Summary
and
Conclusions
Androgen Receptor is a member of the nuclear receptor superfamily and is responsible for executing the biological actions of androgens in target tissues as a ligand-activated transcription factor. It plays important functions in the processes of normal male sexual developmental functions and in the maintenance of male secondary sexual characteristics. AR not only regulates the proper functioning and development of male reproductive organs, such as prostate and epididymis but is also involved in development and function of non-reproductive organs, such as muscle, hair follicles and brain. Malfunction of the AR is associated with many androgen-regulated diseases and their pathogenesis in clinical disciplines ranging from pediatrics (ambiguous genitalia), gynecology (primary amenorrhoea), urology (benign prostatic hyperplasia), neurology (spinal bulbar muscular atrophy), reproductive medicine (male infertility), orthopedics (rheumatoid arthritis), oncology (prostate and male breast cancer) and dermatology (hirsutism, baldness and acne). In recent years it has become evident that in addition to endogenous steroid hormones, many structurally diverse chemicals (pesticides, industrial chemicals, phytoestrogens, flame retardants, plasticizers etc.) present in food and environment are capable of targeting some of the critical nuclear receptors (estrogen, androgen, progesterone, thyroid receptors etc.). These environmental chemicals are hypothesized to be associated with the increased incidents of breast and testicular cancers, altered male:female birth sex ratios, decreased fertility, global decrease in sperm counts, other male reproductive disorders including hypospadias, cryptorchidism etc. These chemicals, as a group are referred to as ‘endocrine disruptors’ or EDs. A number of chemicals under the EDs category have been reported to interrupt reproductive development in wild life and presumably in humans by mimicking or inhibiting the actions of gonadal steroid hormones like estrogens and androgens. The consequences of disrupting the developmental processes by EDs can be more profound during the critical window of hormone responsive phases since steroid hormones are controlling transient and irreversible developmental processes. Being structurally unrelated to
steroids the interaction of these EDs with steroid/nuclear receptors came as an unanticipated surprise.

Unlike AR, the second nuclear receptor PXR, in unliganded state is a predominantly nuclear protein. Emerging evidences have indicated that PXR regulates a large number of target genes, many of which have important roles in steroid and xenobiotic metabolism and elimination. PXR is known to master-regulate some of the key members of the cytochrome P-450 enzymes involved in oxidative metabolism of endogenous and exogenous compounds. Among the different CYPs, CYP3A4 is the predominant isoforms expressed in adult human liver and is reported to be responsible for the metabolism of more than half of the clinical drugs or xenobiotics. The role of PXR-CYP pathway in protecting the body against chemical exposure and its potential involvement in xenobiotic metabolism and clearance is under intensive investigation.

Although a number of screening methods are being used for identifying the potential EDs there is no single method which can accurately predict all the deleterious effects of EDs. Hence, a combination of screening approaches is generally suggested to analyze and implicate a chemical for its potential endocrine disrupting property. EDs can act at multiple sites via multiple mechanisms of action. Receptor-mediated mechanisms have received the most attention, but other mechanisms (e.g., hormone synthesis, transport, and metabolism) have also been shown to be equally important. For most associations reported between exposure to EDs and a variety of biologic outcomes, the mechanism(s) of action are poorly understood. This makes it difficult to distinguish between direct and indirect effects of exposure to EDs. It also indicates that considerable caution is necessary in extrapolating known endocrine disrupting effects by EDs. The effects of these EDs on the transcriptional activity and receptor dynamics of steroid/nuclear receptors have not been extensively studied. It is of great interest, therefore, to determine the effects of EDs on these receptors to understand the mechanism of ED disruption on endocrine systems at the molecular level. In this perspective,
using AR and PXR, we have systematically screened and studied some of the most suspected EDs to implicate their involvements and assess the mode of action in their endocrine disrupting activities. Based on several reports, where these chemicals have been enlisted to be potential threat to endocrine functions, we made a judicious choice for sixteen of the several hundred suspected EDs. We made a stepwise comparison of the potency of these chemicals in modulating the function of two critical NR members i.e. AR and PXR. In our screening approach for EDs, we initially performed the classical promoter-reporter transcription assays followed by analysis of intracellular receptor dynamics in live cells, and finally on modulation of receptor expressions in cultured cells and mice tissues. The results obtained from our studies are outlined below.

- In our initial screening, sixteen potential EDs were studied to establish their androgenic/anti-androgenic activity using promoter-reporter based luciferase assays in a model cell line, CV-1 where cells were transiently co-transfected with AR and its promoter-reporter gene ARE-tk-Luc. Our results revealed that except BCH, none of the selected EDs could activate AR significantly when used alone. Among the sixteen EDs, only BCH was found to be a potent agonist for AR. However, in the presence of sub-saturating concentration of DHT (10^{-10} M), most of the tested EDs inhibited the DHT-induced transcriptional activity of AR except BCH. Strong inhibitory effect of DDT, 2,4’-DDT, 4,4’-DDT, 2,4’-DDE, 4,4’-DDE, procymidone, fenitrothion, vinclozolin and nitrofen were evident. Moderate inhibitions were observed with linuron, methoxychlor, difenoconazole and tetramethrin. However, there was no significant inhibition on DHT-mediated AR transcriptional activity by chlozolinate and metribuzin. The inhibitions by EDs were not the result of EDs toxicities to the cells since general cytotoxicity effects were not observed in CV-1 at identical concentration. In the presence of sub-saturating concentration of DHT, BCH synergistically induced the AR transcriptional activity as compared with the DHT alone. These results implied
that, except BCH, all the suspected EDs behaved like pure antagonists and BCH behaved like agonist.

- To confirm agonistic/antagonistic properties of suspected EDs and assess whether these properties are due to ligand-receptor interactions, we performed subcellular localization studies by expressing GFP-AR in COS-1 or its parental cell line CV-1. Our results from subcellular localization and dynamics of GFP-AR, modulated by EDs, revealed that as compared to un-treated cells, DHT and BCH were most potent (90%) in inducing nuclear translocation of GFP-AR, while 4,4'-DDE, vinclozolin, 2,4'-DDE, 4,4'-DDT, DDT, 2,4'-DDT, fenitrothion and nitrofen translocated the receptor into the nucleus up to 60%, 40%, 30%, 25%, 25%, 20%, 25% and 25% respectively. Other EDs, including procymidone, metribuzin, difenoconazole, chlozoline, methoxychlor, tetramethrin and linuron could not induce nuclear localization of AR.

- To check if selected EDs specificity acted on AR, promoter-reporter based luciferase assay and subcellular localization studies with another nuclear receptor glucocorticoid receptor (GR) were carried out. Our results showed that there were no effects of EDs on GR transcriptional activity when used alone or in combination with dexamethasone. Also, as in the case of GFP-AR, cytoplasmic GFP-GR was not translocated into the nucleus by these EDs. Results suggested that the selected EDs specifically modulate AR activity and not that of another closely receptor i.e. GR.

- We purified full-length human AR and generated polyclonal antibody against it to achieve additional advantages during detection and quantitation of AR. There were several advantages of polyclonal antibody raised against full-length AR i.e. (i) it could recognize AR across the species (ii) it showed high sensitivity due to its ability to detect multiple epitopes on single antigen (iii) could detect chromatin embedded AR (iv) could also detect both denatured and native AR. The antibody successfully recognized AR in western blotting, immunocytology,
immunohistology and AR embedded in condensed chromatin. It also worked across and served as an important tool in the present study.

- We have shown for the first time that AR associates with the mitotic chromatin in agonist-dependent manner. This unanticipated observation prompted us to carry out extensive subcellular dynamics study of AR by potent EDs. To investigate the effect of six of the DDT-related EDs and BCH on dynamic behavior of AR, extensive subcellular dynamics study of GFP-tagged AR were attempted in living COS-1 cells. Our fluorescence microscopy of GFP-AR results showed that DHT- and BCH-bound AR forms ‘nuclear foci’ that are believed to be potential sites of active transcription and also associates with condensed chromatin during mitosis. On the contrary, AR bound to DDT-related EDs localizes homogenously in nucleus and neither forms ‘nuclear foci’ nor associates with condensed chromatin. These observations were further confirmed by immunofluorescence, using anti-AR polyclonal antibody and a cell line stably expressing wild type human AR. Results showed that all the DDT-related EDs, except methoxychlor, act as pure antiandrogens. BCH behaved very similar to pure agonist and when bound to AR, receptor formed ‘nuclear foci’ and also associated with the condensed chromatin during mitosis. Methoxychlor had no effect on subcellular dynamics of AR.

- We performed co-localization study by co-expressing either (i) GFP-AR and RFP-TBP or (ii) RFP-AR and GFP-GRIP1 in living COS-1. Our result showed that DHT and BCH-bound AR is co-localized with TBP/GRIP1 in the interphase nucleus whereas no significant colocalization is observed when AR is bound to 4,4’-DDE. Also, subcellular dynamics studies showed that GFP-AR and RFP-TBP co-localized with mitotic chromatin during mitosis in the presence of agonists (DHT, BCH). On the contrary, in the presence of antagonist (4,4’-DDE), GFP-AR and RFP-TBP did not co-localize with mitotic chromatin. Also, GRIP1 was not associated with mitotic chromatin in untreated or treated (DHT/BCH/4,4’-DDE) cells. This suggested that AR, as a transcription factor, is active only in the
interphase cells and is transcriptionally silenced during mitotic stages due to abortion of some of the critical co-activators from the transcription complex.

- Both DHT and 4,4'-DDE translocate AR to the nucleus. Results from the nucleocytoplasmic studies of AR revealed that nuclear import of AR with agonist (DHT) is faster as compared to pure antagonist(s). On the contrary, ligand withdrawal for nuclear export of AR (pretreated with DHT) is relatively slower as compared to AR export of pure antagonist pretreated cells. These results suggest that higher rate of nuclear export of AR may be due to higher dissociation rate of 4,4'-DDE from bound receptor or unstable receptor-DNA binding due to ligand 4,4'-DDE.

- To determine the repressive actions of NcoR and SMRT (co-repressors for nuclear receptors) on DHT-mediated AR transcriptional activity in the presence of a potent ED (4,4’-DDE), promoter-reporter based luciferase assays were performed in CV-1 cells where cells were co-transfected with AR, NcoR or SMRT and AR promoter-reporter gene. NcoR or SMRT repressed the DHT-induced AR transcriptional activity at the maximum level in the presence of 4,4’-DDE when it was compared with antagonistic activity of 4,4’-DDE in the absence of NcoR or SMRT. This result indicates that 4,4’-DDE increases the recruitment of corepressors thereby potentiating the repressive activity of 4,4’-DDE on AR transcriptional activity.

- Western blot of transiently and endogenously expressed AR in COS-1 and LNCaP cells respectively showed increased levels of AR by DHT while DDT compound (4,4’-DDE) could not show significant alteration in steady state levels of AR. We have also observed decreased expression levels of endogenous AR in LNCaP cells during mitosis as compared to interphase cells. DHT-bound AR during interphase, was at relatively higher level than unliganded or 4,4’-DDE-bound AR. Similar results were obtained in nocadazole arrested mitotic cells. Taken together, these results imply that unlike DHT pure antiandrogen binding to AR does not stabilize the cellular levels of the receptor.
The effect of a potent ED (DDT) on AR expression at mRNA and protein levels was investigated by RT-PCR and Western blot analysis respectively. As compared to un-treated controls, results in mice testis tissue treated with DDT demonstrated decreased level of AR protein and mRNA. On the contrary, levels of AR were unaffected by DDT in prostate cell line and mice liver tissues. These observations provide explanation to the existing literature where deleterious effects of EDs are shown to be primarily related to testicular disorders (testicular dysgenesis, low sperm count, infertility etc.) and not liver and prostate organs.

To check the possibility whether some or all of the selected EDs are metabolized via PXR-CYP pathway, we performed PXR transactivation assays by co-transfection of PXR expression vector and the promoter-reporter gene XREM-CYP-Luc. DDT-related EDs, BCH, linuron, difenoconazole and tetramethrin transactivated PXR strongly while fenitrothion, chlozolinate, methoxychlor, metribuzin and procymidone transactivated PXR with modest potency in our promoter-reporter based luciferase assays. Nitrofen and vinclozolin were either too weak or insignificant activators of PXR in these transactivation assays. Our findings demonstrate that these chemicals can be rapidly metabolized and eliminated by PXR-CYP detoxification pathway.

From the literature it is apparent that a few of the xenobiotics that activate PXR may also up-regulate PXR expression. In this perspective, by using RT-PCR and western blot analysis, we report that DDT up-regulates PXR expression at mRNA and protein levels in mice tissues. Our results showed a significant up-regulation of PXR mRNA and protein in DDT-treated mice liver but not in the testis of the same mice. PXR mRNA was below detectable levels in mice testis. However, our RT-PCR result with CYP3A11 (the most crucial isoform of mouse CYP3A subfamily) showed significant increase of CYP3A11 mRNA expression in both the mice liver and testis.

In conclusion, the present study has utilized classical and alternative screening protocols to study action of EDs on AR to establish androgenic and
antiandrogenic nature of the environmental chemicals. Technological advancement in live cell study using green fluorescent protein (GFP) and its colour variants have contributed significantly to unravel some of the molecular details about receptor-ligand interactions. We have shown that EDs can modulate and AR function and deviate normal receptor dynamics at multiple cellular levels. A novel correlation has been hypothesized that is related to agonist-mediated generation of 'nuclear foci' by AR and its association to mitotic chromatin. Attempt has been made to explain these results by a preliminary 'biopit model' in perspective of emerging concepts of epigenetics and gene-bookmarking. The findings hold substantial importance in view of the current reports implicating EDs in transgenerational action and programming of embryonic transcriptome, male fertility and cancer. Overall the present study has contributed to our understanding about the action of endocrine disrupting chemicals via AR and the study has also offered new avenues that warrant further investigations in the area of receptor-ED interactions.