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Abstract

Endocrine-disrupting chemicals (EDCs) are a broad class of molecules present in our environment that are suspected to cause adverse effects in the endocrine system by interfering with the synthesis, transport, degradation or action of endogenous hormones. Humans are generally exposed to low doses of pollutants, and current researches aim at deciphering the mechanisms accounting for the health impact of EDCs at environmental concentrations. Here, we review different mechanisms through which EDCs might alter hormonal functions by interfering with the nuclear receptors and related pathways, including extranuclear signalling by membrane-bound receptors mediating rapid non-genomic responses, and the aryl hydrocarbon receptor (AhR).

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Mechanisms of endocrine disruption through nuclear receptors and related pathways

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Abstract

Endocrine-disrupting chemicals (EDCs) are a broad class of molecules present in our environment that are suspected to cause adverse effects in the endocrine system by interfering with the synthesis, transport, degradation or action of endogenous hormones. Humans are generally exposed to low doses of pollutants, and current researches aim at deciphering the mechanisms accounting for the health impact of EDCs at environmental concentrations. Here, we review different mechanisms through which EDCs might alter hormonal functions by interfering with the nuclear receptors and related pathways, including extranuclear signalling by membrane-bound receptors mediating rapid non-genomic responses, and the aryl hydrocarbon receptor (AhR).

Keywords: nuclear receptors; dioxin receptor; endocrine-disrupting chemicals.

1. Introduction

Endocrine-disrupting chemicals (EDCs) are exogenous substances that interfere with the function of hormonal systems and produce a range of developmental, reproductive, neurological, immune or metabolic diseases in humans and wildlife [1-3]. Many EDCs are man-made chemicals produced by industry and released into the environment, but some naturally occurring EDCs can also be found in plants or fungi. The group of molecules acting as EDCs is highly heterogeneous and comprises compounds that are often distantly related to endogenous ligands in terms of size or chemical structure. This group contains substances as chemically different as bisphenols, phthalates, parabens, dioxins, alkylphenols, organotins, polychlorinated biphenyls, perfluoroalkyls, or benzophenones, as well as natural compounds such as the phytoestrogens genistein, daidzein or the mycoestrogen zearalenone. Since humans and wildlife are simultaneously and chronically exposed to low doses of multiple contaminants, understanding the molecular mechanisms underlying the physiological consequences of exposure to environmentally relevant concentrations of EDCs is a major challenge [4]. EDCs can affect the endocrine systems of an organism in a wide variety of ways. These include mimicking natural hormones, antagonizing their action or modifying their synthesis, metabolism and transport. As of today, most of the reported harmful effects of EDCs are attributed to their interaction with nuclear or extranuclear receptors that respectively function as transcriptional regulators in the nucleus such as the members of the nuclear receptors (NRs) superfamily [5,6] and the aryl hydrocarbon receptor (AhR) [7], or mediate rapid non-genomic responses, for instance, the membrane-associated nuclear receptors (mbNRs) [8] and the G protein-coupled receptor (GPCR) GPR30/GPER [9]. Herein, we review the main structural and molecular mechanisms used by EDCs to alter these signaling pathways and contribute to the etiology of several diseases.

2. Nuclear receptors are primary targets of EDCs

The 48 human NRs control a plethora of biological processes such as development, organ homeostasis, metabolism, immune function, or reproduction [10,11]. As a consequence, inappropriate exposure to EDCs, can cause proliferative reproductive and metabolic disorders, including hormonal cancers, infertility and obesity. Most of the studies on EDCs have originally focused on NRs involved in reproductive processes, in particular the estrogen (ER α and ER β) and the androgen (AR) receptors. However, mounting data reveal that most NRs are potential targets of EDCs. Among NRs, endogenous ligands have been identified for 24 family members. These receptors are ligand-dependent transcription factors that respond directly to a large variety of hormonal and metabolic substances that are hydrophobic, lipid soluble, and of small size (e.g. retinoic acid or estradiol). The other class of NRs is the group of so-called orphan receptors, for which regulatory ligands are still unknown or may not exist. The transcriptional activity of orphan receptors is thus regulated by post-translational modifications or cell-specific expression of coregulatory proteins, and eventually by pharmacological or environmental chemicals, as for instance the estrogen related receptor γ (ERR γ) which has no endogenous ligand but is one of the main targets of bisphenol-A (BPA) [12]. All NR proteins exhibit a characteristic modular structure that consists of five domains (Fig. 1) [13]. The DNA-binding domain (DBD) is the most highly conserved domain and encodes two zinc finger modules. The ligand binding domain (LBD) is less conserved and mediates ligand binding, dimerization, and hosts a ligand-dependent transactivation function, termed AF-2 which corresponds to transcriptional coregulator interaction surfaces that are modulated by ligands. The remaining domains are the N-terminal region containing a cell- and promoter specific transactivation function termed AF-1, the central hinge region linking

the DBD and LBD, and the C-terminal region that is not present in all receptors and whose function is poorly understood.

2.1 Genomic actions of nuclear receptors

NRs may act either as repressors or activators of gene transcription depending on their ligand-binding status that in turn determines the ability of DNA-bound receptors to recruit coregulators to target gene promoters (Fig. 2). Coactivators and corepressors are large groups of proteins with a varied range of activities and enzymatic functions that contribute to transcription regulation by NRs and many other transcription factors, including AhR. Coactivators contribute to the enhancement of transcription by acetylating histones, a process that plays an important role in the opening of chromatin during transcription activation, whereas corepressors display the opposite activity by recruiting histone deacetylases (HDACs). Coactivator recruitment is usually ligand-dependent, whereas corepressors interact in most cases with unliganded receptors. In the absence of the cognate ligand, some NRs are located in the nucleus, bind to the DNA response elements of their target genes, and recruit corepressors, while others are located in the cytoplasm in an inactive complex with chaperones (Fig. 2). Ligand binding induces major structural alterations of the receptor LBDs leading to (i) destabilization of corepressor or chaperone interfaces, (ii) exposure of nuclear localization signals to allow nuclear translocation and DNA binding of cytoplasmic receptors, and (iii) recruitment of coactivators triggering gene transcription through chromatin remodelling and activation of the general transcription machinery.

2.2 A structural view of the interaction between nuclear receptors and EDCs

The LBD of NRs is composed of a conserved core of 12 α -helices (H1 to H12) and a short two-stranded antiparallel β -sheet (s1 and s2), arranged into a three-layered sandwich fold (Fig. 3A). This arrangement generates a mostly hydrophobic cavity, referred to as the ligand-binding pocket (LBP), which can accommodate the cognate ligand. In hormone-bound LBD

structures, the LBP is sealed by helix H12, thus generating a hydrophobic binding groove for short LxxLL helical motifs (L stands for leucine and x for any amino acid) found within coactivators. Biochemical and cell-based assays have revealed that EDCs bind to NRs with affinities ranging from sub-nanomolar to high micromolar values and structural analyses have revealed some mechanisms by which compounds structurally and chemically unrelated to physiological ligands can bind to NRs at environmentally relevant concentrations [14-16]. Representative examples are the binding of α -zearalanol (α -ZA) to ER α , the covalent interaction of various chemicals with the retinoid X receptor (RXR), the peroxisome proliferator-activated receptor γ (PPAR γ) and the xenobiotic receptor PXR, or the cooperative binding of two chemicals to PXR and PPAR γ . Even though the mycoestrogen α -ZA (Fig. 1) is not a steroidal compound, it recapitulates the key interaction networks observed between the residues of the ER α LBP and the endogenous hormone 17 β -estradiol E₂ (Fig. 3B). As such, α -ZA appears as an E₂ mimic that binds to its target receptor at sub-nanomolar concentrations [17]. In contrast, compounds of the organotin family such as the tributyltin (TBT, Fig. 1) bind with much avidity to many NRs through a mechanism that is totally different from those used by the natural or pharmacological ligands of these receptors. Indeed, the nanomolar affinity of organotins for NRs arises from the formation of a strong covalent bond formed between the tin atom of the chemical and the sulfur atom of a cysteine residue contained in the LBP of the receptors [14] (Fig. 3C). Beside tin compounds, many bioactive chemicals such as pesticides or drugs contain chemical groups that are prone to forming such covalent links with reactive cysteine residues, thereby insuring high affinity binding of the compounds. Structural studies have revealed that the insecticide fipronil and the herbicide pretilachlor bind to and activate PXR *via* this mechanism (our unpublished data). It is thus very likely that part of the low dose effects of a number of environmental compounds can be explained by their covalent interaction with the dozen NRs containing a cysteine residue in their LBP. Compared to

binding sites located at the surface of proteins, the buried and mostly hydrophobic cavity provided by NRs probably offers an environment allowing a residence time long-enough to let the chemical reaction to occur. Finally, recent studies have shown that NR LBPs are more conformable than previously thought and can eventually accommodate several compounds simultaneously [18-22]. In some instances it has been shown that the compounds not only bind concomitantly to the receptor, but they do so cooperatively, *i.e.* the binding of one molecule promotes high affinity binding of the second, with synergistic effects on receptor function. Notably, the contraceptive 17 α -ethinylestradiol (EE2, Fig. 1) and the persistent organochlorine pesticide *trans*-nonachlor (TNC, Fig. 1), both exhibiting low affinity and efficacy when studied separately, were shown to bind and activate the receptor PXR at much lower concentration when used in combination [20] (Fig. 3D).

2.3 Non-genomic actions of nuclear receptors

Beside their interference with the genomic pathways of NRs, EDCs have also been shown to interact with the membrane-associated fraction of these receptors (Fig. 2). Indeed, it is now well accepted that a small amount of NRs (a few percent) are positioned at the membrane (mbNRs) as previously shown for ER α and β , and for many other NRs such as the progesterone, the androgen, the thyroid hormone or the vitamin D receptors [23-26]. As for ER α , the receptor is directed to the plasma membrane/caveolae *via* palmytoylation of an internal cysteine residue (C447) that is located in the LBD. Once localized in caveolae rafts, ligand-bound mbER α is depalmytoylated and interacts with and activates various G α and G β / γ proteins within seconds, thereby triggering a cascade of signal transduction including cAMP production, calcium mobilization, ion channel activation, endothelial nitric oxide synthase and multiple kinases (ERK and PI3K) activation [27]. It is interesting to note that mice expressing ER α mutated in this cysteine 447 (cysteine 451 in mice) are sterile (both males and females) and present differences in E₂ vascular actions, such as abrogation of

endothelial repair and eNOS phosphorylation. In contrast, other responses like endometrial proliferation are similar to wild-type mice [28]. These membrane-localized ERs are thought to mediate the rapid non genomic effects of natural (E_2) or environmental estrogens (BPA or alkylphenols) outside the nucleus [28,29]. Because the membrane-associated receptors are often the same proteins as their nuclear counterparts, one can make the assumption that compounds bind to the two receptor forms with the same mechanisms and thus similar binding affinities. However, it is not excluded that the membrane environment modifies the thermodynamics of the interactions with an impact on the ligand-binding properties of the receptor. Furthermore, it has been postulated that, in contrast to the nuclear pathway requiring ER-ligand complexes of sufficient lifetime to induce a long series of molecular events including dissociation from heat shock proteins, translocation, dimerization and DNA binding, recruitment of coregulators, alteration of chromatin architecture and finally transcription initiation, the extranuclear signalling pathway involving the rapid triggering of kinase cascades could be activated by ER-ligand complexes with shorter half-life [30]. In this context, one can note that the low binding affinity constant (K_d) of compounds such as bisphenols and alkylphenols generally results from a high dissociation rate constant (K_{off}) so that their complexes with ER feature short lifetimes [31]. Although this hypothesis needs further investigations, it could explain that EDCs with about 10,000-fold less affinity than E_2 for ERs can activate the extranuclear-initiated signaling pathway preferentially over the nuclear pathway.

3. The aryl hydrocarbon receptor

3.1 AhR structure and function

The Aryl hydrocarbon Receptor (AhR), also known as the dioxin receptor, is a ligand-dependent transcription factor belonging to the basic helix-loop-helix (bHLH) PER-ARNT-SIM (PAS) protein family that binds to DNA following formation of a heterodimer with the

AhR-nuclear translocator protein (ARNT) [32,7]. Although not members of the NR family, AhR and ARNT display structural and functional similarities with NRs such as a molecular organisation in four major domains including a DBD and LBD, or a transcriptional activity that is regulated by the presence of ligands and coregulators (Fig. 1). The N-terminal bHLH domain is involved in DNA recognition. The PAS domain exists as a tandem of two domains, PAS-A and PAS-B, respectively, each encompassing around 50 amino acids. The bHLH and PAS-A domains of both AhR and ARNT dimerize to form a stable DNA-bound heterodimer. In AhR, the PAS-B domain is responsible for ligand binding. The C-terminal part of the protein also includes a transcriptional activation domain (TAD). Several multi-domain crystal structures of AhR-ARNT heterodimers have been recently reported providing insights onto the modes of DNA binding and dimerization of the complex [33,34]. These structures are characterized by sophisticated domain interplay and reveal a highly intertwined architecture that bind to DNA *via* the positively charged residues of the bHLH domains. None of the structures reported yet contain the PAS-B region of AhR so that the detailed mechanisms of ligand-binding and protein activation remain largely elusive. However, together with the extended interdomain interfaces, the high structural dynamics of the AhR PAS-A domain suggest an allosteric communication route to mediate ligand-induced changes in the PAS-B domain to the DNA-binding bHLH domains, the C-terminal TAD or the nuclear localization sequence (NLS). AhR binds a selection of different endogenous ligands including tryptophan-derived metabolites and several dietary indoles, and is involved in many cellular processes including cell proliferation and differentiation, immune response, inflammation and circadian rhythm regulation [7]. While AhR also functions as a sensor of exogenous chemicals and promotes metabolic clearance through the induction of metabolizing enzymes such as CYP1A1 and CYP1B1 upon activation by external compounds, it is known to mediate most of the toxic effects of a range of environmental contaminants such as polyaromatic

hydrocarbons (PAHs) and dioxins. These toxic effects are numerous and include teratogenicity, immunosuppression, metabolic and endocrine disruption, skin toxicity and cancer. In the absence of ligand, AhR resides in the cytosolic compartment of the cell bound to a molecular chaperone complex containing Hsp90 and XAP2 (Fig. 2). Upon ligand binding AhR translocates from the cytosol to the cell nucleus where it forms a heterodimer with ARNT. The AhR-ARNT complex recognizes specific sequences of DNA called xenobiotic responsive element (XRE) in the promoter region of the target genes and activates transcription of these genes *via* the recruitment of transcriptional coactivators. The activity of the AhR-ARNT complex is negatively modulated by proteasomal degradation and the AhR repressor (AhRR) that interacts with ARNT and forms an AhRR-ARNT heterocomplex down regulating AhR signalling. In addition to its direct interaction with target gene promoters, AhR accomplishes some of its regulatory functions by modulating the activity of other transcriptions factors including ERs, AR, and many others [35]. The liganded AhR-ARNT heterodimer modulates estrogen and androgen signaling positively by physically associating with unliganded ERs and AR and bringing transcriptional coactivators to the promoters of these receptors [36]. In contrast, in presence of estrogens and androgens, the liganded AhR-ARNT represses estrogen and androgen mediated transcription by targeting ERs and AR to the Cul4B-based ubiquitin ligase complex for proteasomal degradation [37]. Thus it appears that AhR acts both as a transcription factor and as an ubiquitin ligase component to mediate different signaling pathways [35].

3.2 AhR ligands and ligand binding

Although AhR was originally discovered as a primary target of the toxic dioxin (2,3,7,8-tetrachloro-dibenzo-p-dioxin, TCDD, Fig. 1), it is now recognized that AhR is able to respond to hundreds of chemically diverse endogenous, dietary and environmental compounds, a number of these compounds interacting with the receptor at (sub)nanomolar concentrations

[38]. AhR ligands include polychlorinated dibenzo-p-dioxins, dibenzofurans (Fig. 1), polychlorinated biphenyls (e.g. PCB126, Fig. 1), polycyclic aromatic hydrocarbons (PAHs) as benzo(a)pyrene (BaP) and related PAHs compounds, including 3-methylcholanthrene (3MC) and β -naphthoflavone. Dietary compounds (3,3'-diindolymethane and indolo(3,2)carbazole) and endogenous metabolites, such as indirubin and 6-formylindolo(3,2)carbazole (FICZ, Fig. 1) are also known to act as AhR ligands. In addition to their high affinity, some AhR ligands such as dioxins are very resistant to metabolism, which appears to confer a greater toxicity when compared to that of compounds such as BAP or 3MC which are rapidly metabolized by CYP1A1 [38]. In contrast, the mechanism of carcinogenicity of some PAHs includes the production of highly reactive genotoxic metabolites. While no structure of the AhR LBD has been reported yet, molecular modelling using the structures of closely related PAS domains has suggested some molecular features accounting for its ligand-binding promiscuity and specificity [39]. In particular, mutational analysis, molecular modelling and molecular dynamics simulations have identified residues that control ligand preferences and the high dynamics of some structural elements rendering the AhR PAS-B domain malleable [40-44]. Interestingly, flexibility of some regions of the LBD is also a characteristic feature of the NR PXR which is another major chemical sensor known to induce the expression of detoxification proteins such as metabolizing enzymes and transporters upon activation by a broad range of chemicals [45]. PXR contains several loops clustering at the bottom of the LBD that confer a high plasticity allowing the ligand binding pocket of the receptor to adopt different shapes according to the bound ligands. Three of these flexible elements are found in a PXR-specific sequence of approximately 60 residues inserted between helices H1 and H3 that display high thermal B factors (indicating structural mobility) or are completely disordered as observed in the crystal structures. However, a striking difference between the two receptors is that AhR is recognized by a number of high affinity ligands like TCDD or

FICZ (with nanomolar affinities), whereas PXR is essentially bound by compounds with affinities in the micromolar to sub-micromolar range [38]. One potential explanation for this difference may reside in the size of the binding cavity of the two receptors. Indeed, while PXR displays the largest binding pocket among all NRs with experimentally-determined volumes ranging from 1280 to 1600 Å³ depending on the bound compound, homology modelling of the PAS-B domain of AhR suggests a volume of the binding pocket in the range 300–400 Å³ [46]. As a consequence, ligands generally occupy only a small fraction of the PXR binding pocket so that compounds appear to interact loosely with the residues lining the cavity, whereas the smaller pocket size of AhR may result in tighter contacts and a more stable ligand-bound complex. Another difference between the two proteins is that AhR is more easily antagonized than PXR. Indeed, a number of potent AhR antagonists have been discovered a while ago [47-49], whereas the first PXR antagonist has been reported only very recently [50,51]. This observation is consistent with a smaller and more constrained binding pocket in AhR.

4. G protein-coupled receptor 30

The G protein-coupled receptor 30 (GPR30) also referred to as G protein-coupled estrogen receptor (GPER) was also proposed to act as a non-classical estrogen receptor localized at the plasma membrane or the endoplasmic reticulum [52,53]. Originally, GPR30 was identified as an orphan receptor belonging to the family of 7-transmembrane-spanning GPCRs. Later on, several studies reported the activation of GPR30 by natural, environmental and pharmaceutical estrogens and antiestrogens (for a review see [54]), leading to its designation as GPER. However contradictory results concerning the ability of this receptor to bind estrogens have been reported. Binding of E₂ and several xenoestrogens to GPR30 was observed by some laboratories [53] whereas others failed to detect any interaction [55,56]. Finally, the transgenic KO for GPR30 do not display phenotypes that result from estrogen

depletion suggesting that GPR30 is not a direct mediator of estrogen effects but could be rather a collaborator in non-nuclear functions of the nuclear ER [57,58]. Several selective GPR30 agonists and antagonists have been recently identified which will probably help characterizing the role GPR30 in these toxic effects in the future [9].

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Figure captions

Fig. 1. Nuclear receptors, AhR and GPR30 are the main known targets of endocrine disruptors. Structural organisation of proteins, and chemical structures of the associated EDCs mentioned in this review (EE2 = ethinylestradiol; TNC = *trans*-nonachlor; TBT = tributyltin; TCDD = 2,3,7,8-tetrachloro-dibenzo-p-dioxin; PCB-126 = polychlorinated biphenyl 126; FICZ = 6-formylindolo(3,2)carbazole).

Fig. 2. EDCs targeted cellular pathways. **1** The genomic pathways of nuclear receptors (NRs) and AhR, **2** the extranuclear signalling pathway of membrane-bound NRs (mbNR), and **3** the G protein-coupled receptor 30 (GPER/GPR30) pathway.

Fig. 3. Different binding modes of EDCs observed with NRs. **A.** Conserved 3D-structure of the ligand binding domain of nuclear receptors (here, ER α in complex with estradiol, PDB_{id} = 3UUD). Close up view of the ligand binding pockets of **B.** ER α in complex with α -zearalanol (PDB_{id} = 4MG8), **C.** RXR in complex with TBT (PDB_{id} = 3E94), and **D.** PXR in complex with EE2 and TNC (PDB_{id} = 4X1G).

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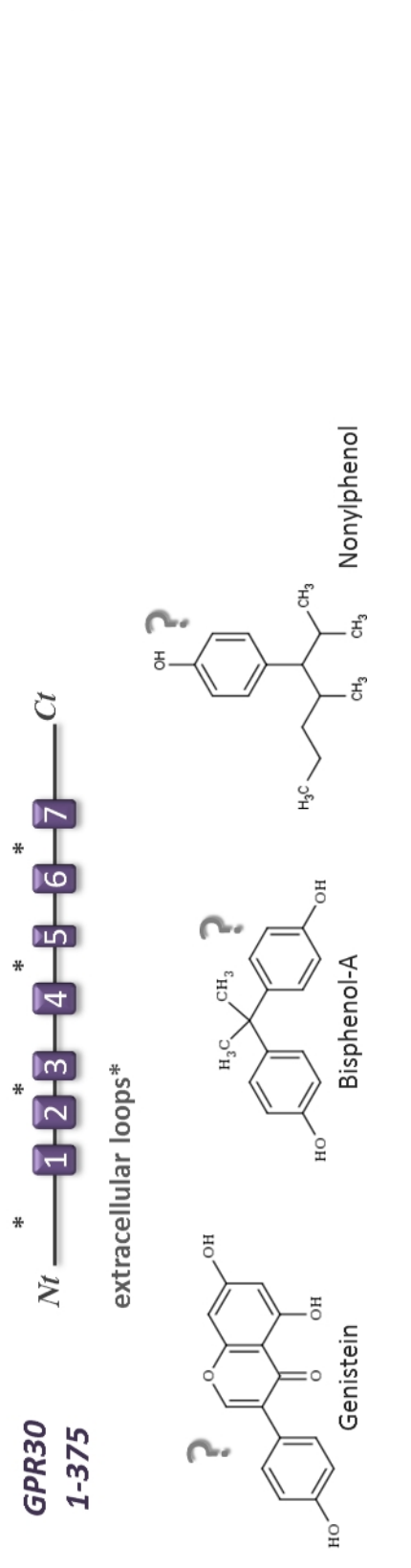
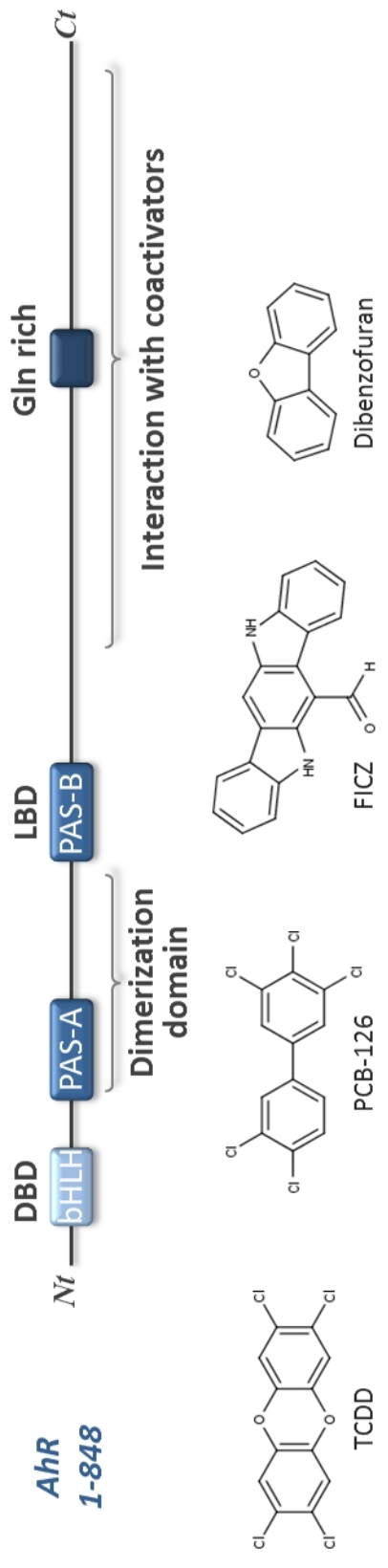
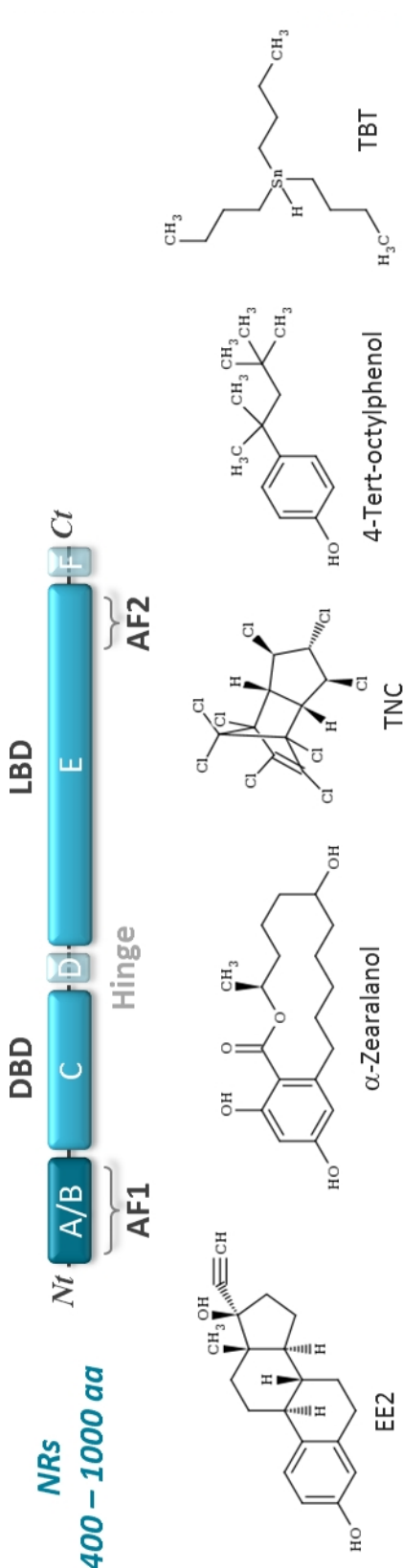
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