3-AR-Luc cells

The effect of potent anti-androgenic compound, (hydroxyflutamide, HF), was then tested on the NIH3T3-hAR-Luc stable cells to evaluate their response to the well established antagonist compounds (Fig. 5.13). HF alone could not activate the transactivation but it inhibited the 0.3 nM testosterone induced luciferase activity in a dose dependent manner. It showed a significant inhibition ($p<0.05$) at 0.01 μM concentration with an IC_{50} value of ~0.7 nM. Later these cells were treated with increasing concentrations of well known testosterone agonists, dihydroxytestosterone (DHT), nandrolone and danazol. These compounds showed an EC_{50} value of 0.19 nM, 0.3 nM and 0.75 nM respectively (Fig 5.14).

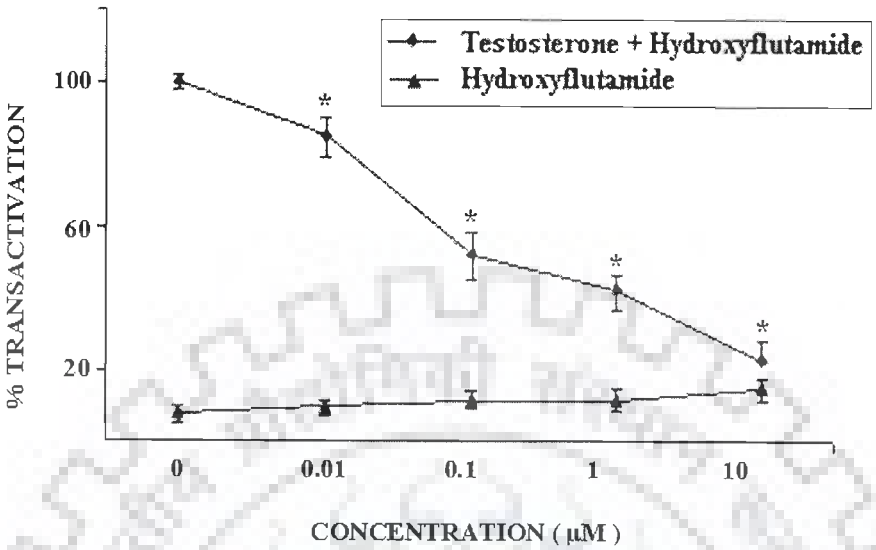


Fig. 5.13 Demonstration of antagonistic activities of hydroxyflutamide in NIH3T3-hAR-Luc cells. Cells were incubated with increasing concentration of anti-androgens alone or in the presence of 0.3 nM testosterone. The mean transactivation obtained with 0.3 nM testosterone was given a value of 100%. The values represent the mean ± S.D. of three similar experiments each performed in quadruplicates.
* Significant inhibition of the testosterone induced luciferase activity as compard to vehicle treated cells ($p<0.05$).

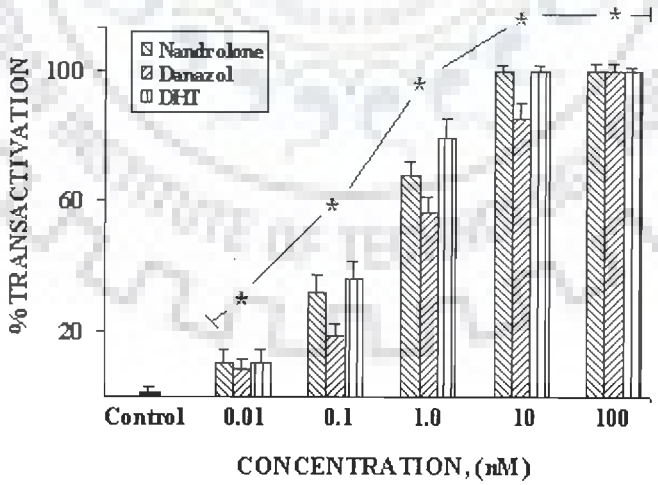


Fig 5.14 Demonstration of agonist activities of nandrolone, danazol and DHT in NIH3T3-hAR-Luc cells. The values represent the mean ± S.D. of three similar experiments each performed in quadruplicates.
* Significant luciferase activity as compared to vehicle treated cells ($p<0.05$).

5.4 Discussion

The major limitation with the binding assay is its inability to distinguish between agonistic and antagonistic biological effects of the test chemicals. In order to address this issue in a better way, *in vitro* cell based bioassays and *in vivo* animal models can be utilized. *In vivo* assays like uterotrophic assays and Hershberger assay in rodents, frog metamorphosis assay, fish gonadal recrudescence assay and many others were based on developmental end points (Willemsen et al., 2004). These assays are time consuming, labor-intensive and above all require the use of live animals. On the other hand, *in vitro* tests based on the ability of the steroid receptors to specifically recognize their ligands offers a good model for rapid screening of the EDCs (Roy et al., 2005). Several *in vitro* assays taking advantage of known steroid hormone signaling pathways have been developed based on different end points like enzyme activities, endogenous protein expression (i.e., pS2, PR, vitellogenin), cell proliferation and gene expression. Among all these *in vitro* models, gene expression assays, monitoring transcription of a recombinant target reporter gene to assess the endocrine potential of a chemical seems to be the most sensitive and easiest tests currently available (Willemsen et al., 2004). In this context of reporter based bioassays, yeast based bioassays have several advantages like: little chance of interference by other hormone receptors as in mammalian cell lines, relatively simple and cost effective allowing the use of media that does not contain steroids (Bovee et al., 2004, Chatterjee et al., 2007). However, they do not discriminate effectively between agonists and antagonists (Shiau et al., 1996; Gaido et al., 1997; Soto et al., 2006). Moreover, yeast cells were identified to have problems of membrane permeability and transport that may give high rate of false results in the measurement of the relative

steroidogenic potency. All this give a high back ground noise in case of yeast based bioassays (Soto et al., 2006) making the mammalian cell based reporter transactivation assays to be the most chosen one.

Therefore, as a part of the tiered screening of endocrine disrupting chemicals in this thesis, we developed two mammalian stable cell lines with the reporter transactivation assay for progesterone and androgen receptors. For this, the NIH3T3 cells that are naïve to either of these receptors were chosen. These cells have all the molecular machinery that can support the luciferase transactivation assay. Also, this cell line is rapid growing, adheres strongly to the substrate, uses the routine DMEM medium for its propagation with no special media substitutes and adapts well to the 96 well format.

Not many reports exist regarding the reporter based transactivation assay development for screening (anti)progestagenic EDCs (Klotz et al., 1997; Schoonen et al., 1998; Willemsen et al., 2004; Molina-Molina et al., 2006; Wilkinson et al., 2008). In this part of the thesis, we initially developed a stable NIH3T3-hPRB-Luc cell line by transfecting pSG5-hPR-B and pPRE-Luc into NIH3T3 cells. Progesterone significantly induced luciferase expression of these NIH3T3-hPR-Luc cells at a concentration of 0.01 nM with an EC_{50} value of 4 nM, the maximum activity was obtained at 50 nM thereafter which it leveled off. The non-transformed cells did not show any response to progesterone which further indicated that the activation was specifically through PR. The sensitivity of this cell line was in good agreement with the earlier reported stable cell lines. RU486, a potent anti-progestin (Beck et al., 1993; Mahajan & London, 1998) exhibited antagonistic activity at a concentration of 0.01 μM and above with an IC_{50} value of ~0.1 μM. Estradiol, testosterone and dexamethasone did not show any luciferase

activity even at a concentration of 100 μM, which suggested that the assay system was quite specific to progesterone with least possible cross-talk with any other steroid receptors. Whatever little non specific cross talk that was detected above 100 μM could be attributed to the lack of receptor specificity by the ligands at a high concentration as has been reported by several authors earlier (Gaido et al., 1997; Michelini et al., 2005).

On the other hand, many recombinant cell lines stably expressing AR and androgen-responsive reporter gene, suitable for screening of (anti)androgenic compounds had been developed earlier. Battman et al. (1998) used the breast cancer cell line T47 D, expressing endogenous AR and stably transfected them with a MMTV-CAT reporter gene. Later, there were reports of using monkey kidney cell line, CV 1 (Fuhrmann et al., 1992) and prostate cell lines (Schrader & Cooke., 2000; Terouanne et al., 2000) In addition to these cells, Raivio et al. 2001 reported a bioassay using the COS 1 cells, where the sensitivity of the assay was improved using the AR-interacting protein 3 coactivator. Subsequently, a number of androgen-responsive stable cell lines were developed by different groups (Korner et al., 2004; Roy et al., 2004, 2006; Sonneveld et al., 2005, Chen et al., 2006). However most of the cell lines reported have some major limitations, for e.g., HEK293 cells, adhere very loosely to the substrate and hence there is a loss of the cells during the screening assays increasing the inter assay variability (Roy et al., 2006; Chen et al., 2006), CHO-K1, MDA-kb-2 cell lines (Wilson et al., 2002; Roy et al., 2004) on the other hand have intrinsic GR leading to a background noise with glucocorticoids; and T47D (Jia et al., 1999; Blankvoort et al., 2001) have intrinsic PR and ER. Moreover, most of the stable cell lines reported earlier for androgen receptor transactivation utilized MMTV-Luc (Dong et al., 2004; Roy et al., 2004; Stroheker et al.,

2005; Sun et al., 2007) which shows a cross reactivity with other steroids, dexamethasone and progesterone (Sonnenveld et al., 2005). These limitations were well addressed in the stable cell line reported in this thesis.

In the development of NIH3T3-hAR-Luc stable cell line, probasin promoter was used which is very specific to the androgen receptor transactivation (Zhang et al., 2000; Andriani et al., 2001). The EC50 value was 0.29 nM which is in good agreement with other stable cell lines reported (Roy et al., 2004; Sonneveld et al., 2005). The specificity of the cells to testosterone was tested by treating the cells with other steroids among which none of them showed any significant ($p<0.05$) cross reactivity. HF showed an antagonistic activity with an IC_{50} ~0.7 μM confirming the cell lines ability to screen AR antagonists (Roy et al., 2004; 2006).

An additional advantage with the developed stable cell lines was that they were adapted for the 96-well format thus making the assay suitable for high throughput screening. The NIH3T3-hPR-Luc cell showed high luciferase activity when progesterone was added to the 96 well plate inoculated with 20,000 cells/well and incubated for 35 h. In the same way, NIH3T3-hAR-Luc cells showed maximum transactivation with 25,000 cells/well incubated for 30 h. These assays showed acceptable repeatability with intra and inter assay CVs being <8% and <16%, respectively, at high concentration which is at par with other sensitive assays reported earlier (Michelini et al., 2005; Xu et al., 2006; Wilkinson et al., 2008).

In conclusion, two stable cell lines were developed for the screening of androgenic and progestagenic EDCs using NIH3T3 cells. These stable cell lines showed high sensitivity and specificity to their respective steroid. Well established antagonists for

these steroids had concentration dependently inhibited the luciferase activity. Finally, the stable cell lines developed were optimized for high throughput screening of EDCs by adapting the assay to the 96 well formats.

CHAPTER-6

6.1 Introduction

An increasing number of natural products, industrial chemicals, pesticides, fungicide etc. have been identified as androgen and progesterone receptor (anta)agonists. Recent studies have shown that the sources of these EDCs are industrial, municipal and surface waste water and even treated effluents (Kumar et al., 2008). An increasing body of evidence reveals associations between various environmental compounds that act as EDCs with the sex hormone sensitive disorders (Chen et al., 2007). For example, in humans, endometriosis (Lebel et al., 1998) and testicular cancer (Ohlson & Hardell, 2000) have been linked to the exposure to organochlorine and plasticizers, respectively. Thus, the damage caused to the endocrine system has its implication on major bodily functions such as reproduction (infertility) and may also lead to the diseases like cancer (Guillette & Gunderson, 2001; Sharpe, 2001; Mendes, 2002). Endocrine disruption effect on sex hormones ranges from feminization in male rainbow trout, masculinisation of females of *Gambusia affinis* to imposex in several buccinideae species (Miyamoto & Klein, 1998). This group reported that compounds including chlorinated pesticides such as DDT, its metabolite DDE, dieldrin, dicofol and hormones such as ethynyl-estradiol, estrone and estradiol released into waste water treatment plants are responsible for these effects. Other organotin compounds like tributyltin hydride and its oxide TBTO, chlorinated biphenyls (PCBs) also were identified to show similar effects (Miyamoto & Klein, 1998; Legler et al., 2002).

Androgens and antiandrogens have also been used in medical applications, for e.g. in the treatment of prostate cancer, as anabolics in hormone replacement therapy,

certain endocrine disorders etc. (Battmann et al., 1998; Sadar et al., 1999; Henderson & Feigelson, 2000; Lu-Yao et al., 2008). Synthetic steroidal progestins also are widely used as therapeutic agents in the control of fertility, combination hormone replacement therapy, and a variety of other endocrine related disorders (De Ziegler & Fanchin, 2000).

In this chapter of the thesis, we utilized the stable cell lines developed- NIH3T3-hAR-Luc and NIH3T3-hPR-Luc, for screening a wide range of chemicals for their androgenic/progesterogenic activities. We also analyzed the WWTP inlet and outlet effluents for the presence of EDCs and quantified their testosterone and progesterone equivalents. To the best of our knowledge, most of the chemicals that were screened in this thesis using our developed assay were being reported for the first time.

6.2 Screening of synthetic (anti)progestative chemicals using NIH3T3-hPR-Luc cells

Fig. 6.1 shows the screening of some known progestagenic EDCs inorder to validate the screening system. These chemicals were collected based on the earlier reports. p,p'-DDT, o,p'-DDT, and nonylphenol significantly ($p<0.05$) inhibited the action of progesterone at a concentration of 1 μM while the compounds like bisphenol A and other DDT metabolites also significantly inhibited the progesterone action but at a slightly higher concentration as compared to the above mentioned chemicals ($p<0.05$). As a whole, all the test chemicals demonstrated anti-progestative activities with varying IC_{50} values ranging from 0.1-10 μM which were at par with the earlier studies (Jin et al. 1997; Scippo et al. 2004; Molina-Molina et al., 2006).

A series of chemicals including pesticides, plasticizers, food additives, cosmetics etc were screened for progesterone EDCs using the NIH3T3-hPR-Luc cells described in the earlier chapter. None of the tested chemicals showed progesterone receptor agonistic

activity (Kojima et al., 2004). However, about twenty different chemicals interfered with the progesterone induced transactivation demonstrating antagonism (Table 6.1). Among the tested chemicals, a few of them like dialifos and lindane (Scippo et al., 2004) were already reported by the previous group. We have screened around fifteen new chemicals that showed progesterone antagonism. Two of them, 4,4'-dichlorobenzophenone and 2,4,6-trichlorphenol showed very strong antiprogestagenic activity with IC_{50} values 0.74 and 0.8 μM, respectively, while 4-octylphenol, 6-hydroxyflavanone, benzoylprop-ethyl, ethiozinpiperophos and 2,3,5-trichlorobenzoic acid showed an IC_{50} ranging between 1-10 μM. Eight of the screened chemicals including chlorpyrifos, nialate, 3-hydroxycarbofuran showed weak to moderate IC_{50} values being more than 10μM.

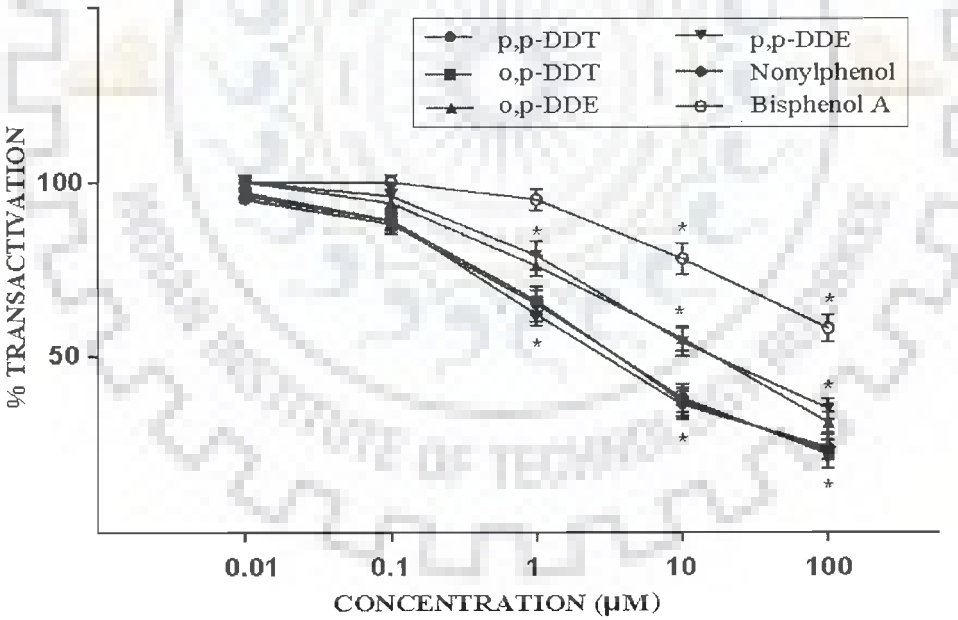


Fig. 6.1 Determination of anti-progestagenic activities of some endocrine disrupting chemicals with clear endocrine disrupting activity. Cells were treated with various concentrations of these chemicals (0.01–100 μM) in the presence of 4 nM progesterone. Luciferase activities were expressed as percentage of that obtained with 4 nM progesterone which was given the value of 100%. The values represent the mean ± S.D. of three similar experiments performed in quadruplicates.
* Significant decrease of luciferase activity as compared to vehicle treated group ($p<0.05$)

6.3 Screening of synthetic chemicals for (anti)androgenic activity using NIH3T3-hAR-Luc cells

When the NIH3T3-AR-Luc cells were treated with increasing concentrations of known testosterone agonists like dihydroxy testosterone (DHT), nandrolone and danazol, these chemicals showed an EC_{50} value of 0.19nM, 0.3nM and 0.75nM respectively (Fig 6.2). On the other hand another group of chemicals like- piperophos, chlorpyrifos, spirinolactone, endosulfan and mancozeb showed significant antiandrogenic activity ($p<0.05$). Piperophos showed a strong antagonistic activity with an IC_{50} of 1.4 μM while spirinolactone, endosulfan, chlorpyrifos and mancozeb also exhibited significant anti-androgenic activities, their IC_{50} values being 7.59, 4.94, 19.3 and 10.6 μM respectively (Fig 6.3) indicating them to be weaker antiandrogenic endocrine disrupting chemicals.

After the chemicals were tested for PR transactivation (Table 6.1), they were also screened for their (anti)androgenic activity. Six of the tested chemicals which includes bicalutamide, fenitrothion, chlorobenzilate, heptachlor, metribuzin and piperophos showed strong antagonism with an IC_{50} value less than 1μM while sixteen other chemicals exhibited an IC_{50} value between 1μM to 10μM (Table 6.1). Fourteen of the test chemicals exhibited a moderate to weak antagonistic activity during this screening. None of the tested pesticides showed androgen agonistic activity which agrees with the earlier reports that almost all the tested pesticides till date showed only antiandrogenic activity (Kojima et al., 2004).

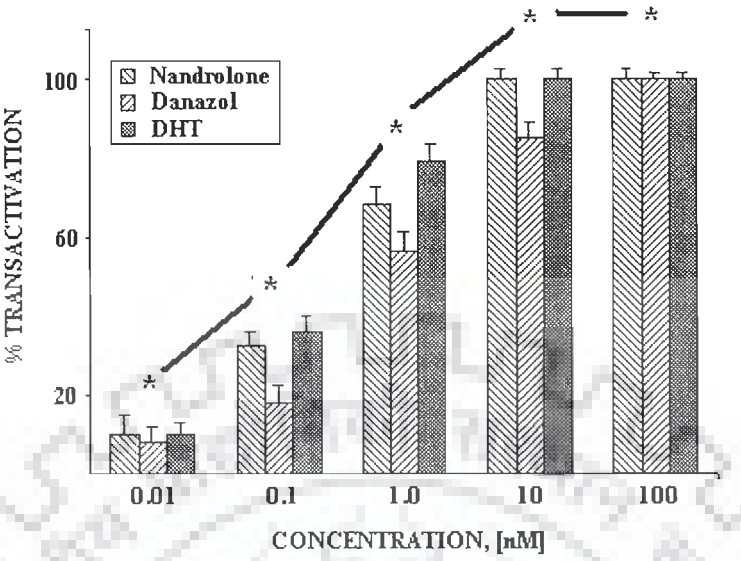


Fig. 6.2 Determination of androgenic activities of some known androgens, DHT, Nandrolone and Danazol. The values represent the mean ± S.D. of three similar experiments performed in quadruplicates.
* Statistically significant induction of luciferase activity ($p<0.05$).

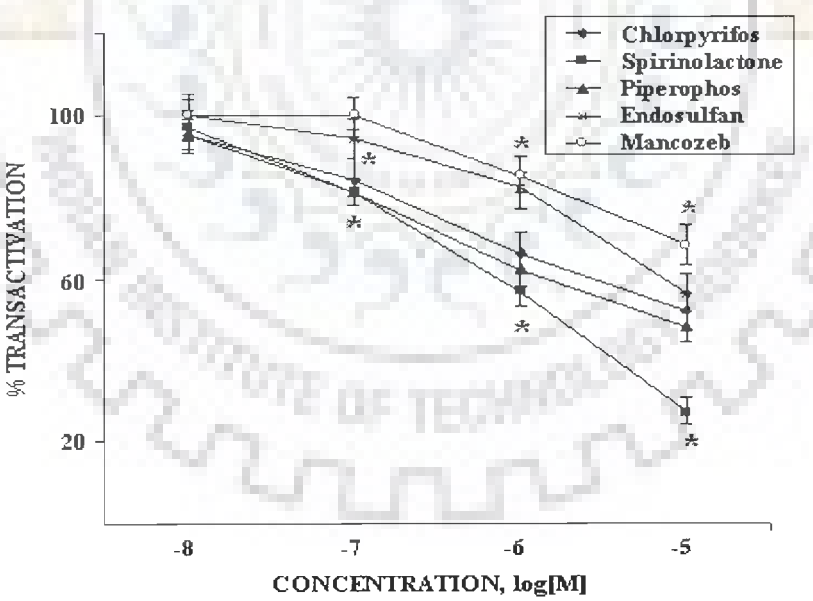


Fig 6.3 Determination of antiandrogenic activities of some endocrine disrupting chemicals. Luciferase activities were expressed as percentage of that obtained with 0.4 nM of testosterone alone which was given the value of 100%. The values represent the mean±S.D. of three similar experiments performed in quadruplicates.
* Significant inhibition of luciferase activity compared to vehicle treated group ($p<0.05$)

Table 6.1 Screening of chemicals for progesterone and androgen antagonists using the transactivation reporter bioassays.

Compound	Androgen receptor (μM)	Progesterone receptor (μM)
Household utilities and other chemicals		
Bicalutamide	+++	+
Hexachlorbenzene	Neg	Neg
3,3',4,4',5-Pentachlorobiphenyl	+	Neg
Naphthalene	+	Neg
4-Octylphenol	++	++
Triclosan	++	Neg
λ-Linolenic acid	+	Neg
3-Methyl hexane	Neg	Neg
Caffeine	Neg	Neg
6-Hydroxyflavanone	++	++
Cinnamic acid	+	Neg
γ-Hexachlorocyclohexane	++	Neg
Phenolphthalin	+	++
Folic acid	+	Neg
Vanillin	+	Neg
Acrylamide	Neg	Neg
Anthracene	+	Neg
Genistein	Neg	Neg
4-Amino-benzonitrile	Neg	Neg
2,4,6-Trichlorphenol	Neg	+++
Pesticides		
4-Aminopyridine	Neg	Neg
Benazolin	Neg	Neg
Benzoylprop-ethyl	Neg	++
Bifenox	++	Neg
Fenitrothion	+++	+
Butachlor	Neg	Neg
Carbendazim	+	+
Carbofuran	Neg	+
Chlorobenzilate	+++	Neg
Chlorpyrifos	++	+
Crimidine	Neg	Neg
Cycloheximide	++	Neg
Dialifos	Neg	Neg
Dichlofenthion	Neg	Neg
Dichloran	Neg	Neg
4,4'-Dichlorobenzophenone	++	+++
Dichloryos	++	Neg
Diphenamid	+	Neg

Edifenphos	+	Neg
Ethiozin	++	++
Ethylene thiourea	+	Neg
Fenamiphos	Neg	Neg
Fenitrothion	++	Neg
Flucythrinate	++	Neg
Helothion	Neg	Neg
Heptachlor	+++	+
3-Hydroxycarbofuran	+	Neg
Leptophos	++	Neg
Lindane	Neg	+
Malathion	Neg	+
Methiocarb	++	Neg
Metribuzin	+++	Neg
1-Naphthol	Neg	Neg
Nialate	++	+
Oxasulfuron	Neg	Neg
Pendimethalin	++	Neg
Phenothrin	++	Neg
Piperophos	+++	++
Probenazole	Neg	Neg
Pyron	Neg	Neg
Spiroxamine	Neg	Neg
Thiabendazole	Neg	+
2,3,5-Trichlorobenzoic acid	Neg	++
Clorohene	Neg	Neg
Phenanthrene	+	Neg
Pentyl phenol	Neg	Neg

+ IC50 > 10μM
++ 10μM ≥ IC50 > 1μM
+++ IC50 ≤ 1μM

6.4 Detection of (anti)progestative and (anti)androgenic activity of waste water treatment plant (WWTP) effluents on 96-well Plates

Using the stable cell lines, we finally screened the inlet and outlet effluents from five different waste water treatment plant (WWTP) for the presence of EDCs. The stable cell lines were incubated in 96-well plate in the presence of various dilutions of extracted water samples from five different WWTP effluents (both inlets and outlets). Our data

showed detectable levels of progesterone and androgen receptor mediated transcriptional activity at levels significantly greater than the vehicle treated group by both inlet and outlet water samples from all the WWTP effluents tested by us ($p<0.05$). As shown in the Fig 6.4, there is a gradual increase in the progesterogenic activities by varying dilutions of WWTP effluents. The effluents resulted in an average of 20-25% increase in progesterone transactivation (as compared to 100% caused by only progesterone) (Fig 6.4) and around 32-38% increase in androgen receptor transactivation (data not shown) at 1 and 2 ml equivalent/well samples from both inlet and outlet effluents. It is calculated from the transactivation data that the inlet and outlet water samples collected from different WWTP plants had a range of, 5-12 ng/l and 4-9 ng/l respectively progesterone equivalents and 9-15 ng/l and 7-14 ng/l testosterone equivalents respectively. This further confirmed that though the treatment plants are able to remove majority of contaminating toxic chemicals, yet some minor amount of EDCs remains in the treated effluents which bypass the treatment process.

Table 6.2 Progesterone and testosterone equivalents in the inlet and outlet effluents of the tested five different WWTP samples.

	Progesterone Equivalents (ng/l)		Testosterone Equivlents (ng/l)	
	Inlet	Outlet	Inlet	Outlet
WWTP I	9.11	7.23	12.26	11.08
WWTP II	5.97	5.91	9.81	8.28
WWTP III	11.93	8.92	15.64	13.98
WWTP IV	6.59	4.08	10.25	8.18
WWTP V	6.59	4.71	8.58	6.98

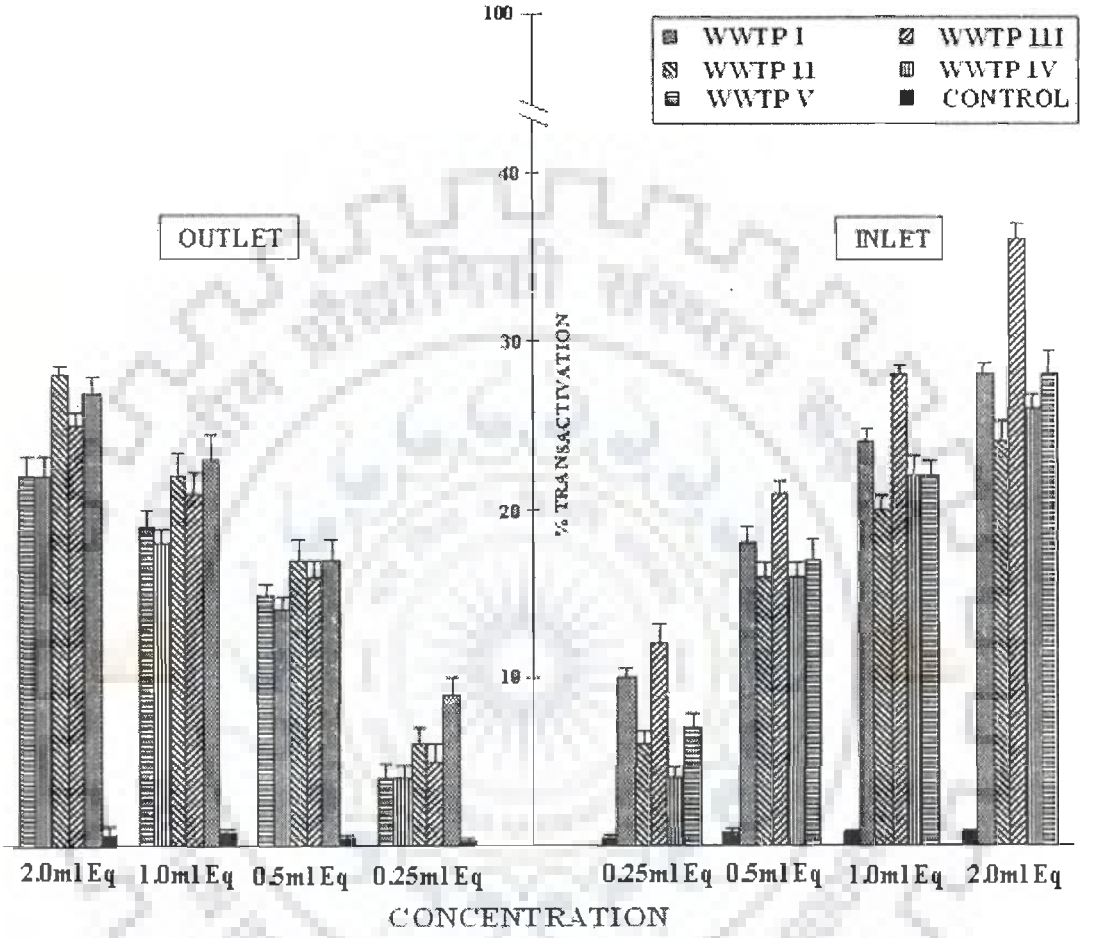


Fig. 6.4 Detection of progestative effects of various dilutions of extracted water samples from both inlet and outlet streams of five different waste water treatment plants. The transfected cells were treated with various dilutions (0.25, 0.50, 1 and 2 ml equivalent/well) of extracted water samples (as described in methodology) from inlet and outlet streams of WWTP. The induction of luminescence was expressed as percentage of that obtained with 100 nM progesterone which was given the value of 100%. The values represent the mean ± S.D. of three similar experiments each performed in quadruplicates.

6.5 Discussion

The main aim of this study was to screen different chemicals for their (anti)progestagenic and (anti)androgenic activities. Using the transactivation assay system described in the earlier chapter, more than 65 synthetic chemicals representing various classes of chemicals like pesticides, plasticizers, food additives and organochemicals were tested for endocrine disruption. Bicalutamide which is generally used as non-steroid drug in prostate cancer treatment and; fenitrothion, chlorobenzilate, heptachlor, metribuzin, piperophos which were widely used pesticides in India exhibited strong androgen antagonistic activity with their IC_{50} less than 0.1 μM. These chemicals except chlorobenzilate also exhibited significant weak antiprogestagenic activity. Piperophos showed moderate antiprogestagenic and strong antiandrogenic activities with an IC_{50} of 7.1 μM and 1.4 μM respectively. 4-octylphenol (Murray et al., 2001), triclosan (Kumar et al., 2008), bifenox, fenitrothion, methiocarb (Kojima et al., 2004) were earlier reported to exhibit antiandrogenic activity. The IC_{50} values determined by us were in good agreement with those reported earlier. Cycloheximide (Masuyama et al., 2002) and phenothrin (Kim et al. 2005) are two common pesticides which were earlier shown to activate the pregnane and estrogen receptor, respectively, were also found to act as antiandrogens with an IC_{50} of 9.21 and 5.84 μM, respectively. Among the test chemicals that exhibited both androgenic and progesterogenic activities, bicalutamide, 4-octylphenol, 6-hydroxyflavanone, chlorpyrifos, fenitrothion, ethiozin, heptachlor and nialate showed a stronger antiandrogenic activity while phenolphthalin, 4,4'-dichlorobenzophenone exhibited strong antiprogesterogenic activity. Andersen et al. (2008) showed that green house pesticides like dichloryos and chlopyrifos caused

impaired development in the sons of the women exposed to this pesticide during their pregnancy. These chemicals tested with this assay interfered with the androgen receptor transactivation showing IC_{50} values of 4.33 and 9.82 μM, respectively.

Progesterone receptor was not as extensively studied as androgenic and estrogen receptor in terms of EDCs. Only few reports exist regarding the screening of progesterogenic EDCs either using binding assay or transactivation assay (Laws et al., 1996; Jin et al., 1997; Koltz et al., 1997; Scippo et al., 2004; Molina-Molina et al., 2006, Viswanath et al., 2008). Except for a very few chemicals screened here like DDT and its metabolites, nonylphenol and bisphenolA, to the best of our knowledge, the other chemicals were not reported earlier either by *in vitro* or *in vivo* assay for their (anti)progesterogenic activities. However, surprisingly none of the pesticides and chemicals selected and tested by us showed agonist activity to either androgen or progegterone receptors. A similar pattern of the response was also reported earlier by another group (Kojima et al., 2004).

The other important source of EDCs that directly affect the humans and the aquatic life is the waste water that is either discharged into the water bodies or reused for secondary usages after processing. It is suggested that industrial and municipal effluents as well as urban and agricultural runoff are the major sources of EDC discharged into the aquatic environment (Snyder et al., 1999; Nasu et al., 2001; Boyd et al., 2003; Björkblom et al., 2008). Elevated levels of vitellogenin and decreased serum testosterone in male carp (*Cyprinus carpio*) (Folmar et al., 1996) and gonadal intersex in roach (*Rutulis rutulis*) and flounder (*Platichthys flesus*), are some of the effects observed in fishes caught near WWTP discharge sites (Jobling et al., 1998; Allen et al., 1999). Further,

several studies have reported the correlation between reproductive abnormalities in fish and exposure to WWTP effluents even several kilometers downstream from outfalls (Jobling et al., 1998; Sumpter, 1998). Recent studies shows that these EDCs are required in a minor quantity to cause endocrine disruption (Sarmah et al., 2006), for instance, concentrations as low as 1 ng/l of estrogen (natural estrogen) led to induction of vitellogenin in male trout (Hansen et al., 1998). Metcalfe et al. (2001) observed the formation of ova in the testis of Japanese medaka starting at a concentration of 4 ng/l of estradiol and 0.1 ng/l of 17α-ethnylestradiol. All these indicate that even a minor quantity of the contaminant is sufficient to bring about changes in the endocrine physiology of wild life and human beings. Therefore, in the second phase of our study the (anti)progestative and (anti)androgenic activities of waste water treatment plant effluents were evaluated using our assays. Although there are many reports regarding the estrogenic activity (Snyder et al., 2001; Aerni et al., 2003; Campbell et al., 2006; Fernandez et al., 2007) in the WWTP effluents, limited reports exist showing the androgenic activity (Urbatzka et al., 2006) and almost none for (anti)progesterogenic activity except one from our lab (Viswanath et al., 2008). To the best of our knowledge, this is the first report where these effluents have been tested *in vitro* for their (anti)progestative activities from WWTP.

In this report, the influents and effluents were collected from five different WWTP complexes of Northern part of India. Our data showed a significant dose dependent increase of 20-25% in progesterone receptor dependent transactivation of the reporter gene and 32-38% of androgen transactivation in both inlet and outlet water samples from all the five WWTP effluents, albeit the effect was lower in the outlet

samples as compared to inlet ($p<0.05$). This was in accordance with the binding studies discussed in chapter 4. This indicates that although the treatment of inlet water samples in WWTP reduces majority of offending chemicals, EDCs are still retained in the outlet samples to the extent that it can demonstrate toxic effects (Ternes et al., 1999; Cargouët et al., 2004). GC-MS analysis of the water extracts from WWTP effluents demonstrated that it is rich in several aromatic compounds like nonylphenol (NP), hexachlorobenzene (HBC), isoandrosterone and dehydrepiandrosterone (DHEA) (results shown and discussed in the next chapter). At this point though it is difficult to speculate the exact amounts of every contaminating EDC within the effluents, the inlet and outlet effluents were determined to have a range of 5-12 ng/l and 4-9 ng/l progesterone equivalents; 9-15 ng/L and 7-14 ng/L testosterone equivalents, respectively. The variations in the amount of androgenic and progestagenic equivalents in various WWTP effluents could be attributed to pattern of life style in different populations of sampling. Thus all these data indicates the need for further understanding their mode of action of these effluents in the *in vivo* system.

In conclusion, the 96 well format of the reporter bioassay optimized earlier was used for screening a wide array of synthetic chemicals of various categories. Later the WWTP effluents were shown to possess androgenic and progesterogenic activity and their corresponding hormonal equivalents were found to be in significant amount that has the potential to cause endocrine disruption in the aquatic life which are directly exposed to these chemicals. Thus, there is a further need to analyze the endocrine disrupting and toxic effects of offending chemicals present in the effluents of WWTPs.

CHAPTER-7

7.1 Introduction

Majority of the effects observed in the aquatic environment concerning the reproductive system, for instance, the feminization of male fish with sewage treatment plant effluents, are attributed to the presence of EDCs (Ternes et al., 1999). The individual compounds that are responsible for these harmful effects are currently unknown, whereas many substances like nonylphenols, phthalic esters, PCBs, dioxins, polycyclic aromatic hydrocarbons (PAHs), phytoestrogens, contraceptives, estrogens, androgens, and progesterone are suspected to influence the hormonal systems (Ternes et al., 1999; Leusch et al., 2006; Oh et al., 2006).

WWTPs receive a large spectrum of molecules from domestic and/or industrial waste which are not completely eliminated during the treatment process (Ternes et al., 1999; Cargouët et al., 2004). In this context, WWTP discharges are considered as a major source of EDC rich pollution that plays a significant role in environmental contamination. Several studies have reported the correlation between reproductive abnormalities in fish and exposure to WWTP effluents even several kilometers downstream from outfalls (Jobling et al., 1998) and these are even required in a minor quantity to cause endocrine disruption (Sarmah et al., 2006). However, except for a few reports (Kumar et al., 2008), these effluents were not evaluated for their androgenic and progestagenic effects in *in vivo* system.

Apart from interfering with the hormone binding to the receptor, EDCs were well studied to interfere with the steroid biosynthesis pathway. A wide range of chemicals like plasticizers, pesticides, fungicides, pharmaceuticals were shown to effect steroidogenesis

and hypothalamic - pituitary-gonadal axis thereby altering the serum testosterone and luteinizing hormone (LH) in *in vivo* rat models. Exposure to the phthalates - benzylbutyl (BBP), di(n)butyl (DBP), and diethylhexyl (DEHP) (Howdeshell & Wilson, 2008), fungicides like procloraz (Blystone et al., 2007), iprodione (Blystone et al., 2007), ketoconazole (Ankley et al., 2005; Perkins et al., 2008), pesticides like fenarimol, dieldrin (Fowler et al., 2007), dioxin (Moore et al., 1991) decreases testicular testosterone production.

Therefore, after the WWTP effluents were tested for their endocrine disruption activity by receptor binding assay and transactivation studies, in the present study, the effect of these effluents on *in vivo* rat models was tested. Similarly, chlorpyrifos and piperophos which showed prominent antiandrogenic and antiprogestative effect in both the receptor binding and transactivation assays were also included in the present study to understand their effect in *in vivo* models. As a part of this study, the (anti)androgenic activity of the test compounds was evaluated by Hershberger assay and also in the intact rats. On the other hand the (anti)progestagenic activity was evaluated by rabbit endometrial proliferation assay.

7.2 In vivo determination of androgenicity in WWTP effluent

7.2.1 Determination of androgenicity of WWTP effluents by Hershberger Assay

At a dose of 150 and 200 ml equivalent of water samples, both inlet and outlet water from WWTP induced a statistically significant increase in the weight of all accessory sex organs viz. prostate gland, seminal vesicle, vas deference, glans penis, and Cowper's gland, in all treated groups as compared to the reference vehicle control ($p<0.05$) (Table 7.1). Thus, the significant increases in absolute sex accessory tissue

weights were attributed to the specific androgenic effects of the WWTP inlet and outlet water. Water samples at a concentration of 100 ml equivalent per day, induced a significant increase only in ventral prostrate, glans penis and Cowper's gland, while no significant increase was observed in other accessory sex structures checked by us. However, treatment of the animals with 50 ml equivalent of water samples did not result in any significant change in the weight of the organs (Table 7.1).

Table 7.1 Effect of WWTP inlet and outlet water samples on weight of accessory sex tissues from castrated rats given 20 consecutive daily treatments WWTP water samples (Hershberger assay).

	Ventral prostate (mg)	Glans penis (mg)	Cowper's gland (mg)	Seminal vesicles (mg)	Vas defference (mg)
Control	10.55±0.35	44.55±0.12	4.40±0.18	47.85±0.18	64.45±1.23
50ml/150µl					
Inlet treated	13.25±0.26**	44.32±0.39*	4.72±0.22*	48.37±0.16*	63.30±1.27*
Outlet treated	11.35±0.23**	44.77±0.47*	4.30±0.19*	47.95±0.29*	63.00±1.46*
100ml/150µl					
Inlet treated	15.00±0.35**	48.02±0.27**	5.45±0.17**	48.62±0.24*	63.52±0.95*
Outlet treated	14.00±0.35**	46.47±0.31**	4.37±0.18*	47.75±0.28*	63.22±0.97*
150ml/150µl					
Inlet treated	30.35±0.70**	54.32±0.23**	8.38±0.20**	67.72±0.24**	98.52±0.28**
Outlet treated	27.80±0.25**	52.55±0.21**	8.17±0.11**	63.47±0.19**	92.82±0.27**
200ml/150µl					
Inlet treated	90.50±0.28**	62.00±0.13**	12.72±0.24**	89.10±1.23**	111.0±0.41**
Outlet treated	86.30±0.55**	59.25±0.22**	11.10±0.17**	80.85±0.23**	102.0±0.48**

Each value denotes mean±S.E.M. of eight animals.
* Not significantly different from vehicle control group at $p<0.05$.
**Significantly different from vehicle control group at $p<0.05$ level.

7.2.2 Determination of antiandrogenicity of chlorpyrifos and piperophos by Hershberger Assay

Based on the already available data of reporter based bioassay, chlorpyrifos and piperophos were studied for their antiandrogenic activity using the Hershberger assay. As a part of this assay, castrated rats primed with 4 mg/kg/day testosterone were

administered with three different doses of the pesticides. Piperophos exhibited a strong antagonistic activity at a concentration of 5 mg/kg/day dosage and above leading to a significant decrease in the weights of the SATs ($p<0.05$). On the other hand, chlorpyrifos did not show any significant decrease in the SATs at 5 mg/kg/day dosage except for cauda epididymis. However, this chemical showed a significant effect at the higher dosages tested (10 and 15 mg/kg/day) ($p<0.05$) (Table 7.2).

Table 7.2 Effect of increasing doses of chlorpyrifos and piperophos on weights of SATs from (0.4 mg/kg) testosterone primed castrated rats administered for 20 consecutive days.

	Testo--sterone (0.4 mg/kg)	Seminal vesicles (mg)	Vas defference (mg)	Glans penis (mg)	Cauda Epididymis (mg)
Control	–	52±1.31	70±0.92	49±2.01	3.4±0.95
Control	+	396±2.10	272±1.97	87.5±1.56	32.2±1.09
Chlorpyrifos (5 mg/kg/day)	+	395±1.87	270±2.05	88.3±1.84	24.7±1.18*
Chlorpyrifos (10 mg/kg/day)	+	245±2.08*	177±1.16*	57.7±2.32*	18.9±2.05*
Chlorpyrifos (15 mg/kg/day)	+	205±1.46*	158±0.85*	52.7±1.85*	14.1±2.16*
Piperophos (5 mg/kg/day)	+	285±2.32*	199±1.65*	71.8±1.88*	19.4±1.91*
Piperophos (10 mg/kg/day)	+	187±1.84*	142±2.05*	49.2±0.95*	10.8±0.92*
Piperophos (15 mg/kg/day)	+	102±1.18*	128±1.99*	42.5±1.19*	6.5±2.01*

Each value denotes mean±S.E.M. of eight animals.
*Significantly different from vehicle control group at $p<0.05$ level.

7.2.3 Determination of (anti)androgenicity of WWTP effluents, chlorpyrifos and piperophos in treated intact rats

7.2.3.1 Gene expression profile of steroidogenic enzymes by semi-quantitative RT-PCR.

As shown in Fig 7.1A, a marked upregulation of mRNA of cytochrome P450scc,

cytochrome P450C-17, 3β-HSD and 17β-HSD in testis was detected in both inlet- and outlet- treated groups. It is to be noticed here that although the effect on expression is less evident in the outlet treated group as compared to the inlet-treated group, the former was around 2-folds more as compared to control. Testis of the inlet-treated group showed a 3.5, 2.5, 2.6 and 2-fold upregulation of mRNA of P450scc, 3β-HSD, 17β-HSD and P450C-17, respectively; while in the outlet-treated group upregulation was 2.3, 1.9, 2 and 1.7-fold, respectively, for the foresaid genes (Fig 7.1B). Further, 2.3- and 1.7-fold increases were found in AR expression in testis of inlet- and outlet-treated group of animals demonstrating its autoregulation.

On the other hand, when chlorpyrifos and piperophos were orally administered to the rats in the concentration of 10 mg/kg body weight, both the pesticides showed a significant decrease in the expression of the important steroidogenic enzymes- P450scc, 3β-HSD, 17β-HSD, tested in testes of the treated groups of rats in comparison to the control group (Fig 7.2A). P450scc showed an approximate 0.3-0.35 fold decrease in the expression in both the treated groups. There is almost 0.4 fold decrease in the 3β-HSD in chlorpyrifos treated rats while an ~0.65 fold decrease of this gene expression is observed in case of piperophos treatment. Both the treated groups showed a significant 0.6-0.5 fold decrease in the 17β-HSD gene expression in comparison to the control group (7.2B). In case of the AR expression, although piperophos treatment group showed a 40% decrease in the receptor's expression, chlorpyrifos did not show any significant change. StAR protein which is important in the cholesterol transport into mitochondria during steroid biosynthesis did not show any significant change in its protein expression as seen from the immunoblot of this receptor protein from the control and treated groups (Fig 7.2C).

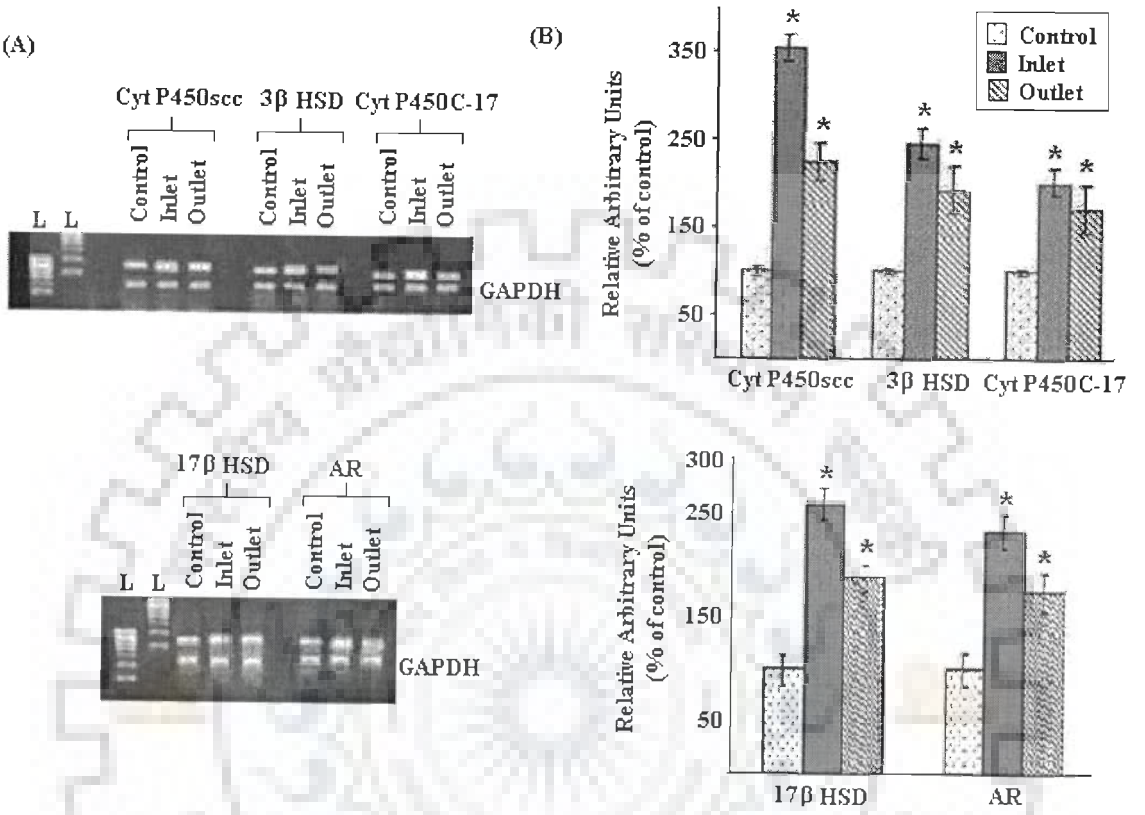


Fig 7.1 (A) RT–PCR analysis of testicular mRNA expression of some major steroidogenic enzyme (P450scc, P450C-17, 3β-HSD, 17β-HSD and AR) genes in rats treated with 200 ml equivalent of sewage inlet and outlet water samples. (B) The relative intensity of the signals were quantified by densitometer and normalized against the internal control (GAPDH). The values are mean±S.E.M. of three separate experiments.
* Significant increase in the gene expression compared to vehicle control ($p<0.05$).

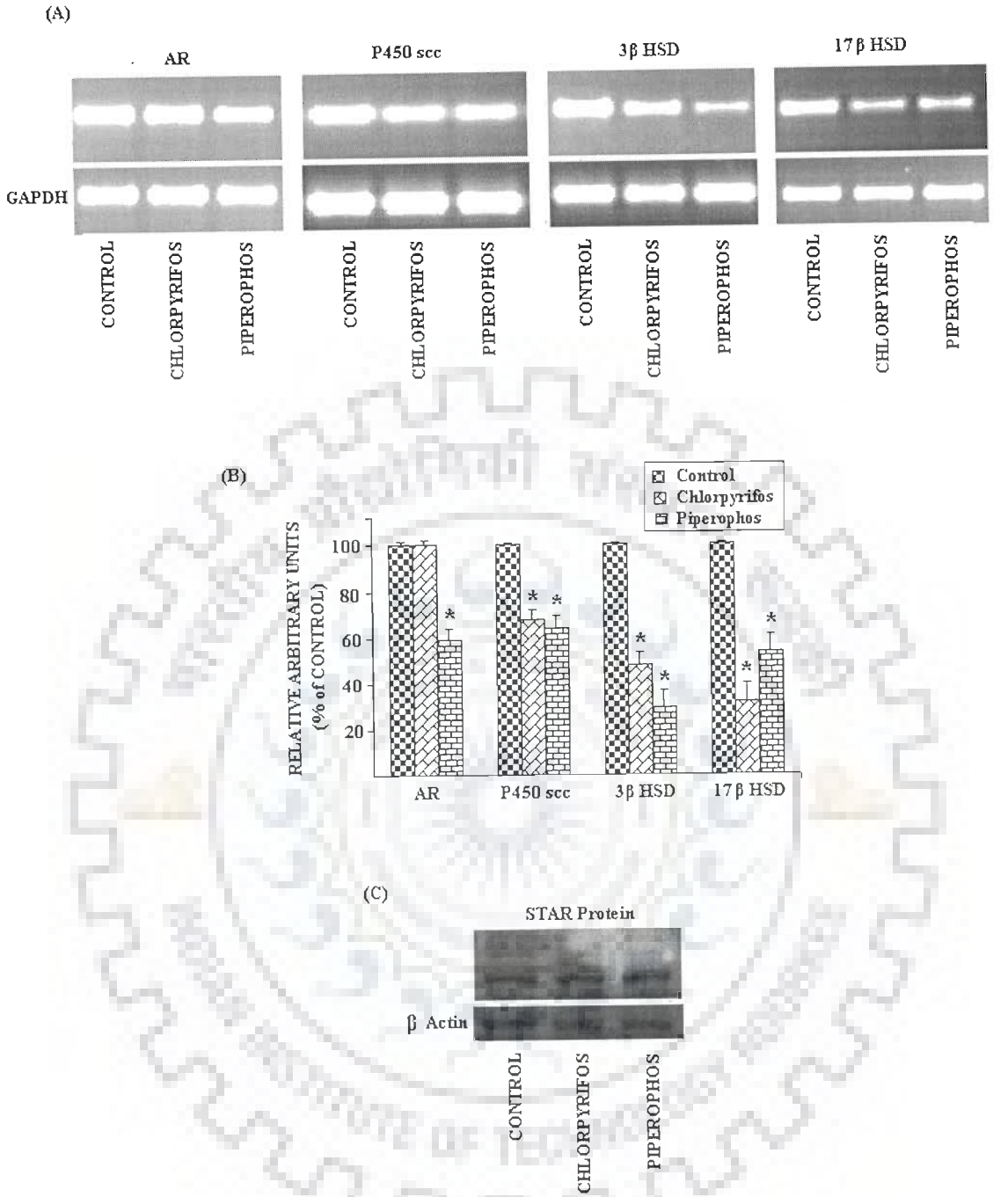


Fig 7.2 (A) RT–PCR analysis of testicular mRNA expression of cytochrome P450scc, 3β-HSD, 17β-HSD and AR genes in intact rats administered with 10mg/kg/day chlorpyrifos and piperophos, respectively. (B) The relative intensity of the RT-PCR products were quantified by densitometer and normalized against the internal control (GAPDH). (C) Western blot showing the expression pattern of StAR protein in testicular protein of these treated groups. The mRNA and protein of the vehicle treated groups served as control.

*Significant decrease in the gene expression as compared to vehicle control ($p<0.05$).

7.2.3.2 Testicular 3β-HSD and 17β-HSD levels determined in vitro

Spectrophotometric enzyme assays for 3β-HSD and 17β-HSD demonstrated that 50 and 100 ml equivalent of water samples could not induce any significant change in the level of these two enzymes (Fig 7.3A). At 150 ml equivalent of the samples there was a significant increase of testicular 3β-HSD and 17β-HSD levels. Finally, treatment with 200 ml equivalent of water sample induced dramatic effects causing a 2.5-fold increase of testicular 3β-HSD, while almost 3-fold increase of 17β-HSD activity in inlet-treated groups. In outlet-treated groups, the effect was 2.3- and 2.5-fold for 3β-HSD and 17β-HSD, respectively (Fig 7.3B). However, no significant changes were observed in the activity of these enzymes in response to chlorpyrifos treated group while piperophos treatment showed a marginal 1-1.5- fold decrease in both the enzyme activities in comparison to the control animals (Fig 7.3C).

In the present investigation, 17β-HSD activity was estimated for its reverse catalyzing activity by using testosterone as substrate and androstenedione as product. Since this enzyme is active in both the directions, i.e., converting androstenedione to testosterone and vice versa, a similar pattern of enzyme activity was obtained when androstenedione was used as substrate instead of testosterone (data not shown).

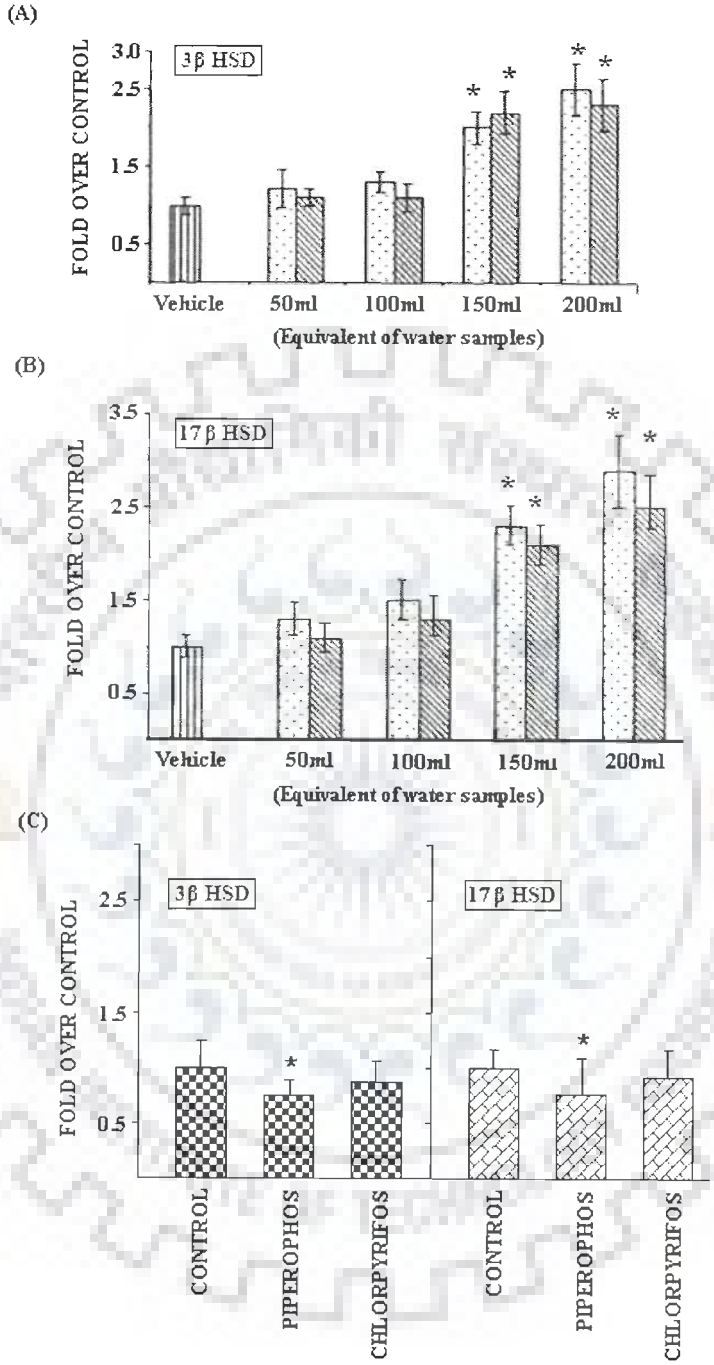


Fig 7.3 Effects of WWTP water samples from inlet (dotted bar) and outlet (thatched bar) on testicular (A) 3β-HSD and (B)17β-HSD enzyme activity *in vitro*. (C) Effect of 10 mg/kg/day chlorpyrifos and piperophos, on testicular 3β-HSD and 17β-HSD enzyme activity *in vitro*. The results are expressed as fold increase of enzyme activity over vehicle-treated groups. Data are mean±S.E.M.; n=8.

*Significant level of differences in enzyme levels as compared to vehicle-treated groups for both the enzymes ($P<0.05$).

7.2.3.3 Serum hormone levels

As shown in Table 7.3, in comparison to control group, there is an apparent increase in the level of testosterone in the intact rats treated with WWTP effluents (31% in inlet- and 21% in outlet treated groups). Also, there is a decrease in the levels of LH (33% in case of inlet and 24% in case of outlet) and FSH (26% in case of inlet and 19% in case of outlet) in the serum samples, confirming further the androgenicity of the contaminants in the WWTP (Table 7.3). Decrease in the levels of LH and FSH could be attributed to the initiation of feedback mechanism by the androgenic contaminants in the water samples.

In case of chlorpyrifos and piperophos treatment, they did not show any significant alteration in the level of serum LH and FSH. However, the testosterone level however decreased significantly by 21% and 32% in case of chlorpyrifos and piprophos administration, respectively (Table 7.3). The decrease in the serum testosterone level could be attributed to the reduction in the expression and activation of key steroidogenic enzymes in those treated rats (Fig 7.2A).

Table 7.3 Serum levels of LH, FSH and testosterone from intact rats treated with WWTP water samples and pesticides [chlorpyrifos and piperophos (10 mg/kg body weight)].

	LH (ng/mL)	FSH (ng/mL)	Testosterone (ng/mL)
Control	6.52±1.79	0.83±0.09	7.50±1.10
200 ml/150 µl			
Inlet treated	4.32±0.94**	0.61±0.08**	10.40±1.70**
Outlet treated	4.93±1.27**	0.67±0.02**	9.80±1.60**
Chlorpyrifos	6.49±0.97	0.81±0.04	5.95±0.98**
Piperophos	6.22±1.18	0.84±0.06	5.12±1.81**

Each value denotes mean±S.E.M. of eight animals.
** Significantly different from vehicle control group at $p<0.05$ level.

7.2.3.4 Analysis of systemic toxicity in WWTP-treated animals

Systemic toxicity that was presumed from the decrease in the weight of liver and kidney in the above section was confirmed by about 2- to 3-fold increase in the serum level of alkaline phosphatase, SGPT and SGOT in both inlet and outlet water treated groups of animals and the effect was more pronounced in inlet stream (Fig 7.4). The WWTP did not show any effects on the levels of Acid phosphatase.

The administered dosage of pesticides did not show significant changes in the levels of serum acid phosphatase and alkaline phosphatase. However, SGPT and SGOT levels increased by 1- to 1.5- fold indicating noticeable toxicity in the liver and kidney (Fig 7.4).

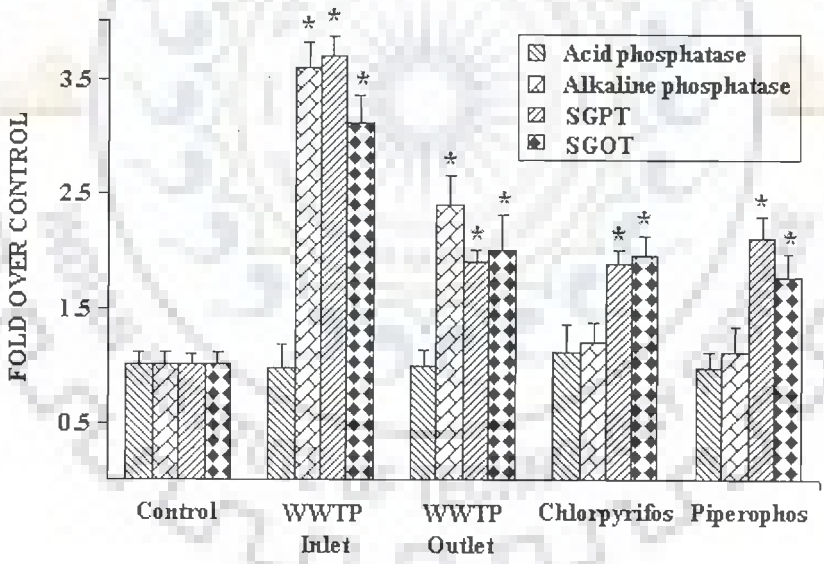


Fig 7.4 Effect of WWTP inlet, outlet water samples (200 ml equivalent), chlorpyrifos and piperophos (10 mg/kg/day) on ALP, ACP, SGOT and SGPT levels in the blood serum of the treated rats. The values are mean±S.E.M. of three separate experiments.
* Significantly different from vehicle treated group ($p<0.05$)

7.2.3.5 GC–MS analysis

To detect the possible (anti)androgenic and (anti)progestagenic compounds in the

WWTP effluents that are responsible for their endocrine disrupting activity, GS-MS analysis of the water samples was carried out. Four compounds, viz. 4-nonylphenol, hexachlorobenzene, dehydroepiandrosterone (DHEA) and isoandrosterone, having retention times of 10.72, 11.80, 16.6 and 17.65 min, respectively, were identified by GC–MS as shown in Fig 7.5A.

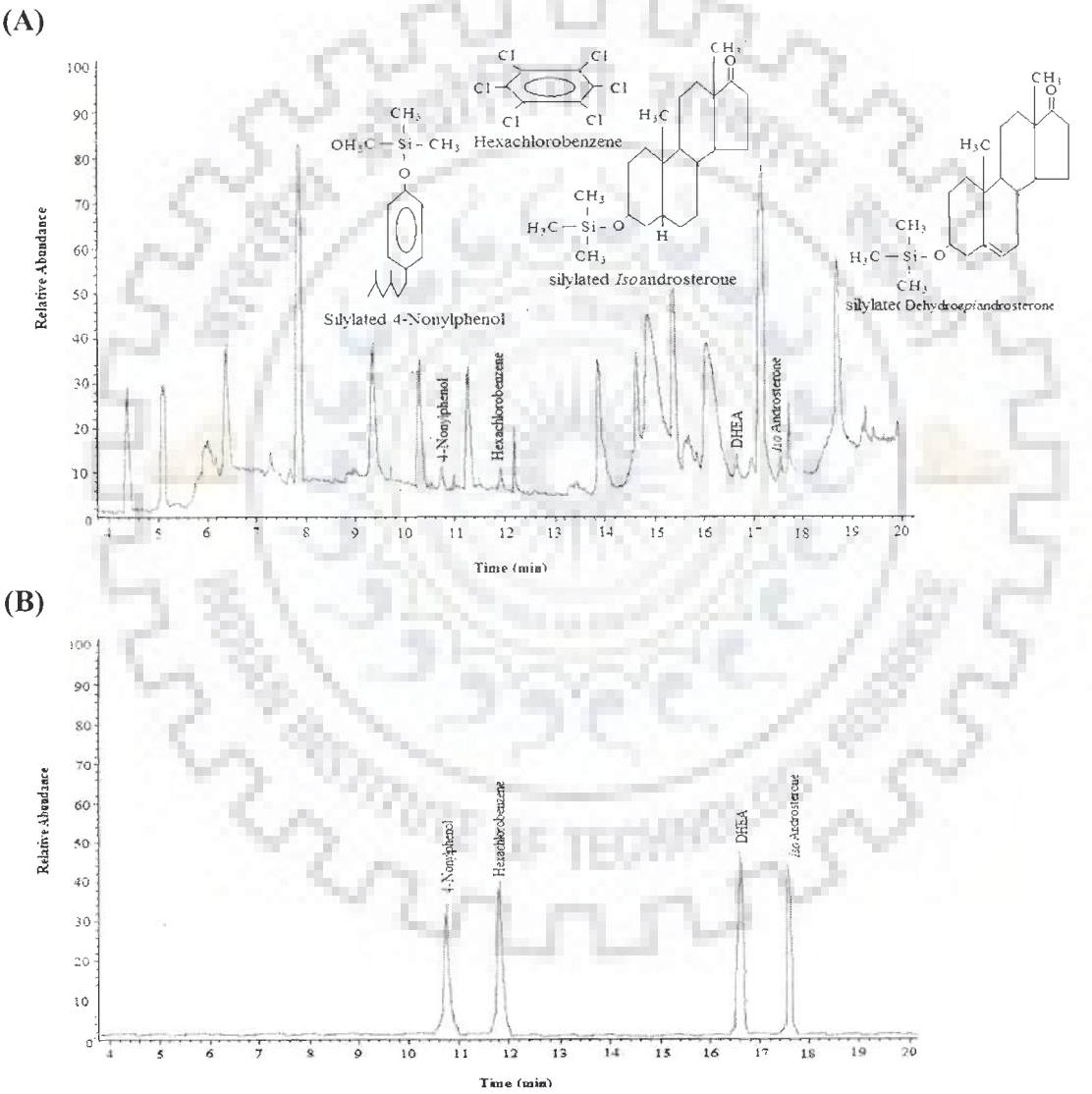


Fig 7.5 Representative GC–MS chromatograms of final sewage effluent sample (A) and its comparison with solutions of known standards (B) in full acquisition mode. The WWTP outlet effluents were derivatized and analyzed by GC–MS. The insets in Panel A show the structure of derivatized (silylated) forms of target chemicals except hexachlorobenzene that is not derivatized by BSTFA.

The inset shows the structures of the derivatized compounds. The elution profile of the effluent samples matched closely with the known standards (Fig 7.5B). All the detected contaminants were well within the detection limit.

7.3 *In vivo* studies for the determination of (anti)progestagenic activities

7.3.1 (Anti)progestational activity in rabbit endometrial transformation test

Earlier, reporter based bioassay results showed progestative effect of WWTP effluents and antiprogestative effect of chlorpyrifos and piperophos. These effects were confirmed in *in vivo* model using rabbit endometrial transformation test.

Estrogen-primed immature rabbits receiving 5 days of progesterone treatment without the test compound demonstrated an increased McPhail index. Daily oral administration of 5 mg/kg/day and 10 mg/kg/day doses of piperophos inhibited this progesterone-dependent transformation of the uterus in a dose-dependent manner (Table 7.4). Treatment with chlorpyrifos did not result in the inhibition of endometrial transformation by progesterone at a concentration of 5 mg/kg bodyweight, however, there was a slight inhibition observed at a higher concentration of pesticide administration of 10 mg/kg body weight (Table 7.4).

On the other hand, for testing WWTP effluentsfor progestagenic activity, the effluents were administered to rabbits only primed with estrogen without any progesterone treatment. WWTP effluents showed progesterogenic effect by inducing the proliferation of the estrogen primed endometrium indicating a significant progesterone endocrine disrupting activity (Table 7.4).

Table 7.4 Effects of test chemicals and WWTP effluents on progesterone induced-endometrial transformation.

Compound	Daily dose for 5 days (mg/kg) body weight	Progesterone (0.1 mg per day for 5 days, s.c.)	Progestational index, Mc Phail units, 0-4 (mean ± S.D)
Vehicle1	-	-	0.0±0.0
Vehicle2	-	+	4.0±0.0
Chlorpyrifos	5	+	4.0±0.0
	10	+	3.5±0.4**
Piperophos	5	+	3.4±0.2**
	10	+	2.7±0.3**
WWTP effluent			
Inlet	200ml Eq	-	2.9±0.5*
Outlet	200ml Eq	-	1.8±0.4*

Each value denotes mean±S.E.M. of six animals.
*Significant increase of Mc Phail units compared to vehicle control ($p<0.05$).
**Significant decrease of Mc Phail units compared to vehicle control ($p<0.05$).

7.4 Discussion

Several reports suggest the presence of a number of potential EDCs as well as toxicants in finally treated sewage effluent and in receiving water bodies with synthetic and natural androgens/estrogens, insecticides, pharmaceuticals, phenols, detergents, etc. (Ternes et al., 1999; Lucy et al., 2002; Thomas et al., 2002; Oh et al., 2006). A dramatic effect of this effluent at the endocrine disruption as well as toxicity level has been shown in the aquatic animals exposed to the sewage water (Jobling et al., 1998; Svenson and Allard, 2005; Leusch et al., 2006; Sarmah et al., 2006; Subramanian and Amutha, 2006). Thus, there is a need to analyze the endocrine-disrupting chemicals present in finally treated effluent of WWTP on terrestrial animals. In this study, complex mixture of chemicals from inlet and outlet water samples of WWTP and two common pesticides used extensively in Inida, chlorpyrifos and piperophos, which have shown moderate and

strong antagonism, respectively in transactivation studies, were chosen to be tested for their (anti)androgenic effects in rodent models. The dose administered to the rats was below the LD_{50} value of the pesticides.

Both inlet and outlet water samples were screened by Hershberger assay to determine their androgenic effect on the male reproductive system. This is a simple, short and specific assay to screen the compounds for (anti)androgenic effect (Gray et al., 1997; Kennel et al., 2004). The Hershberger assay caused a dose-dependent increase in the weight and size of the SATs, depicting a clear androgen agonistic activity in both inlet- and outlet-treated castrated rats. Since the animals were castrated, the source of endogenous testosterone in rats could be the adrenal glands. Testosterone is also known to be produced in adrenal glands in response to hormonal signals and it is called adrenal androgen, but the content of testosterone produced in the adrenal gland is negligible in normal status (Rainey et al., 2002; Carson & Rittmaster, 2003; Kang et al., 2005). Since the water samples tested by us contained some androgen precursors or its analogs (DHEA and isoandrosterone), it could be argued that they might have contributed to the serum androgen levels that is needed for the maintenance of SATs in the castrated treated animal groups. On the other hand, chlorpyrifos and piperophos showed a significant decrease in the weights of the SATs in testosterone primed castrated rats exhibiting their clear antiandrogenic activity.

After the Hershberger assay, the probable mechanism of action of these (anti)androgenic contaminants was determined by administrating the samples to intact rats. RT–PCR analysis of the major steroidogenic enzymes namely cytochrome P450scc (P450scc), cytochrome P450C17 (P450C17), 17β-HSD, and 3β-HSD demonstrated a

significant upregulation of testicular mRNA transcripts in WWTP treated groups of animals as compared to control. In case of the pesticides treated groups, as expected, these genes expression were found to be significantly down regulated. These observations were further supported by the *in vitro* estimation of testicular 3β and 17β-HSD enzyme activities, which showed a significant increase in enzyme activity in WWTP treated groups while there was a decreased activity of these enzymes observed in case of pesticide treated groups, compared to the control group of animals ($p<0.05$). The decrease in the steroidogenic enzymes expression and activity in piperophos treated group was more prominent than the chlorpyrifos treatment. However, there was no significant change in the StAR protein expression in either of the pesticide treated animal groups. The up/downregulation of these steroidogenic enzymes, P450scc, P450C17, and 3β-HSD, by xenobiotics and the low dose of testosterone have already been reported by some groups (Lin et al., 2006; Kim et al., 2007; Kortner & Arukwe, 2007). Xenobiotics-dependent direct up/downregulation of steroidogenic enzymes and steroidogenesis could be attributed to several factors: (i) their action through arylhydrocarbon receptor (AhR) (Indarto and Izawa, 2001); (ii) direct binding of these chemicals to steroid receptors, steroidogenic enzymes and proteins associated with steroidogenesis (like Steroidogenic Acute Regulatory protein) (Indarto & Izawa, 2001; Eertmans et al., 2003; Kang et al., 2005; Rice et al., 2006); and (iii) increased stability of transcripts and transcriptional rate of the promoter of steroidogenic enzymes (Lin et al., 2006; Lyssimachou et al., 2006). Although adrenal gland is also a source of steroid biosynthesis, the level of testosterone synthesized is very low by this gland. Therefore the effect of the test chemicals in case of this organ steroidogenesis was ignored in the present study. Both the pesticides and the

WWTP effluents showed significant androgen receptor binding and transactivation in the earlier chapters. This conclusively proved that these chemicals may be directly interfering with the expression of these genes as the steroidogenic genes' expression was auto-regulated by the androgen receptor itself. At this point, it is difficult to make a precise conclusion on how these test chemicals and effluents affected the steroidogenic enzyme levels as the transcription of steroidogenic enzymes is a very complex process. Since many of the EDCs and steroid agonists act directly on the receptor and steroidogenic enzymes (as mentioned above), it is possible that the increase in the expression and activity of these steroidogenic enzymes in our study is independent of the levels of LH and FSH which are known to play a significant role in steroidogenesis in normal instances. LH-independent regulation of steroidogenesis has also been reported earlier by some authors where treatment of rats with testosterone or its analogs resulted in upregulation of steroidogenesis even without any significant changes in LH levels (Kennel et al., 2004). These further support our finding that the low levels of LH did not interfere with up/downregulation in steroidogenesis. However, the exact cross talk between the gonadotrophic hormone, xenobiotics, and testosterone remains an intriguing question that needs further research in this direction. An upregulated mRNA of androgen receptor in the testes of WWTP treated group of animals is another interesting feature of this study that may be caused due to structural similarity of the offending chemical in the test sample with that of androgens. However, autologous regulation of the AR gene in the testes is still a matter of controversy (Ohsako et al., 2003) since reports exist on both regulation and non-regulation of AR mRNA expression by the androgen itself (Shan et al., 1997; Ohsako et al., 2003). In case of the chlorpyrifos and piperophos treatment, they

did not show significant changes in the AR gene expression patterns. All these data confirmed that the androgenic potential of the contaminating chemicals in the WWTP effluents and the antiandrogenic activity of chlorpyrifos and piperophos pesticides, involved the changes in the expression pattern of genes crucial in the steroidogenic cascades. However, it still remained elusive if these effects are either by the direct interaction of these chemicals with the genes or not.

The next obvious question was to check whether, in addition to endocrine disruption, the effluent also demonstrated toxic effects in treated animals. Upon performing the toxicity analysis, it was found that there was a decrease in the weight of liver and kidney, which was further assessed by histopathological studies (data not shown). Further, serum alkaline phosphatases, acid phosphatase, SGOTand SGPT were analyzed as indices of systemic toxicity. In the present study, the decrease in the weight of liver and kidney of both inlet and outlet water-treated and pesticides-treated rats could be attributed to the degeneration of cells in these organs. These data were further supported by an increased level of serum ALP, SGOT and SGPT in the treated groups as compared to control. These enzymes have been extensively used as marker enzymes for toxicological studies of liver and kidney (Sato et al., 2004; Bhattacharjee & Sil, 2006). It could be argued that the change in testosterone levels in treated animals is simply secondary to xenobiotic-induced liver/kidney injury thereby reducing testosterone metabolism. However, at this point, this fact cannot be overruled completely, but earlier reports confirmed that the systemic toxicity has also been noted in rats treated with methyltestosterone (MT) and paper mill effluents (rich in several anti-androgenic chemicals) along with significant change in absolute SAT weights and other male

reproductive structures. These have been attributed to the specific (anti)androgenic effect of MT and contaminants of leather industry effluents rather than systemic toxicity (Kennel et al., 2004; Rana et al., 2004).

In the next phase of experiment, we wanted to identify the offending chemicals responsible for the androgenic and progestagenic effects in the WWTP effluents. For this, the WWTP effluents were analyzed by GC-MS. The water samples were found to contain several aromatic compounds like nonylphenol (NP), hexachlorobenzene (HBC), isoandrosterone and dehydroepiandrosterone (DHEA). All these chemicals have been known to be used in various household and industrial applications; that is, HBC is a commonly used insecticide, NP is a breakdown compound of different synthetic product while isoandrosterone and DHEA are natural androgens found in human body and percolates to the WWTP through human/animal use or excretion (Soto et al., 2004). All these four compounds identified by us have already been well documented as androgen receptor agonists in various reports (Ralph et al., 2003; Leusch et al., 2006; Kortner & Arukwe, 2007).

Earlier studies showed that WWTP inlet and outlet effluents exhibit progesterogenic effect while chlorpyrifos and piperophos show an antiprogesterogenic activity. In the chapter, we studied their (anti)progestagenic activity effect in the female immature rabbits by endometrial proliferation assay. In this endometrial transformation test, chlorpyrifos and piperophos showed comparable anti-progestational effects when they were administered at 10 mg/kg per day orally. Piperophos showed a stronger antagonism than chlorpyrifos which is in good agreement with the transactivation results shown earlier. WWTP inlet and outlet effluents showed an agonistic effect on the

estrogen primed endometrium causing proliferation in it. In case of WWTP effluents, the inlet effluent showed a higher rate of proliferation than the outlet effluent indicating the stronger agonistic activity of inlet effluent over the outlet effluent. This could be attributed to the presence of proliferation agents in higher proportion in inlet as compared to the outlet. At this point, it is difficult to speculate what chemicals exactly are causing this effect. However, several androgenic chemicals have been shown to act as progestagenic chemicals especially at higher concentrations due to loss of specificity at that elevated concentrations (Chatterjee et al., 2008). Since GS-MS analysis revealed the presence of DHEA, isoandrosterone in addition to nonylphenol and hexachlorobenzene, they either combinationally or singly might be resulting in this progestagenic effect by WWTP effluent.

In conclusion, the synthesis of steroid hormones is one of the crucial processes in the endocrine regulation consisting of sensitively regulated steps that are affected by different endocrine-disrupting chemicals. The data presented here conclusively provides evidence of WWTP effluents (both inlet and outlet), chlorpyrifos and piperophos acting as endocrine disruptors in rats and demonstrates their potential impact on androgen metabolism. The test chemicals can interfere with transcriptional activity of major steroidogenic enzymes and the downstream effects. Further NP, HCB and androgen agonists (DHEA and isoandrosterone) were shown to be present in WWTP effluents by GS-MS analysis. These pesticides and effluents also showed progestagenic endocrine disruption in the rabbit *in vivo* system similar to the earlier studies. To our knowledge, this is the first study reporting the progesterogenic activity in WWTP effluents as well as the pesticides chlorpyrifos and piperophos by *in vivo* model as described in this chapter.

CHAPTER-8

8.1 Introduction

Androgens are the hormones that play a pivotal role in the development and maintenance of the male sex characteristics. Their biological effects are mediated by the ubiquitously expressed androgen receptor (AR). The levels of AR change in different pathological conditions such as malignancies or in response to physiological changes of the endocrine system (Roy et al., 2004). AR bound to the testosterone translocates to the nucleus where it binds to the regulatory regions of androgen-responsive genes, recruits the transcriptional machinery and co-regulators and subsequently leads to the transcription of the target genes (Blankvoort et al., 2001). Anti-androgenic compounds however bind to the AR, but block its transcriptional activity.

The AR-ligand complex bound to the ARE recruits a number of co-activators that are needed for the expression of the downstream target genes. Some of the co-activators include p160 family proteins like steroid receptor coactivator-1(SRC-1), TIF2, acetyltransferase (ACTR)/thyroid hormone receptor-activated molecule 1 (TRAM1) P300/CBP-associated factor (PCAF) (Ma et al., 1999; Slagsvold et al., 2001), CREB binding protein (CBP/P300) (Aarnisalo et al., 1998; Fronsdal et al., 1998) etc. Co-activators, CBP/P300 and P/CAF possess strong histone acetyl transferase (HAT) activity and also help in the recruitment of additional transcriptional factors. *In vitro* experiments have demonstrated a direct, SRC-independent interaction between CBP, P/CAF and the AR (Fu et al., 2000). In addition to their effects on histones, CBP and P/CAF can acetylate proteins such as transcription factors and co-regulators including AR where it acetylates three lysine residues in its DBD-hinge region (Fu et al., 2000). Further, these

co-factors function as a direct bridge between DNA-bound AR and the basal transcriptional machinery (Hannelore et al., 2007). Thus CBP/P300 plays an important role in the androgen receptor mediated gene expression.

Apart from interfering with the hormone binding to the receptor, EDCs have also been known to interfere with the steroid biosynthesis pathway. A wide range of chemicals like plasticizers, pesticides, fungicides, pharmaceuticals were shown to affect steroidogenesis and hypothalamic - pituitary-gonadal axis thereby altering the serum levels of testosterone, luteinizing hormone (LH) and follicle stimulating hormone (FSH). Exposure to the phthalates benzylbutyl (BBP), di(n)butyl (DBP), and diethylhexyl (DEHP), fungicides like procloraz, iprodione, ketoconazole, pesticides like fenarimol, dieldrin, dioxin decreases testicular testosterone production (Moore et al., 1991; Ankley et al., 2005; Blystone et al., 2007; Fowler et al., 2007; Howdeshell & Wilson, 2008; Perkins et al., 2008). Studies showed that the decrease in testosterone production by Leydig cells was due to several reasons, to name a few, due to the alterations in the cholesterol availability in the mitochondria for steroidogenesis, altered steroidogenic enzyme activities and feed back mechanisms (Kumar et al., 2008). Chen et al. (2007) had shown the interference of triclocarban in cAMP pathway activated by LH receptor. All these support the notion that these EDCs acts at various levels of steroidogenic cascade to disrupt the endocrine system.

The main aim of the present study was to understand the role of two common pesticides (chlorpyrifos and piperophos) mode of action *in vitro* at cellular level as endocrine disruptors. These chemicals showed prominent interaction with AR as well as PR in receptor binding and transactivation assays in the earlier chapters. Their

antiandrogenic and antiprogestagenic activities were also studied in *in vivo* models. These pesticides were studied further to check if they interfere with the recruitment of the co-activator CBP and its direct cross talk with the transactivation of AR. Further they were studied for their effects on steroid biosynthesis in rat Leydig cell cultures and subsequent analysis for their cross-talk with LH receptor mediated functions.

In the regard of this study, pTAL, pAP1-Luc, pNFkB-Luc and pCRE-Luc plasmid vectors were used. These plasmids were described in detail in the methodology, chapter-3 of this thesis. Briefly, these vectors have specific *cis*-acting DNA binding sequences located upstream of the TATA-like promoter (pTAL) region from the herpes simplex virus thymidine kinase (HSV-TK) promoter driving a luciferase reporter gene. The vector pTAL was used as null vector, which did not have any *cis*-acting elements in its promoter region and was a negative control in the assay. pAP1-Luc, pCRE-Luc and pNFkB-Luc has *cis*-acting elements, activator protein 1 (AP1) binding element, cAMP response element (CRE) and nuclear factor of kB (pNFkB) binding element, respectively. The increase in the expression of any of these secondary molecules leads to the increase in the luciferase induction thus making them quantifiable.

8.2 Role of CBP in the chlorpyrifos treated NIH3T3 cells

8.2.1 Crosstalk of the androgen receptor pathway with other signal transduction pathways

Four batches of NIH3T3 cells were transfected with pSG5-hAR and one of the four *cis*-acting elements, pTAL, pAP1-Luc, pNFkB-Luc or pCRE-Luc plasmids, representing different signal transduction pathways that were previously reported to have a crosstalk with androgen receptor pathway. Cells transfected with pTAL was used as

negative control since it did not have any *cis* acting element. These transfected batches of cells were then treated with chlorpyrifos and piperophos. It was observed that in the chlorpyrifos treatment, there was a ~5 fold increase in lucifersae activity of pNFkB-Luc transfected cells (Fig 8.1A).

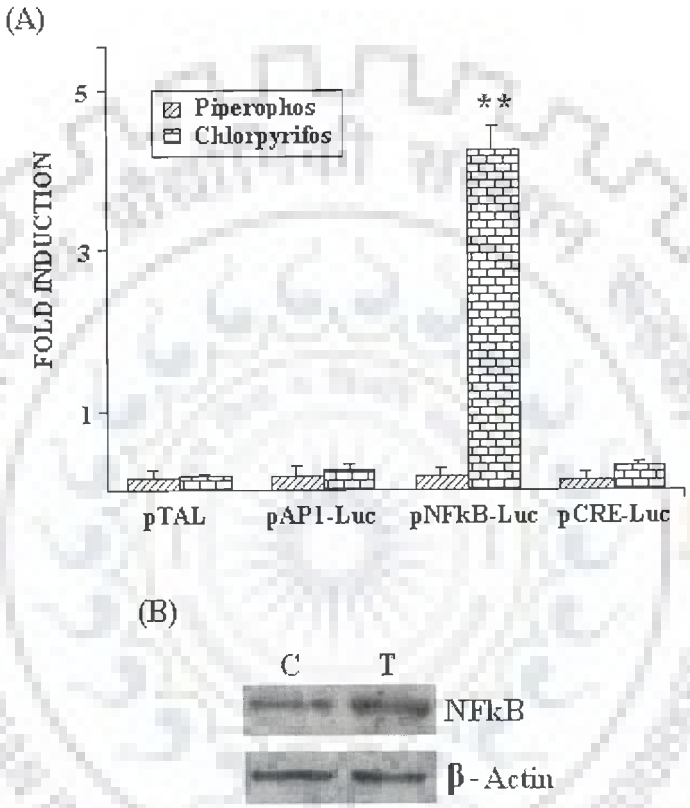


Fig. 8.1 (A) NIH3T3 cells were transfected with three *cis*-acting elements, representing different signal transduction pathways, upstream of a luciferase reporter gene that were previously reported to have a crosstalk with androgen receptor pathway. The values represent the mean±S.D. of three similar experiments performed in triplicates. [** Significant luciferase activity as compared to the pTAL transfected cells ($p<0.05$)]. (B) Immunoblot analysis of NIH3T3 cells in response to chlorpyrifos. Lane C, Protein extract from vehicle treated NIH3T3 cells. Lane T, Protein extract from chlorpyrifos treated NIH3T3 cells.

This indicated that probably in these cells; NFkB was interacting with the *cis* acting element. Thus, the increase in NFkB in these chlorpyrifos treated cells was confirmed by the immunoblot of the NFkB protein from both the treated and control cells (Fig 8.1B). However, the other two groups of treated cells transfected with pAP1-Luc and pCRE-Luc did not show any significant change. Piperophos on the other hand did not show any significant change in any of these pathways studied (Fig 8.1A).

8.2.2 Over expressed CBP in NIH3T3-AR-Luc rescue the chorpyrifos weak antagonistic activity.

When chlorpyrifos and piperophos were added to the NIH3T3-AR-Luc cells, these chemicals did not induce any luciferase activity; however these chemicals inhibited the testosterone stimulated transactivation showing their clear antagonistic activity (Fig 6.3). Chlorpyrifos showed weak antagonism with an IC_{50} of 19.3 μM while piperophos exhibited a strong antagonistic activity with an IC_{50} of 1.4 μM in NIH3T3-AR-Luc stable cells. In order to elucidate the exact mode of AR inhibition by these chemicals, we studied if at all these chemicals interfere with the recruitment of co-activators by AR.

As a part of this study, the receptor complex ability to recruit the CBP cofactor that is earlier reported to be important during the initiation of the transactivation of steroid receptor genes was studied. NIH3T3-AR-Luc cells were transfected with exogenous pCMV-CBP to over express the CBP protein within the recombinant cell line. These cells were then treated with the test chemicals, piperophos and chlorpyrifos at a dose as determined earlier, in the presence and absence of 0.3 nM testosterone (to study the antagonistic and agonistic activities, respectively) and the luciferase activity was measured.

Chlorpyrifos, in the presence of excess CBP failed to inhibit the testosterone stimulated luciferase gene expression in NIH3T3-AR-Luc cells, treated with both 0.3 nM testosterone and chlorpyrifos (Fig 8.2A). In contrary, when only chlorpyrifos (without testosterone) was added to these CBP over expressing NIH3T3-AR-Luc cell line, there was about 3-4 fold up regulation of the luciferase activity (Fig 8.2B). Thus most interestingly, the over expressed CBP has rescued the weak antagonistic activity of the chlorpyrifos. This shows that there is a limitation for the intracellular CBP available for the AR bound chlorpyrifos complex in NIH3T3-hAR-Luc cells not transfected with exogenous CBP. Piperophos however did not show any such activity with the over expressed CBP (Fig 8.2B). Probably this could be due to the strong androgenic inhibition activity involving multi cascades.

Both NFkB and AR need CBP for their transactivation activity in the cells during their biological response. Therefore, the increased NFkB in the chlorpyrifos treatment earlier studied and the CBP limitation in chlorpyrifos treated transactivation indicate that the increased NFkB in the chlorpyrifos treated cells might be utilizing the CBP for its activity causing a limitation for the co-factor by AR bound chlorpyrifos complex leading to its weak anti-androgenic activity.

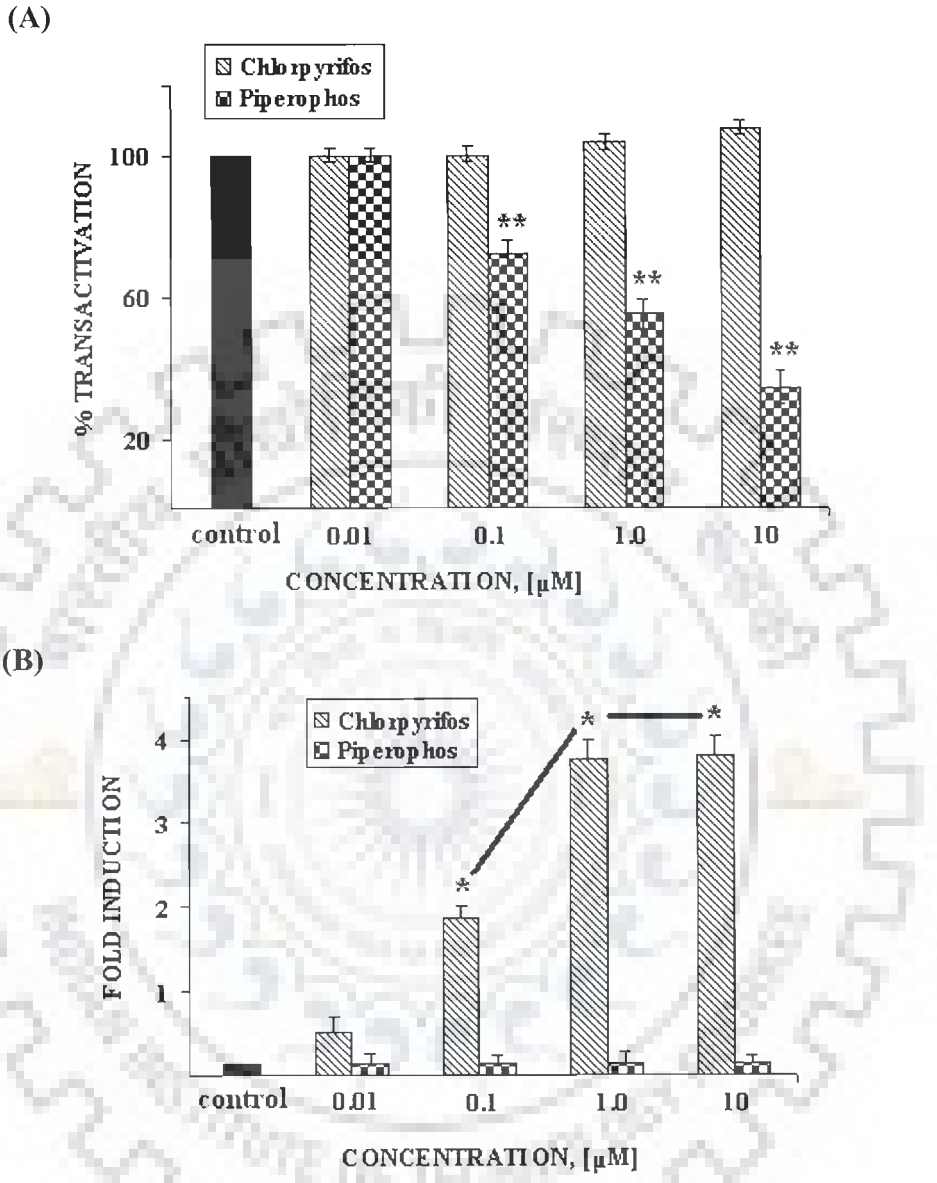


Fig 8.2 pCMV-CBP was transfected into NIH3T3-AR-Luc cells to over express CBP protein. (A) The CBP over expressing NIH3T3-AR-Luc cells treated with increasing concentrations of both piperophos and chlorpyrifos in the presence of 0.3 nM testosterone. (B) The CBP over expressing NIH3T3-AR-Luc treated with increasing concentrations of chlorpyrifos or piperophos in the absenc of testosterone. The values represent the mean±S.D. of three similar experiments performed in triplicates.
**Significant decrease of luciferase activity ($p<0.05$) with respect to vehicle treated cells.
*Significant increase of luciferase activity ($p<0.05$) with respect to vehicle treated cells.

8.2.3 Co-immunoprecipitation of CBP

To further confirm if chlorpyrifos have any direct interaction with CBP in causing weak antagonism by chlorpyrifos in the AR transactivation, co-immunoprecipitation studies were carried out to understand the physical recruitment of CBP by AR bound chlorpyrifos complex. As piperophos did not exhibit such a limitation for CBP in the former studies, hence it was not included in this study.

Whole cell extracts of the NIH3T3-AR-Luc cells with or without transfected with exogenous CBP following their treatment with chlorpyrifos were immunoprecipitated with excess of antiserum for androgen receptor and followed by immunoblotting with anti CBP antibody. The cell extract transfected with pCMV-CBP was included as control. As shown in Lane 2 of Fig 8.3, in the absence of exogenous CBP, chlorpyrifos although interacts with AR (as shown by binding and transactivation studies) but fails to recruit CBP. It is also evident in Lane 6, that 10 μM chlorpyrifos in the presence of 0.3 nM testosterone decreased the CBP recruitment as shown by the significant decrease in the CBP protein band. Thus, this consolidates the idea that chlorpyrifos treatment decreased the CBP recruitment by the AR complex. On the other hand, in the presence of excess CBP, chlorpyrifos recruited CBP, as shown by distinct immonoblot for CBP pulled down by AR antibody (Lane 4).

Thus, all this data clearly indicated that the AR bound chlorpyrifos complex was unable to recruit CBP in the treated cells. However, if the CBP was over expressed in the treated cells, the AR-chlorpyrifos complex was able to recruit the CBP showing that excess CBP in the cell was able to rescue the complex from the shortage of CBP in the treated cells.

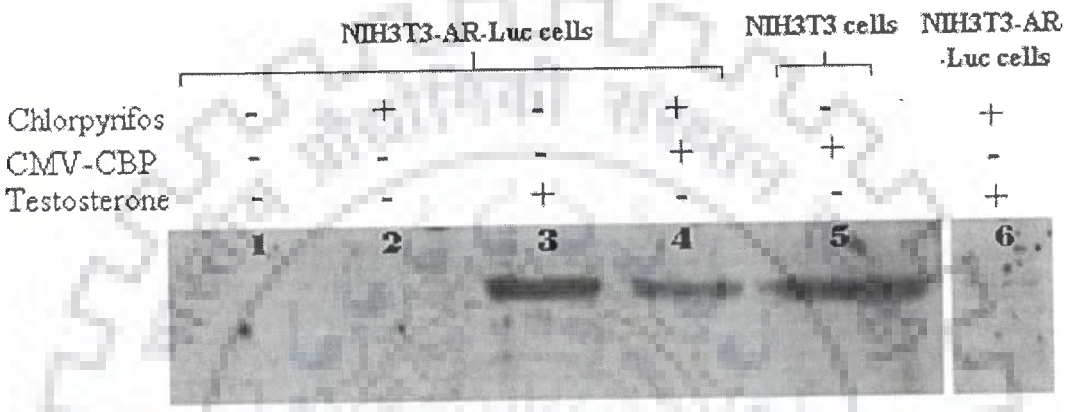


Fig 8.3 The androgen receptor was co-immunoprecipitated from the NIH3T3-AR-Luc cells and CMV-CBP transfected NIH3T3-AR-Luc cells overexpressing CBP co-factor, treated with chlorpyrifos (10 μM). Immunoprecipitated proteins were subjected to SDS-PAGE and immunoblotted with anti CBP antibody. Lane 5, Cell extract from CBP transfected NIH3T3 cells without prior immunoprecipitation was ran and immunoblotted with CBP antibody (+ve control).

8.3 Effect of chlorpyrifos and piperophos on steroidogenesis in isolated Leydig cells

8.3.1 Effect of test chemicals on Leydig cell viability and steroidogenesis

The Leydig cells were isolated from the testes of rat and the purity of the established primary culture was checked by 3β-HSD immunocytochemistry (Fig 8.4). Initially, MTT assay was carried out to check the test pesticides effect on this Leydig cell viability. As shown in Fig. 8.5, there was no significant reduction in the cell viability till the concentration of 10 μM of the test chemicals. However, both chlorpyrifos and piperophos showed significant cytotoxicity at a concentration of 100 μM (Fig. 8.5) ($p<0.05$). This further confirmed that the doses used by us were below the toxicity level. In the next set of experiments these chemicals were tested for their effect on LH induced testosterone production by the isolated rat Leydig cells.

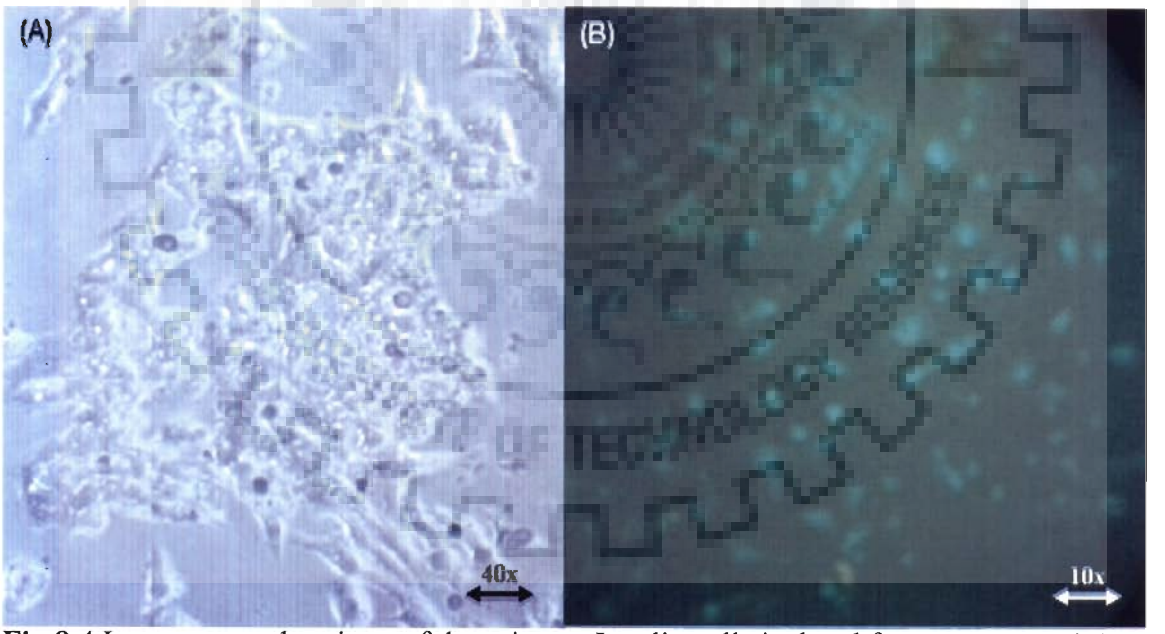


Fig 8.4 Immunocytochemistry of the primary Leydig cells isolated from rat testes. (A) Formaldehyde fixed primary Leydig cells. (B) Isolated Leydig cells stained for 3β-HSD protein by immunocytochemistry.

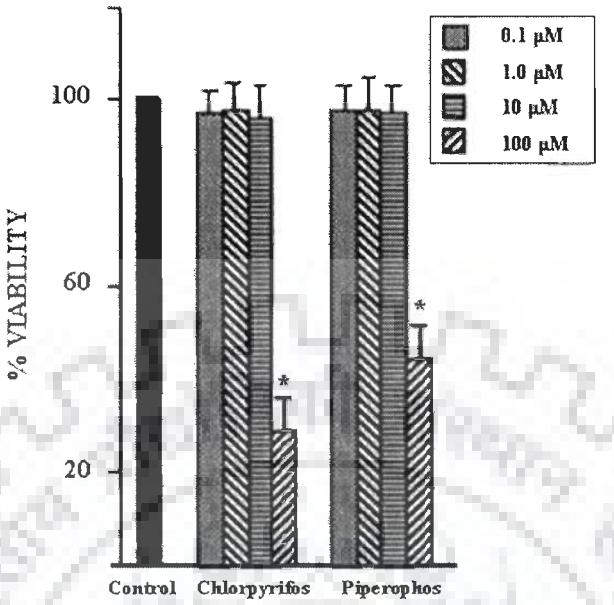


Fig. 8.5 Effect of the test chemicals on the cell viability of Leydig cells by MTT assay. The cells were treated with different concentrations of the compounds (0.1-100µM). The cells treated with the vehicle control were taken as 100% viable. The values represent the mean±S.D. of three similar experiments performed in triplicates.
* Significant decrease in cell viability as compared to vehicle treated Leydig cells ($p<0.05$).

As shown in Fig. 8.6, both the chemicals showed a significant reduction in the testosterone production at a concentration of 10 µM. Mean testosterone produced when the cells are treated with 100 ng/ml hCG was 148 ng/10^6 cells, while the cells treated with 10 nM chlorpyrifos and piperophos in the presence of 100 ng/ml hCG showed a decrease in testosterone production to 96 and 115 ng/10^6 cells, respectively. This accounts to a decrease of 35% and 23% by chlorpyrifos and piperophos, respectively. To further investigate the effects of chlorpyrifos and piperophos on the steroidogenesis machinery within the Leydig cells, their effects on various steroidogenic gene expression patterns were analyzed. RT-PCR analysis of some of the crucial steroidogenic enzymes, P_{450}scc, 3β-HSD and 17β-HSD, showed that both chlorpyrifos and piperophos resulted in

a significant down regulation in the expression of all the three genes as measured by densitometric analysis (Fig. 8.7A&B). Further steroidogenic acute regulatory (StAR) protein, a crucial factor for steroidogenesis, was then evaluated in response to these chemicals. As shown in Fig. 8.7C, the immunoblot analysis with StAR antibody demonstrated a significant decrease in the expression of StAR protein in the cells treated with chlorpyrifos while there was no significant change observed in the piperophos treatment.

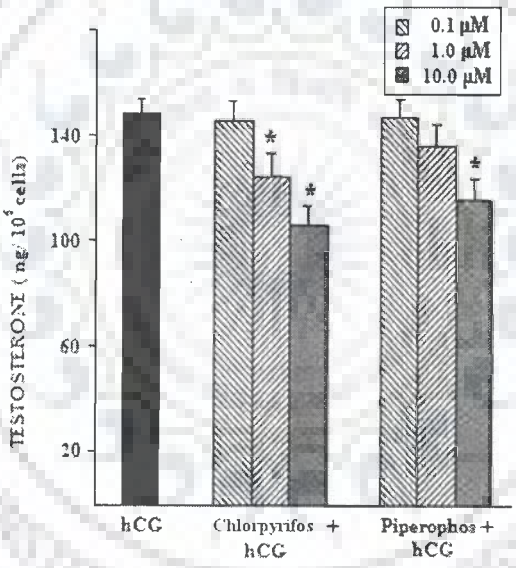


Fig. 8.6 Effect of various concentrations of test chemicals on testosterone production by Leydig cells in the presence of 100 ng/ml hCG. The cells treated with hCG alone were considered as control. The values represent the mean ± S.D of three similar experiments performed in triplicates.
* Significant decrease in the testosterone production by Leydig cells as compared with the vehicle treated cells ($p<0.05$).

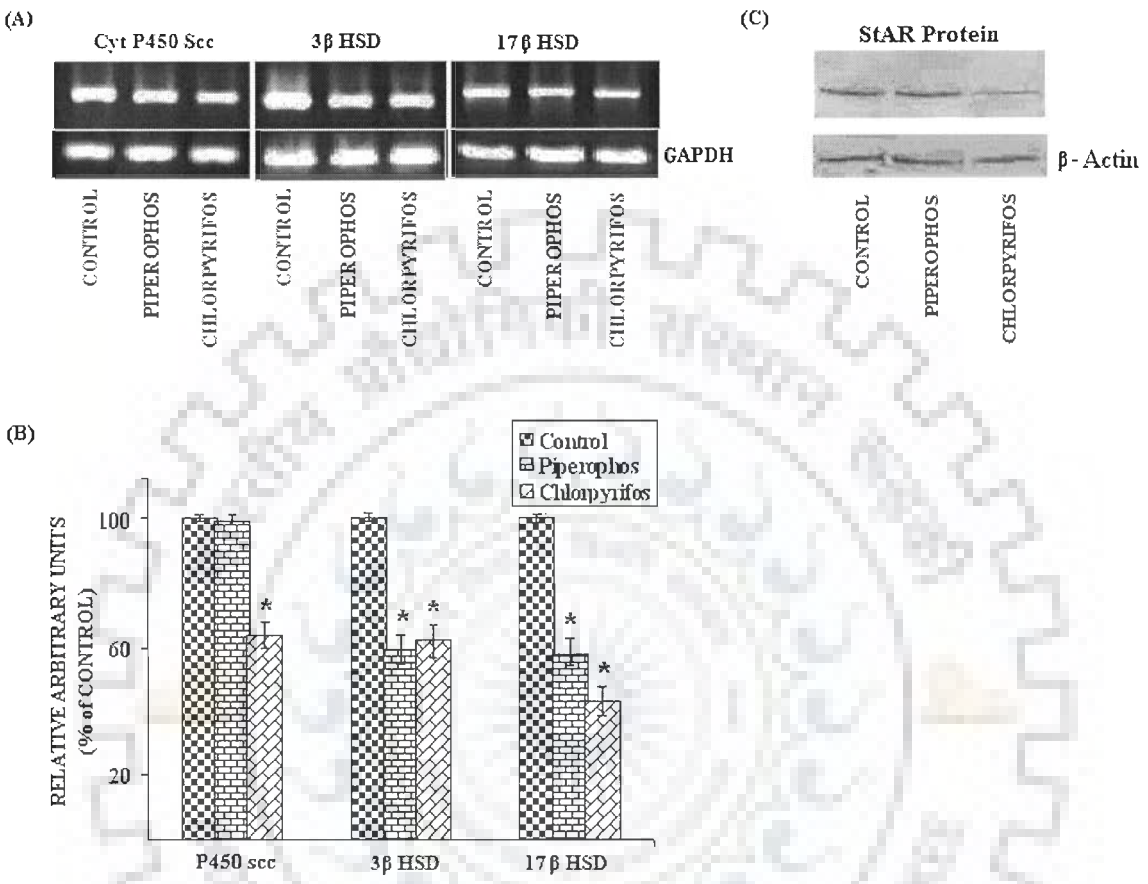


Fig 8.7 (A) RT–PCR analysis of Leydig cells mRNA expression of some major steroidogenic enzyme (cytochrome P450Scc, 3β-HSD and 17β-HSD) genes treated with 10μM of pesticides, piperophos and chlorpyrifos. (B) Western blot showing the expression pattern of StAR protein in these treated group of Leydig cells. (C) The relative intensity of the RT-PCR products were quantified by densitometer and normalized against the internal control (GAPDH).
*Significant decrease in the gene expression as compared to vehicle control ($p<0.05$).

8.3.4 Effects of chemicals on intracellular cAMP production mediated by hLH receptor

LH receptor is a transmembrane receptor that acts via cAMP increase on binding to luteinizing hormone. Here we checked the effect of piperophos and chlorpyrifos by indirect cAMP assay. The CHO-K1 cells were transiently transfected with hLH receptor and pCRE-Luc (Viswanath et al., 2007) and then treated with increasing concentrations of these chemicals both in the presence and absence of (10U) hCG. Chlorpyrifos showed a significant reduction of hCG induced cAMP production at 1 μM concentration which declined further at 10 μM concentration as estimated by the reduction of luciferase transactivation (Fig. 8.8A) ($p<0.05$).

At a concentration of 10 μM, chlorpyrifos showed approximately 15-18% decrease in luciferase induction of pCRE-Luc. However, piperophos did not show any significant change when compared with the vehicle treated cells. This confirmed that chlorpyrifos not only interacts with AR as antagonists but it also inhibits LH/hCG induced cAMP production. Later, the intracellular cAMP levels were measured in the pesticides treated primary Leydig cells using cAMP ELISA kit (Sigma, St Louis, CA, USA). It was found that there was a significant ($p<0.05$) decrease of 15 pM cAMP concentration at 1 μM chlorpyrifos treatment which further decreased by ~20 pM at 10 μM chlorpyrifos treatment (Fig 8.8B).

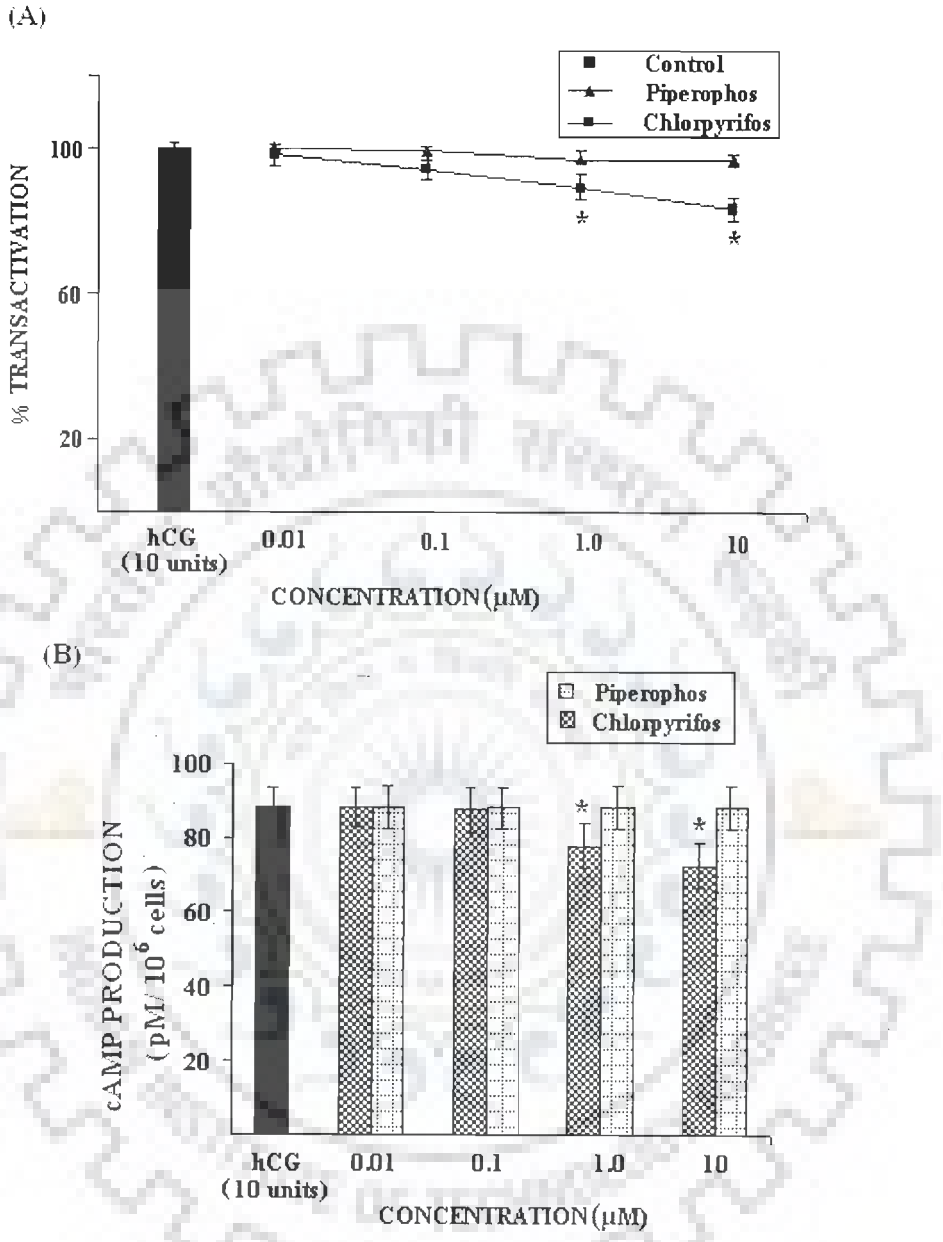


Fig 8.8 (A) Effect of the chlorpyriphos and piperophos on intracellular cAMP production in hCG (10 units) treated CHO-K1 cells transiently transfected with human luteinizing hormone receptor (hLHR) and CRE-Luc. (B) Effect of chlorpyriphos and piperophos on intracellular cAMP in hCG (10 units) stimulated primary Leydig cell culture. The cells treated with only hCG was taken as control whose response was considered 100%. The values represent the mean±S.D of three similar experiments performed in quadruplicates. * Significant decrease in the cAMP dependent luciferase activity by chlorpyrifos as compared with vehicle treated group ($p<0.05$).

8.4 Discussion

Steroid receptors associate with different co-activators or co-repressors that act as bridging factor for basal transcription machinery. These receptors were identified to interact with several co-regulators for their function (McKenna et al., 1999; Lee et al., 2000). Aarnisalo et al. (1998) have shown that CBP is an important androgen receptor co-activator and its inactivation with adenoviral protein 12S E1A prevents the receptor association with RNA polymerase II complex and P/CAF histone acetyl transferase leading to the inhibition of androgen dependent transactivation. There were earlier reports of EDCs interfering with the co-regulators transcription levels and availability to the steroid receptors leading to the inhibition of steroid dependent transactivation. For example, Lonard et al. (2004) showed that 4-hydroxytamoxifen and raloxifene are able to increase the SRC-1 and SRC-3 co-factor protein levels effecting the ERα transactivation. Similarly, bisphenol A was shown to increase expression levels of the thyroid hormone receptor activator protein 220 (TRAP220) co-activator and ERβ in mouse uterus (Inoshita et al., 2003). All these show that there is a positive cross talk between EDCs, steroid receptors and their co-activators.

In the present study chlorpyrifos showed weak anti-androgenic activity with an IC_{50} value of 19.3 μM when it was screened using the stable NIH3T3-hAR-Luc reporter based bioassay. It was also shown in chapter 4 that it physically binds to the receptor in the competitive binding studies. Once it was confirmed that chlorpyrifos physically interacts with AR and also participates in regulating its transactivation, naturally it was interesting to understand its mode of action. Towards this end, the first set of experiments to be performed was to check the cross talk of this chemical with other known pathways

with which AR interacts. As a part of it, AP1, NFkB and cAMP dependent pathways were studied. It was observed that there was a ~5 fold increase of NFkB dependent transactivation activity in these treated cells which was later supported by the increase in NFkB protein expression in the immunoblot analysis. Later, it was observed in the co-immunoprecipitation study that the androgen receptor-chlorpyrifos complex was unable to recruit an important co-integrator protein, CBP that is needed in the transcriptional initiation. CBP has been known to associate with a number of factors in the cell like AP1, NFkB (Supakar et al., 1995, Jänne et al., 2000) for their corresponding response during the normal physiological functioning in the cell. Aarnisalo et al. (1998) reported that CBP becomes a limiting factor for AR in the presence of overexpressed NFkB, AP1, CREB in a cell as all these competes for the common cofactor (CBP) for their transactivation. Till this point, from the existing data, it appeared that chlorpyrifos resulted in the upregulation of NFkB which in turn competes for CBP along with AR for its transactivation, resulting in the shortage of CBP within the cell. Probably this was responsible for down regulation of AR transactivation within these treated cells. Naturally the next hypothesis could be to supplement CBP to cells and check the rescue effect on the antagonism shown by chlorpyrifos treatment. To investigate this, the NIH3T3-AR-Luc cells were transfected with exogenous CBP (pCMV-CBP) to overcome the CBP limitation in the treated cells. Once CBP was over expressed within the cells, chlorpyrifos did not show antagonistic activity any further in these treated NIH3T3-AR-Luc cells in the presence of testosterone. Moreover, when the CBP transfected NIH3T3-AR-luc cells were treated with increasing concentration of chlorpyrifos, there was a dose dependent increase of luciferase activity induction to about 3-4 folds indicating its

marginal agonistic activity. Dose dependent increase ruled out the possibility of any background or non-specific induction of the reporter activity (Aarnisalo et al., 1998). In the co-immunoprecipitation studies, when the treated cells were over expressed with CBP, AR was able to efficiently recruit the cofactor even in the presence of chlorpyrifos. This further conclusively proved that limitation of CBP in the chlorpyrifos treated cells was responsible for androgen induced inhibition of transactivation. This fact actually supported that in the presence of exogenous CBP, the chlorpyrifos was a partial agonist. Min et al, (2002) reported the existence of a similar competition for GRIP-1 co-activator between CAR xenobiotic receptor and ER, and the over expression of this co-activator did rescue the inhibitory effect of ER transactivation. This study consolidates the fact that EDC may affect the efficient recruitment of co-regulators and thus block the corresponding gene expression (Tabb & Blumberg, 2006). However, the affinity/efficiency of the chlorpyrifos bound AR in comparison to the testosterone bound AR complex with which it can recruit CBP has to be investigated further in future.

On the other hand, piperophos did not either show the CBP rescue effect or the cross talk with cell signal molecules like NFkB, AP1, cAMP as analysed in the study. The strong anatagonism of this chemical treatment may be attributed to two factors. Firstly, the AR bound piperophos complex probably fails to attain a conformation that can recruit the coactivator complex and secondly, they may associate with co-repressors which strongly block the transactivation further supporting the strong antagonism of this chemical. Both these possibilities need further investigation to understand its mode of action. In a similar context, Hodgson et al. (2007) showed that bicalutamide, a strong antagonist, used in the treatment of prostate cancer enhance the recruitment of co-

repressors NCoRI and SMRT thus preventing the transactivation of AR. The same group also showed the recruitment of these corepressors by mifepristone which exhibits strong antagonism for AR. In summary, this part of study depicted that recruitment of co-activators/co-repressors play a major decisive role in the mode of action of various steroid hormone disruptors.

In the next part of the study, the effect of chlorpyrifos and piperophos on steroidogenesis was studied in Leydig cells. Many pesticides and fungicides, apart from their direct interaction with AR has also been shown earlier to directly interact and modulate the steriodogenesis pathway leading to the decrease/increase in the testosterone production both *in vivo* (rat and fish models) and *in vitro* (Leydig cell cultures). Dieldrin (Fowler et al., 2007), Octylphenol (Murono et al., 1999), atrazine (Friedmann, 2002), Vinclozolin (Murono & Derk, 2004), ketoconazole and related imidazole anti-fungal chemicals (Schurmeyer & Nieschlag, 1984; Kan et al., 1985) were some of the pesticides/ fungicides which were shown to reduce the testosterone production in Leydig cells. Using similar approach, we studied the interferences in the steroidogenesis by these chemicals in the primary cultures of rat Leydig cells. In our study, chlorpyrifos and piperophos showed a decrease in the testosterone production in the Leydig cell cultures in the presence of hCG. Recent reports demonstrate that xenobiotics- dependent direct up/downregulation of steroidogenesis could be attributed to several factors: (i) their action through arylhydrocarbon receptor (AhR) (Indarto & Izawa 2001);(ii) direct binding of these chemicals to steroid receptors, steroidogenic enzymes and proteins associated with steroidogenesis (like Steroidogenic Acute Regulatory protein) (Kang et al., 2005; Rice et al., 2006) and (iii) increased stability of transcripts and transcriptional

rate of the promoter of steroidogenic enzymes (Kumar et al., 2008). In this regard, the decrease in the testosterone synthesis by these two chemicals (chlorpyrifos and piperophos) could be attributed to the reduction in the expression of the key steriodogenic enzymes - P450scc, 3β and 17β-HSD. StAR gene expression is an important rate limiting step of steriodogenesis. This protein helps in the transport of cholesterol from cytoplasm into mitochondria during steroid biosynthesis (Stocco, 2000, 2001). Chlorpyrifos showed a significant decrease in the level of StAR protein expression while piperophos did not show any effect.

LH hormone acts via G-coupled receptor leading to the increase in cAMP. This increase in cAMP induces the cholesterol transport into the mitochondria suggesting an increase in the StAR gene expression in a cAMP responsive manner (Clark et al., 1995; Sugawara et al., 1995; Caron et al., 1997). In our study, chlorpyrifos showed a 15-18% decrease in the intracellular cAMP in the hCG stimulated CHO-K1 cells transiently transfected with hLH receptor and CRE-luc reporter construct. This was further confirmed by the decrease in cAMP in these treated cells using ELISA based assay. In summary, probably the reduction in cAMP resulted in the decrease in StAR and P450scc expressions in chlorpyrifos treated Leydig cells finally leading to the decrease in the testosterone biosynthesis. Although the decreased steroid biosynthesis by these chemicals is understood, the chemical's interference with the cAMP decrease has to be further investigated.

In conclusion, chlorpyrifos and piperophos are shown to be androgen antagonists. Chlorpyrifos bound AR was unable to recruit CBP coregulator protein and the overexpression of this cofactor could rescue the antagonistic activity by binding to the

receptor-ligand complex. Piperophos, however, could not show a similar effect although it was far more a potent antiandrogen than chlorpyrifos atleast in terms of IC_{50} value. This warrants further study on this chemical, since it might involve some other mode of inhibition of androgenic action. These chemicals also showed an inhibitory effect on testosterone biosynthesis in Leydig cells by altering the expression pattern of some of the crucial steroidogenic enzymes. To the best of our knowledge, this is the first ever report to show the CBP involvement in antiandrogenic action of some EDCs and understanding these pesticides mode of action in isolated rat Leydig cells. However, the crosstalk of different pathways that is responsible for the CBP limitation in these EDCs treated cells and the further addressing the co-repressors involvement would help in better understanding of the mode of endocrine disruption action and their reproductive toxicology.

A Hypothetical schematic representation of the various targets of chlorpyrifos, piperophos and WWTP effluents causing endocrine disruption was depicted in the Fig 8.9 (based on the results of this thesis).

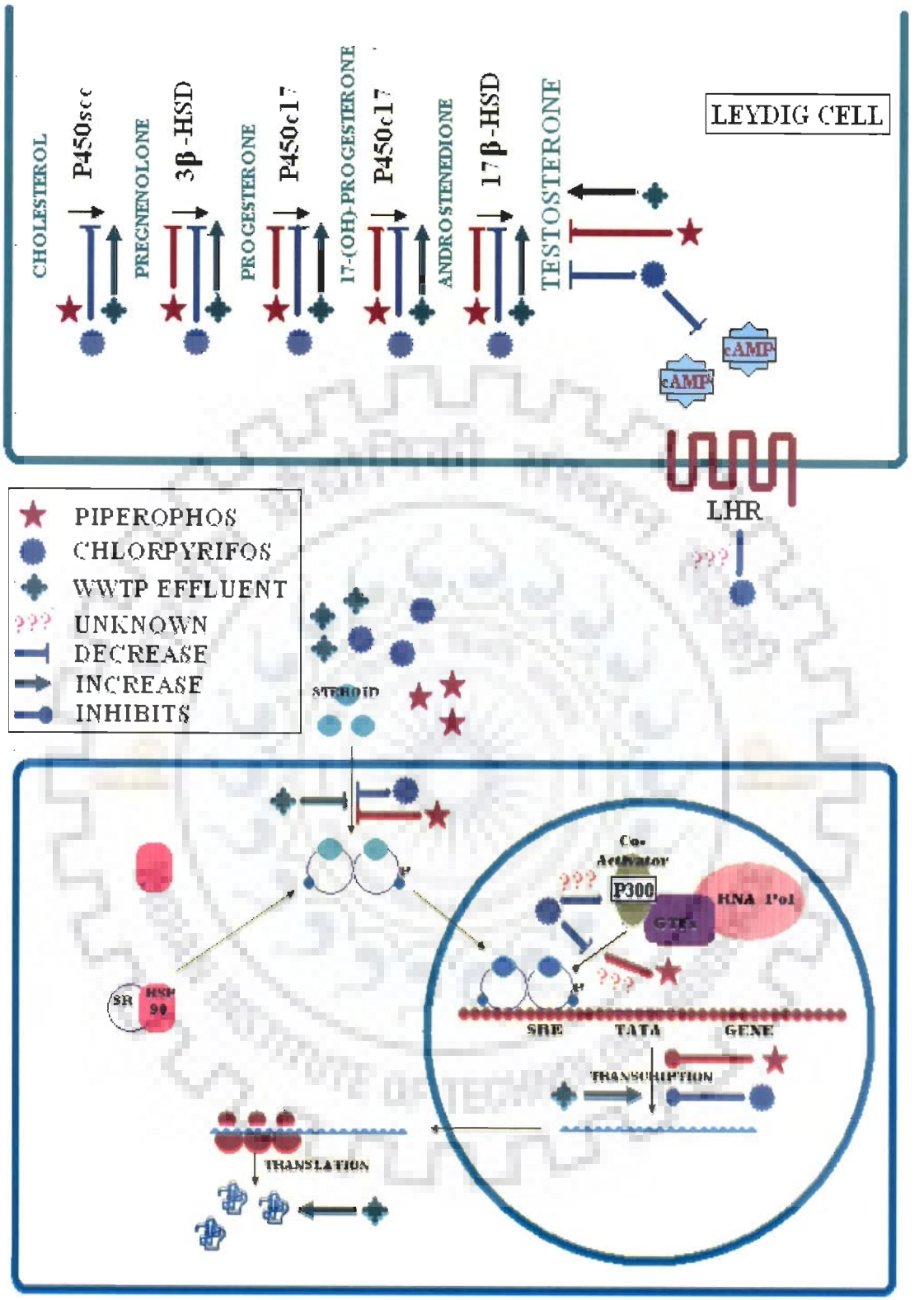


Fig 8.9 Hypothetical schematic representation of the various targets of chlorpyrifos, piperophos and WWTP effluents causing endocrine disruption (based on the results of this thesis.

CHAPTER 9

SUMMARY

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Endocrine disruptors are those chemicals that have the ability to modulate or deregulate the endogenous endocrine system. In humans, potential adverse health effects include (1) infertility (fecundity, reduced semen quality, endometriosis), (2) abnormal prenatal and childhood development (spontaneous abortions, male reproductive tract abnormalities and other birth defects, altered sex ratios, precocious puberty) and (3) reproductive cancers of prostate, breast, ovaries, endometrium, or testes (U.S. EPA, 1997; National research council, 1999; EM-COM, 2002; IPCS, 2002; Phillips et al., 2003; Phillips et al., 2008). Although the risk posed by endocrine disruptors represents an important area of environment health, it represents a considerable controversy regarding the nomenclature of the term "endocrine disruptor". An increasing number of chemical compounds from the environment have been identified as endocrine disruptors utilizing *in vitro* and *in vivo* bioassays. These include pesticides, industrial chemicals, waste water effluents, house utilities, cosmetics and pharmaceuticals that behave like ligands for the steroid or aryl hydrocarbon receptors producing effects that mimic the natural hormone (Roy & Pereira, 2005).

The present thesis describes the development of a multi-tier bioassay approach for the endocrine disruptors screening using both *in vitro* and *in vivo* models with a major focus on androgenic and progestagenic EDCs as they were not as extensively studied as estrogenic EDCs. A wide range of chemicals were screened using the competitive receptor binding assays and reporter based cell bioassays. Some of the chemicals were studied for their effect in *in vivo* models and further studied to elucidate their mode of action. The chemicals or environmental samples studied in this thesis, were selected

keeping in consideration the wide range of sources of EDCs that the human population and other living organisms are exposed to.

The receptor binding assay represents an important component of the US EPA tier 1 screening battery and of level 2 of the conceptual frame work. For this, the ligand binding domain (LBD) of human progesterone receptor and (DBD & LBD) of human androgen receptor were cloned into the bacterial expression vectors and their recombinant proteins were purified. These recombinant receptors showed good affinity and specificity to their respective steroids with their K_D values 0.89 nM and 8.9 nM for hAR(DBD & LBD) and hPR(LBD) respectively. The use of recombinant receptors offers a number of advantages which include an inexhaustible source of material, homogeneity of the binding proteins and stability of the binding properties compared to receptors prepared from animal tissues (Scippo et al., 2004). A wide range of chemicals were tested for their androgen and progesterone receptor binding abilities. Most of the chemicals selected for screening in this thesis were not reported earlier either by the *in vitro* or *in vivo* assays. Regarding the progesterone receptor especially, there are only a few reports on the EDCs screening (Dong et al., 2004; Scippo et al., 2004). Thus the chemicals screened in this thesis add to the existing database of the androgenic and progestagenic EDCs.

Although the receptor binding assay gives important information about the physical interaction of the chemical with the receptor, it is less sensitive and also fails to distinguish whether the bound chemical is an agonist or an antagonist (Hartig et al., 2007). Matsui, (2007) attempted to use mutated androgen receptor in binding studies to distinguish between AR agonist and antagonists but failed to validate the assay with all

the known EDCs. However, the reason for this discrepancy could not be elucidated. On the other hand, cell based reporter bioassays address these limitations of the binding assays making them a very reliable system for screening EDCs. Stable reporter cell lines developed for the screening of these EDCs were found to be more advantageous than the transient transactivation assays. Transient transfections do not reflect physiological conditions because the target DNA sequences are over expressed and maintain their responsiveness only for a limited time. Moreover, there is a high inter assay and intra assay error (Roy et al., 2006).

There were already several reports of the stable cell lines developed for screening (anti)estrogenic EDCs, however there were comparatively less reported cell lines for screening (anti)androgenic (Roy et al., 2004; Sonneveld et al., 2005; Stroheker et al., 2005; Chen et al., 2006; Roy et al., 2006; Sun et al., 2007) and (anti)progestagenic EDCs (Schoonen et al., 1998; Dong et al., 2004; Molina-Molina et al., 2006; Wilkinson et al., 2008). These reported cell lines have their limitations, for e.g., stable cell lines reported by Chen et al. (2006) and Roy et al. (2006) used HEK293 cells which adhere very loosely to the substrate and hence there is a loss of the cells during the screening procedures increasing the inter assay variability. On the other hand, CHO-K1, MDA-kb-2 cell lines as was reported earlier (Wilson et al., 2002; Roy et al., 2004) had intrinsic GR leading to a background noise with glucocorticoids. Similarly, T47D cells (Blankvoort et al., 2001) have endogenous PR and ER thus interfering with the EDCs screening. On the other hand, yeast based bioassays have several advantages like: little chance of interference by other hormone receptors as in mammalian cell lines, relatively simple and cost effective allowing the use of media that does not contain steroids (Bovee et al., 2004, Roy et al.,

2004). However, they do not discriminate effectively between agonists and antagonists (Shiau et al., 1996; Gaido et al., 1997; Soto et al., 2006). Moreover, yeast cells were identified to have problems of membrane permeability and transport that may give high rate of false results in the measurement of the relative steroidogenic potency. All these give a high back ground noise in case of yeast based bioassays (Soto et al., 2006).

In order to address all these limitations in a better way, mouse fibroblast NIH3T3 cells that are completely naïve to steroid receptors were selected by us for the development of the stable cell lines. These cells have all the machinery that can support the luciferase transactivation assay. Further, this cell line is rapid growing, adheres strongly to the substrate and uses the routine DMEM medium for its propagation with no special media substitutes. Two stable cell lines were established - NIH3T3-hPR-Luc and NIH3T3-hAR-Luc for the screening of progesterone and testosterone respectively. Most of the stable cell lines reported earlier utilized MMTV as the steroid response element (Dong et al., 2004; Roy et al., 2004; Stroheker et al., 2005; Sun et al., 2007). This promoter can bind AR, PR and GR thus decreasing the specificity of the assay (Betrabet et al., 2004; Sonneveld et al., 2005). However in our assay, we used specific probasin promoter for androgen receptor (Kasper et al., 2003) and progesterone response element for progesterone receptor, stable cell line developments to address this problem. Sonneveld et al. (2005) developed the AR CALUX cell line by stable transfection of the human U2-OS osteosarcoma cell line with the androgen receptor and a luciferase reporter gene construct containing three androgen-responsive elements coupled to a minimal TATA promoter to make their reporter more androgen-specific. Both the stable cells developed in this thesis were well adapted to screen the compounds in a 96 well format

for high through put screening. The cell lines showed significant response to 0.01 nM of their corresponding steroids with their EC_{50} being 0.4 nM and 0.29 nM for NIH3T3-hPR-Luc and NIH3T3-hAR-Luc respectively. The sensitivity was found to be in good agreement with earlier reported assays. These cells also showed high specificity to their corresponding steroids and responded very well with already established antagonists and reported EDCs. All these characteristics make these stable cell lines very efficient models to screen (anti)androgenic and (anti)progestagenic EDCs.

In addition to the already discussed limitations for binding studies, all-*trans* retinoic acid which did not bind the receptor was shown to be an EDC by both transactivation and *in vivo* study (Takeyoshi et al., 2002) which further emphasizes the need for transactivation assays for the EDC screening. Therefore, all the chemicals that were screened by receptor binding studies in the first part of our study were again screened using transactivation assay to validate the binding results. Almost all the binding assay results were in good agreement with the transactivation assay except for a few exceptions. 3,3',4,4',5-Pentachlorobiphenyl which did not show any androgen receptor binding showed weak antiandrogenic activity with an IC_{50} value of 28 μM. Similarly, nialate that showed weak receptor binding showed strong antiandrogenic activity in the transactivation assay (Dong et al., 2004). Initially DDT, nonylphenol, bisphenol A, endosulfan (Scippo et al., 2004) which were already well established as antiandrogenic and antiprogestagenic chemicals were used in the screening for validation of the established stable cell lines. More than ten chemicals which included diphenamid, nialate, pentachlorobiphenyl, etc. were screened for their antiandrogenic activity for the first time in this thesis to the best of our knowledge. Similarly we screened a whole lot of

new chemicals using the stable NIH3T3-hPR-Luc cell line developed for their anti progestagenic activity. The IC_{50} values of the chemicals obtained in the screening by NIH3T3-hAR-Luc and NIH3T3-hPR-Luc were in good agreement with the earlier reports. However, slight variations can be attributed to the differences in the procedures followed, cell lines and DNA constructs used by different groups (Roy et al., 2006; Sun et al., 2007). Like wise in the other groups screened for androgenic EDCs also, we did not find even a single agonist amongst screened chemicals either for androgen or progesterone receptors (Kojima et al., 2004). The main limitation of the transactivation assay is that, pro-steroids, which are compounds that are metabolized *in vivo* into steroids, cannot be detected. For example methoxychlor does not show estrogenic activity until it is metabolized to the free phenolic product, which is estrogenic (Soto et al., 2006). The receptor in the transactivation assays becomes insensitive on chronic exposures to the xenobiotics (Zysk et al., 1995) and some times shows unspecific activity at high concentrations of the xenobiotics (Cato et al., 2002; Kuiper et al., 1997). Certain agonists, such as resorcylic acidlactones, result in a significantly higher luciferase activity (overactivation) than that obtained with E2, making it difficult to define partial and full agonistic activity using the cell based transactivation assay. Therefore this suggests that the *in vivo* studies would give a better understanding regarding the chemicals endocrine disrupting potential. Therefore, for this, two pesticides, chlorpyrifos and piperophos which showed remarkable binding with both progesterone and androgen receptors were selected to be studied further in *in vivo* system to understand their mode of action.

Another major source of EDCs that was discussed in this thesis was the WWTP effluent. There were almost no reports available on the endocrine disruptors in WWTP

effluents from Indian subcontinent except one by Senthilkumar et al., (1999) which demonstrates the presence of several potential EDCs like PCBs, DDT, hexachlorocyclohexane isomers (HCHs), chlordane compounds (CHLs) in dolphins, fishes, benthic invertebrates and sediments collected from the river Ganges, one of the major rivers in India. To the best of our knowledge, this is the first report on the presence of progestagenic EDCs in WWTP effluents using cell based reporter bioassay and *in vivo* studies. In this thesis, we screened for the presence of EDCs from five different WWTP plants located in the Northern Province of India. Our data showed that these effluents contained a complex mixture of various aromatic compounds exerting both androgenic and progestagenic activities. Our study also demonstrated that the average levels of progesterone and testosterone equivalents in the extracted water sample from these WWTPs were in the range of 7-9 ng/l and 8-15 ng/l respectively as determined by the *in vitro* reporter assay. Earlier, several authors showed similar though not same levels of these contaminants from various WWTP effluents across the world (Leusch et al., 2006; Van Der Linden et al., 2008). Whatever marginal variations obtained can be attributed to the lifestyle of the population of the area under consideration. The androgenic activities found in these studies can be explained by the presence of some of the known androgens like DHEA, isoandrosterone, hexachlorobenzene (HBC) (Ralph et al., 2003) and nonylphenol (Roy et al., 2004; Scippo et al., 2004) that were shown by the GC-MS analysis of the WWTP effluents. Further efforts have to be made to separate and identify other possible EDCs in these effluents to understand the possible contaminants that contribute to the androgenic and progestagenic activities.

The synthesis of steroid hormones is one of the crucial processes in the endocrine

regulation. It consists of sensitively regulated steps that were earlier shown by several authors to be the potential targets for different endocrine disrupting chemicals. The data presented in this thesis conclusively provides evidence that the two pesticides, chlorpyrifos and piperophos, and WWTP effluents tested by us act at various target sites of testosterone biosynthesis confirming the potential impact on human androgen axis. Chlorpyrifos and piperophos administration/treatment led to the decrease in the testosterone production while the WWTP effluents increased the testosterone biosynthesis leading to the increased serum testosterone. These chemicals have also shown to affect the serum gonadotropins levels, cholesterol transport and interfere with transcription of major steroidogenic enzymes and the downstream effects, thus amplifying their potential endocrine-disrupting impact. Similar results were obtained in the chemicals' *in vitro* treatment of primary Leydig cells supporting the effect of these chemicals on steroidogenesis. At this point, it could be speculated that majority of the offending chemicals act through their cognate receptors as has been reported earlier by several authors (Ralph et al., 2003; Chen et al., 2007; Kortner & Arukwe, 2007). In addition these chemicals were also shown to interfere with the LH dependent pathway involving adenylate cyclase and cAMP as shown by chlorpyrifos in our study. The mechanisms of cAMP decrease and the possible physical interaction of the chlorpyrifos with the LH receptor have to be addressed in future to further understand the different targets of the EDCs.

The steroid receptor-ligand complex bound to the SRE interacts with various co-regulators to activate the expression of target genes leading to the altered cellular functioning (Chang & McDonnell, 2002; McDonnell & Norris, 2002). During the last few

years, different mechanisms of action of EDCs apart from the conventional interference in the receptor binding have been reported. EDCs were shown to interact with the xenobiotic metabolism receptors (Moore et al., 2002; Wyde et al., 2003) and compete for different co-regulator proteins that are needed for the steroid receptor transactivation, finally resulting in the down regulation of steroid metabolism itself without directly competing for the binding of steroid receptor (Tabb et al., 2006). In this regard, bisphenol A is found to increase the expression of TRAP 220 co-activator (Lonard et al., 2004). 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene increased the expression of CAR which competed with the ER for GRIP-1 co-factor resulting in antagonism (Min et al., 2002). Recently, Hodgon et al. (2007) showed that mifepristone and bicalutamide recruits NCoRI and SRMT co-repressor protein. All this shows that the chemicals can exhibit endocrine disruption by interfering with the co-regulator proteins. In this context, chlorpyrifos treatment of NIH3T3 was shown to increase the NFkB protein intrinsic level. In the later part of the study, chlorpyrifos bound to androgen receptor was shown unable to compete for the recruitment of CREB binding protein (CBP) resulting in a weak androgen antagonism. However, interestingly, increase in the expression of cellular CPB protein rescued the antagonistic activity of the chlorpyrifos. All this reinforces the concept of the EDCs interference with the co-regulator molecules. However further studies are needed to reconfirm the exact mode of interaction between these EDCs and the co-regulator molecules.

In conclusion, in the present thesis, various chemicals were screened for their endocrine disrupting activity and an attempt was made to understand the mode of action of few chemicals both by *in vitro* and *in vivo* studies. The present work especially

reinforces the concept of the EDCs interference with the cofactors required for the steroid receptor transactivation. However, given the complexities in the steroid biosynthesis pathways and biological activities of hormones, together with unknown biokinetic properties of these EDC for systemic toxicology, further investigation with *in vivo* and *in vitro* experimental models are required to define a clear-cut picture on this aspect of endocrine disruption research. The holistic approach as presented in this thesis in understanding the mode of action of a diverse variety of EDC using *in vitro* and *in vivo* models shows that environmental xenobiotics or various daily usable chemicals (pharmaceuticals, cosmetics and toiletries), though present in low doses, yet may pose a serious threat to human health. Based on this information and considering the serious impact of these chemicals on human health, further investigations with adequate screening systems and *in vivo* confirmation are urgently needed to fully appreciate the spectrum of these endocrine disrupting properties.

CHAPTER 10

REFERENCES

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REFERENCES

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