CHAPTER 5

TRANSCRIPTIONAL ACTIVATION POTENTIAL OF DEOXYBENZOINS AND ISOFLAVONES

This chapter presents and discusses the experiments carried out to evaluate the transcriptional activation potential of the DOBs and IFs.

ER agonists can modulate the expression of several genes through the ‘classical’ genomic pathway (Björnström and Sjöberg, 2005; Jagannathan and Robinson-Rechavi, 2011; Katzenellenbogen and Katzenellenbogen, 2000). Some the genes modulated by estrogens are common for both ERα mediated and ERβ mediated transcriptional activation, due to the presence of common ERE in the promoter region of these genes (Bhamre et al., 2010; Cowley and Parker, 1999; Jagannathan and Robinson-Rechavi, 2011; Klinge, 2001; Kulakosky et al., 2002; Leitman et al., 2010). Introduction of ERE upstream of non-endogenous genes (reporter genes) like luciferase in expression vectors can also be used for sensitive evaluation of the transcriptional potential of ER agonists (Gutendorf and Westendorf 2001).

In this study, the transcriptional activation potential of the DOBs and IFs were estimated by the ability of the compounds to upregulate the expression of endogenous genes (TFF1 and CTSD) and ERE dependent luciferase.

5.1 EFFECT OF DOBs AND IFs ON ERE DRIVEN LUCIFERASE EXPRESSION

The *in vitro* ER transcriptional activation assay is a direct method for evaluating the ER mediated transcription activation potential of the test compounds. Measuring the enzyme activity of luciferase, whose expression is
controlled by ERE mediated activation, gives an opportunity to quantitatively express the transcriptional activation potential of compounds. In the present study, Hep2 cells (having low levels of endogenous ER) were transiently transfected with ERE-luc reporter construct and an expression plasmid for either ERα or ERβ. This allowed the study of ER agonist activity and specificity of the test compounds effectively. It was found that the DOB derivatives, viz. CMPD3, CMPD6 and CMPD9, were non-subtype specific, while showing good transcriptional activation potential through ERα as well as ERβ (Figure 5.1, Figure 5.2, Table 5.1). Among the isoflavone derivatives, CMPD1 was ERα selective, while CMPD7 was strongly ERβ selective. The transcription activation potential of all the tested compounds was lesser than that of genistein and estradiol. The effect of DOBs was more potent than the induction effect of the IFs (except CMPD7).
Figure 5.1  Ligand induced luciferase expression in Hep2 cells transiently transfected with ERα and 3XERE-TATA-luc

Figure 5.2  Ligand induced luciferase expression in Hep2 cells transiently transfected with ERβ and 3XERE-TATA-luc
Table 5.1 Transcriptional activation potential of the ligands

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<tr>
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<th>Control</th>
<th>CMPD3</th>
<th>CMPD6</th>
<th>CMPD9</th>
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<tr>
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<td>Estradiol</td>
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Transcriptional activation potential was calculated by the ratio of normalized luciferase activity in ERα transfected Hep2 cells to normalized luciferase activity in ERβ transfected Hep2.

The single point transcriptional activation potential values (Table 5.1) suggested that CMPD1, CMPD3 and CMPD9 were marginally ERα selective (values >1), while CMPD7 was strongly ERβ selective (value 0.38). CMPD2 and CMPD11 also showed moderate ERβ selectivity (values <1). Interestingly, comparison of Table 5.1 with Table 3.2 and Table 3.3 confirms the lack of parity between the computational docking scores and the observed biological activity of the molecules. This disparity can be attributed to the fact that docking calculation is limited with the calculation of complementarities between the ligand and a small portion of the receptor; whereas in the biological context, the ligand binding may influence the receptor configuration, enabling or disabling the recruitment of coactivators necessary for the transcription. Current molecular docking methods cannot predict such large scale post-docking changes occurring in the receptor due to constraints in molecular mechanics (Seeliger and de Groot, 2010; Warren et al. 2006).

The *in vitro* ER transcriptional activation assay is a widely accepted method for measuring the activities of the ER binding ligands (Gutendorf and Westendorf, 2001). The expression plasmids used for *in vitro* ER transcription assay will have reporter protein gene controlled by a promoter and the ER recognition site (ERE). Multiple copies of the EREs are usually included to
increase the sensitivity of the transcription induction. Various reporter proteins can be used as the end points for the activation potential of the screened ligands. These include fluorescent proteins and enzymes.

The use of fluorescent protein (such as Green Fluorescent Protein) as the reporter protein enables the visualization of the induced expression in live cells or whole animals (Gutendorf and Westendorf, 2001; Lee et al., 2012a, 2012b; Schaeffer et al., 2011). While this method is useful in visually determining the estrogenic effects, there are ambiguities in expressing the endpoints in quantitative terms. Luciferase is routinely used as a reporter protein in majority of the ER transcription activation assay methods (Sedlák et al, 2011). Luciferase assays have advantage that the endpoints can be quantified reliably on luminometers, but can be performed only after lysing the cells. For assessing the ER transactivation potential, transient transfections are more widely used, because of the larger reporter plasmid content in the cells during the assay which increases the sensitivity of the assay (Gutendorf and Westendorf, 2001). The current study was able to successfully use the luciferase assay as a measure of transcriptional activation property of the tested compounds. This study was useful in comparing between the active test compounds near their IC$_{50}$ and EC$_{50}$ values, and a more detailed study across a suitable concentration range could be attempted.

5.2 EFFECT OF DOBs AND IFs ON EXPRESSION OF TFF1 AND CTSD

The expression of estrogen responsive genes, TFF1 and CTSD, in PC3 cells and MCF7 cells was also additional evidence to the estrogenic effect of the test compounds. In line with the cell proliferation and ER transcriptional activation assay, the gene expression studies suggested that the DOBs (CMPD3, CMPD6, and CMPD9) were able to differentially up-
regulate the expression of estrogen responsive genes in MCF7 cells and PC3 cells (Figure 5.3).

Figure 5.3  Expression of estrogen responsive genes, TFF1 and CTSD, in PC3 and MCF7 cells treated with DOBs, as determined by a RT-PCR in a multiplex mode. GAPDH transcript acts as the internal control.

Among the IFs, CMPD7 showed significant upregulation of TFF1 and CTSD in PC3 cells, while the remaining IFs showed moderate upregulation of these genes in PC3 (Figure 5.4). CMPD5 failed to show any response.

In MCF7 cells, the CMPD1, CMPD2, CMPD10 and CMPD11 showed marked upregulation of TFF1 and CTSD, while CMPD7 did not show any effect (Figure 5.5).
Figure 5.4 Expression of estrogen responsive genes, TFF1 and CTSD, in PC3 cells treated with IFs, as determined by RT-PCR in multiplex mode.

Figure 5.5 Expression of estrogen responsive genes, TFF1 and CTSD, in MCF7 cells treated with the IFs, as determined by a RT-PCR in multiplex mode.

The ER-agonist complex induces early upregulation of several genes (Frasor et al., 2003; Jagannathan and Robinson-Rechavi, 2011; Kininis and Kraus, 2008) which causes cell proliferation or inhibition dependant on the differential amount of ER subtype and available levels of coactivators and corepressors. The expression of TFF1 and CTSD are two well known examples of estrogen induced early gene expression in mammalian cells, which share highly similar ERE (Bretschneider et al., 2008; Lau et al., 2000). These two genes are well known as markers for malignant tumours, especially breast cancer (Jagannathan and Robinson-Rechavi, 2011; Kininis and Kraus, 2008; Wang et al., 1997). TFF1 encodes for a small peptide which may have
role in angiogenesis of the tumors (Lazennec et al 2001, Van Meeuwen et al 2007), while the cathepsin-D is a protease, which increases the tumor invasion (Wang et al 1997). Both these genes are driven by an ERE, which may be activated by ligand bound ER\(\alpha\) or ER\(\beta\).

The multiplex RT-PCR demonstrated that the expression of these two marker genes were markedly higher (~4 fold) in PC3 and MCF7 cells treated with CMPD3 and CMPD9 than the untreated cells, while the CMPD6 treated cells resulted in a moderate stimulation (Figure 5.3), showing that these compounds were acting through the predominant ER subtype present in each cell type. However, the induction of these genes by the test compounds occurred at a higher concentration (10 \(\mu\)M) when compared to estradiol (1 nM) and genistein (1 \(\mu\)M) suggesting their moderate estrogenic effect.

The expression of TFF1 and CTSD was higher in PC3 cells treated with CMPD7 when compared to the treatment the treatment with other IFs (Figure 5.4). At the same time, the effect of CMPD7 on expression of TFF1 and CTSD was not significant in MCF7 cells (Figure 5.5). These results were largely in agreement with the results of the luciferase transcriptional activation assay, wherein the CMPD7 showed strong luciferase induction in ER\(\beta\) transfected Hep2 cells (Figure 5.2).

5.3 SUMMARY OF TRANSCRIPTIONAL ACTIVATION POTENTIAL

The transcriptional activation potential of the DOBs and IFs were evaluated by luciferase reporter assay and upregulated expression of TFF1 and CTSD. The DOBs (CMPD3, CMPD6 and CMPD9) were found to be moderately potent in activating the transcription of ER dependent genes. Also, the DOBs did not exhibit significant ER subtype selectivity in mediating the expression ER dependent genes. Among the IFs, CMPD1 was moderately
potent and ERα selective, while CMPD7 was a strong activator of ERβ mediated transcriptional activation.

More accurate transcriptome studies with quantitative techniques like Real Time PCR or Microarray analysis can be used to further study the effect of these DOBs and IFs in more detail.