Non-monotonic dose–response relationship in steroid hormone receptor-mediated gene expression

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Abstract

Steroid hormone receptors are the targets of many environmental endocrine active chemicals (EACs) and synthetic drugs used in hormone therapy. While most of these chemical compounds have a unidirectional and monotonic effect, certain EACs can display non-monotonic dose–response behaviors and some synthetic drugs are selective endocrine modulators. Mechanisms underlying these complex endocrine behaviors have not been fully understood. By formulating an ordinary differential equation-based computational model, we investigated in this study the steady-state dose–response behavior of exogenous steroid ligands in an endogenous hormonal background under various parameter conditions. Our simulation revealed that non-monotonic dose–responses in gene expression can arise within the classical genomic framework of steroid signaling. Specifically, when the exogenous ligand is an agonist, a U-shaped dose–response appears as a result of the inherently nonlinear process of receptor homodimerization. This U-shaped dose–response curve can be further modulated by mixed-ligand heterodimers formed between endogenous ligand-bound and exogenous ligand-bound receptor monomers. When the heterodimer is transcriptionally inactive or repressive, the magnitude of U-shape increases; conversely, when the heterodimer is transcriptionally active, the magnitude of U-shape decreases. Additionally, we found that an inverted U-shaped dose–response can arise when the heterodimer is a strong transcription activator regardless of whether the exogenous ligand is an agonist or antagonist. Our work provides a novel mechanism for non-monotonic, particularly U-shaped, dose–response behaviors observed with certain steroid mimics, and may help not only understand how selective steroid receptor modulators work but also improve risk assessment for EACs.

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Introduction

Steroid hormone receptors (SHRs) comprise a superfamily of transcription factors that are activated by steroid hormones to regulate specific gene expression. They play critical roles in a variety of physiological processes including homeostasis, metabolism, reproduction, and behaviors. Widely studied SHRs include androgen receptor (AR), estrogen receptor (ER), progesterone receptor (PR), and glucocorticoid receptor (GR). The overall structure of these SHRs is highly conserved and contains four major functional domains: a conserved DNA-binding domain (DBD), a C-terminal ligand-binding domain (LBD), an N-terminal transactivational domain (NTD), and a hinge region connecting DBD and LBD (Ruff *et al.* 2000, Gelmann 2002, Bledsoe *et al.* 2004, Rogerson *et al.* 2004). In its most general form, the classical genomic action of steroid hormones involves the following intracellular processes. In the absence of natural ligand, SHRs in the cytoplasm or nucleus are associated with chaperone complexes, which function to stabilize SHRs and enhance their ligand-binding affinity (Fang *et al.* 1996, Pratt & Toft 1997). During ligand binding, SHRs first dissociate from the chaperone complexes, followed by nuclear translocation of those SHRs initially located in the cytoplasm (Htun *et al.* 1996, Georget *et al.* 1997, Tyagi *et al.* 2000). Liganded receptor monomers then homodimerize with each other to form receptor dimers (Kumar & Chambon 1988, Wrange *et al.* 1989, Wang *et al.* 1995). Depending on the type of SHRs, receptor dimers recognize and bind specifically to the DNA of target hormone response elements (HREs), with each monomer recognizing one half-site of the HRE (Nordeen *et al.* 1990, Langley *et al.* 1995, Klinge *et al.* 1997). The receptor dimer–DNA complex then recruits a battery of nuclear coregulators to alter the local chromatin structures (Heinlein & Chang 2002, Smith & O'Malley 2004). With a relaxed chromatin structure, polymerase II gains access to the promoter to initiate gene transcription. Ligands that allow recruitment of corepressors (CoR) can induce condensation of the chromatin, thus turning off gene transcription (Fernandes & White 2003).

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SHRs that are normally activated by endogenous ligands can also respond to exogenous substances, including synthetic drugs and environmental chemicals. Acting on an endogenous hormonal background, these chemical compounds can potentially modulate endocrine events, resulting in altered SHR-mediated biological functions. Many therapeutic drugs are designed to interact directly with SHRs as agonists or antagonists to alter gene transcriptional activities in target tissues. The effects exerted by many of these drugs may vary from tissue to tissue (Dutertre & Smith 2000, Giannoukos *et al.* 2001, Berrevoets *et al.* 2002). For example, while both tamoxifen and raloxifene reduce the risk of invasive breast cancers by acting as ER antagonists in mammary tissues, they have a strong agonistic activity in bones that helps maintain bone density in women (Dutertre & Smith 2000, Francucci *et al.* 2005). Because of the opposing effects, these drugs are more appropriately termed as selective receptor modulators (SRMs). Besides synthetic drugs, a large set of chemicals that can interfere with endocrine functions are environmental pollutants termed as endocrine active chemicals (EACs). EACs may interfere with the synthesis and metabolism of endogenous hormones, or in many cases, interact with SHRs directly (Amaral Mendes 2002, Markey *et al.* 2002). An important aspect of health risk assessment for EACs is understanding dose–response curves at low doses that are relevant to human exposure in the environment. A large body of evidence indicates that SHR-mediated adverse effects of EACs are sometimes nonlinear or even non-monotonic (i.e. U-shaped or inverted U-shaped) in dose ranges exerting no overt cytotoxicity (Kemppainen & Wilson 1996, vom Saal *et al.* 1997, Maness *et al.* 1998, Putz *et al.* 2001*a*,*b*, Almstrup *et al.* 2002, Terouanne *et al.* 2003, Kohlerova & Skarda 2004).

The molecular basis for the bidirectional actions of SRMs and non-monotonic or hormetic effects of EACs is not completely understood. A variety of mechanisms have been proposed to explain these observations. For instance, the ratio of coactivators (CoA) to corepressors in a cell may determine whether an exogenous ligand behaves primarily as an agonist or antagonist (Smith *et al.* 1997, Szapary *et al.* 1999, Smith & O'Malley 2004). Alternatively, the opposing effect of SRMs in different tissues may result from the involvement of different SHR subtypes (McInerney *et al.* 1998, Zhou & Cidlowski 2005). Kohn and Melnick found that an inverted U-shaped dose–response can arise from conditions where there are unoccupied receptors by endogenous hormones and recruitment of CoA by xenobiotic ligands is relatively weak (Kohn & Melnick 2002). Conolly and Lutz hypothesized that a U-shaped dose–response curve can result from transcriptionally inactive mixed-ligand receptor dimers (Conolly & Lutz 2004). Possibilities also exist that the non-genomic effect of steroid ligands, which often leads to the activation of kinases such as mitogen-activated protein kinase, may modulate the genomic actions of the same ligands in opposite directions via receptor or coregulator phosphorylation, resulting in non-monotonic responses in gene expression (Acconcia & Marino 2003, Rochette-Egly 2003).

The present study focused on the steady-state dose–response for gene expression mediated through SHRs. Using a computational modeling approach, we demonstrated that non-monotonic dose–responses can readily arise within the classical framework of steroid signaling. Our results indicated that the inherently nonlinear process of receptor homodimerization in SHR signaling plays an important role in rendering U-shaped dose–response curves, which can be further modulated by mixed-ligand heterodimers.

Methods

Model structure

Definitions for a pure agonist, antagonist, and partial agonist in particular, in the endocrine literature have been largely observational rather than mechanistic. For modeling purposes, we need to be more explicit, and so these terms are defined as follows. A pure agonist is a ligand that is able to recruit CoA exclusively to activate gene transcription. If a ligand is able to recruit CoR exclusively to deactivate gene transcription, it is termed as an active antagonist, whereas if a ligand can recruit neither CoR nor CoA after binding to a steroid receptor, it is termed as a passive antagonist. A partial agonist is a ligand that is able to recruit both CoA and CoR, albeit not simultaneously. In this way, when a partial agonist acts alone in our model, it always activates gene transcription but with a reduced maximal response when compared with a pure agonist. It is generally believed that whether a receptor is able to recruit CoA or CoR depends on its conformational changes after ligand binding, particularly in the LBD domain (Brzozowski *et al.* 1997, Shiau *et al.* 1998), and the phosphorylation status of the receptor also modulates this recruiting process (Atanaskova *et al.* 2002, Rochette-Egly 2003).

Since there are always background levels of endogenous hormones in a physiological state, we simulated gene expression driven by exogenous ligand X in the presence of endogenous ligand L (Fig. 1). In the absence of X, endogenous ligand L first binds to a receptor SHR to form a liganded receptor complex LR. Two LRs then associate with each other to form a receptor homodimer LRRL. LRRL in turn binds to the HRE in the promoter of target genes. While bound to HRE, LRRL is able to recruit CoA to the local promoter site and together these molecules produce an activational complex CoALRRLH. Since L mimics an endogenous

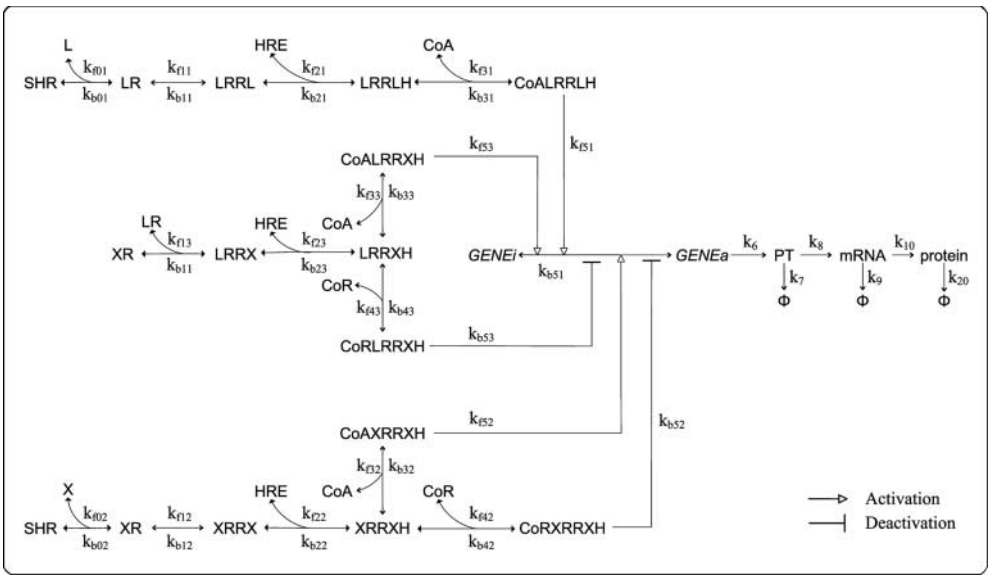

Figure 1 Structure of the ODE-based model for SHR-mediated gene expression. For a detailed description of the signaling pathway, see Methods. For ODEs, parameter values, and supporting references, see Tables S1 and S2 in the Supplementary Material. k_{f01}, k_{f11}, k_{f21}, k_{f31}, k_{f02}, k_{f12}, k_{f22}, k_{f32}, k_{f42}, k_{f13}, k_{f23}, k_{f33}, and k_{f43} are the association rate constants; k_{b01}, k_{b11}, k_{b21}, k_{b31}, k_{b02}, k_{b12}, k_{b22}, k_{b32}, k_{b42}, k_{b13}, k_{b23}, k_{b33}, and k_{b43} are the dissociation rate constants.

hormone here, we assumed that L acts only as a pure agonist, thus by our definition, recruiting no CoR to the local promoter. Exogenous ligand X follows a similar signaling process as the endogenous ligand L. However, X may function as either a pure agonist by recruiting CoA, a partial agonist by recruiting both CoA and CoR, or a passive or active antagonist. When both endogenous ligand L and exogenous ligand X are present, it is also possible that L-bound receptor LR may interact with X-bound receptor XR to form mixed-ligand heterodimers (LRRX). Similar to XRRX, LRRX may regulate gene transcription differentially, depending on whether CoA, CoR, or both are recruited. Although Fig. 1 indicates that recruitment of CoA or CoR occurs after a receptor dimer binds to HRE, the dimer may also interact with CoA or CoR directly prior to occupying HRE (Thenot *et al.* 1999, Margeat *et al.* 2001). We found that the inclusion of these DNA-independent interactions between receptor dimers and coregulators did not qualitatively change the simulation results obtained in the absence of these interactions, except for the circumstance of receptor overexpression, in which excessive receptors may serve to scavenge the free coregulators resulting in repression of gene expression at high doses of X. This auto-inhibitory effect of receptor overexpression has been observed *in vitro* with ERs (Bocquel *et al.* 1989, Webb *et al.* 1992). Therefore, the present study presents only the results considering DNA-dependent recruitment.

The control of gene activation at the promoter was modeled based on the current understanding of gene induction in eukaryotic cells (Zhang *et al.* 2006). At any given time, a gene could be in one of two discrete transcriptional states, inactive ($GENE_i$) or active ($GENE_a$), corresponding to compact and relaxed chromatin structures respectively. Once in the active state, gene transcription proceeds at a relatively constant rate, whereas in the inactive state, no transcription occurs. Transitions between the inactive and active states are controlled by rate constant k_{f5} and k_{b5} as indicated below:

$$\mathrm{GENE_i} \underset{k_{b5}}{\overset{k_{f5}}{\rightleftarrows}} \mathrm{GENE_a}$$

where

$$k_{f5} = k_{f51}[\mathrm{CoALRRLH}] + k_{f52}[\mathrm{CoAXRRXH}] + k_{f53}[\mathrm{CoALRRXH}], \quad (1)$$

$$k_{b5} = k_{b51} + k_{b52}[\mathrm{CoRXRRXH}] + k_{b53}[\mathrm{CoRLRRXH}]. \quad (2)$$

Notably, the transition from the inactive to active state is regulated by coactivator-bound receptor–DNA complexes CoALRRLH, CoAXRRXH, and CoALRRXH (Eq. (1)). These complexes would work to relax local chromatin structures by acting as or recruiting histone acetyltransferase, a process not explicitly modeled. Conversely, transition from the active to inactive state is regulated by corepressor-bound receptor–DNA complexes CoRXRRXH and CoRLRRXH (Eq. (2)),

which presumably convert relaxed chromatin into a compact structure by acting as or recruiting histone deacetylase. The term k_{b51} serves as a constitutive repressor activity, which turns off gene transcription in the absence of ligand-induced corepressor recruitment. Once in the active state, the gene transcribes primary transcripts (PTs). PTs are processed to become mature mRNAs, followed by protein translation. By modeling the process of gene regulation with these steps, we were able to incorporate both positive and negative transcriptional controls in a mechanistically more accurate manner, rather than relying on empirical equations.

Model parameters

Ordinary differential equations (ODEs) and parameter values are listed in the Supplementary Material (Tables S1 and S2), including references and rationale for the choice of parameter values. For direct comparison, the default parameter values for exogenous ligand X-initiated processes and mixed-ligand heterodimer-initiated processes were set the same as for endogenous ligand L. But these parameters were varied systemically in the present study to investigate their effects on dose–response curves. Since we are interested only in the steady-state dose–response behavior, only the forward association rate constants of reversible reactions were varied to investigate the effects of these processes.

Modeling tools

The computational model was first constructed and parameterized in PathwayLab (InNetics Inc., Linköping, Sweden) and then exported into MatLab (The Mathworks, Inc., Natick, MA, USA). Dose–response curves were obtained by running the model to steady state in MatLab. The model in the Systems Biology Markup Language (SBML) and MatLab format is provided as additional supplementary materials.

Results

Since exogenous ligand X can be either a pure agonist, an antagonist, or a partial agonist, we explored the steady-state dose–response relationship between gene expression and X for each of the three possibilities in this order. Moreover, with each possibility, we also considered situations in which mixed-ligand heterodimers either do not form at all, act as an activator, or as a repressor.

Exogenous ligand X as a pure agonist

In the absence of mixed-ligand heterodimer LRRX

Contrary to the intuition that an agonistic exogenous ligand X would add to the basal gene expression sustained by endogenous ligand L, simulations surprisingly revealed that X, acting on top of L, exhibits non-monotonic U-shaped dose–response curves (Fig. 2, left panels). X at relatively low doses first depresses the basal gene expression, and after reaching a minimum expression, the steady-state protein level reverses the downtrend as the dose of X continues to increase and finally reaches a saturated phase. This U-shaped profile was preserved in most of the conditions where parameter values associated with the signaling events were varied (see details below). To quantify the U-shape, we regard the magnitude of U-shape as the difference between the expression level at the nadir and the lesser of the basal and saturated expression levels. Conversely, the magnitude of inverted U-shape is the difference between the expression level at the peak and the greater of the basal and saturated expression levels. For continuity, the difference in positive values denotes a U-shaped response and negative values an inverted U-shaped response.

Since the physiological level of an endogenous steroid hormone (represented by L), such as testosterone and estrogen, varies between individuals or fluctuates through various physiological states, we investigated the dose–response curve for exogenous ligand X in the presence of different L levels. As indicated in Fig. 2A, with higher L levels, the nadir of the U-shaped dose–response curve shifts progressively to the right, indicating increasing difficulty for X to initially repress gene expression activated by L. However, with higher L levels, the magnitude of U-shape becomes more prominent, although it eventually levels off (Fig. 2A, right panel). Exogenous ligands often differ in their binding affinity towards target SHRs. By varying k_{f02}, the association rate constant between X and SHR, our simulation demonstrated that an increase in the binding affinity merely results in a parallel, leftward shift of the dose–response curve without affecting the magnitude of U-shape (Fig. 2B). Another important variable is the intracellular concentration of SHRs, which may be at different levels among different cell types and tissues (Kuiper *et al.* 1997) or at different developmental and physiological stages, thus affecting cellular responses to endogenous hormones and exogenous ligands. By increasing the initial abundance of SHR in the model, we found that the dose–response curve generally shifts upward, with the dose of X associated with the expression nadir remaining largely unchanged (Fig. 2C). Increasing the abundance of SHR initially enhances the magnitude of

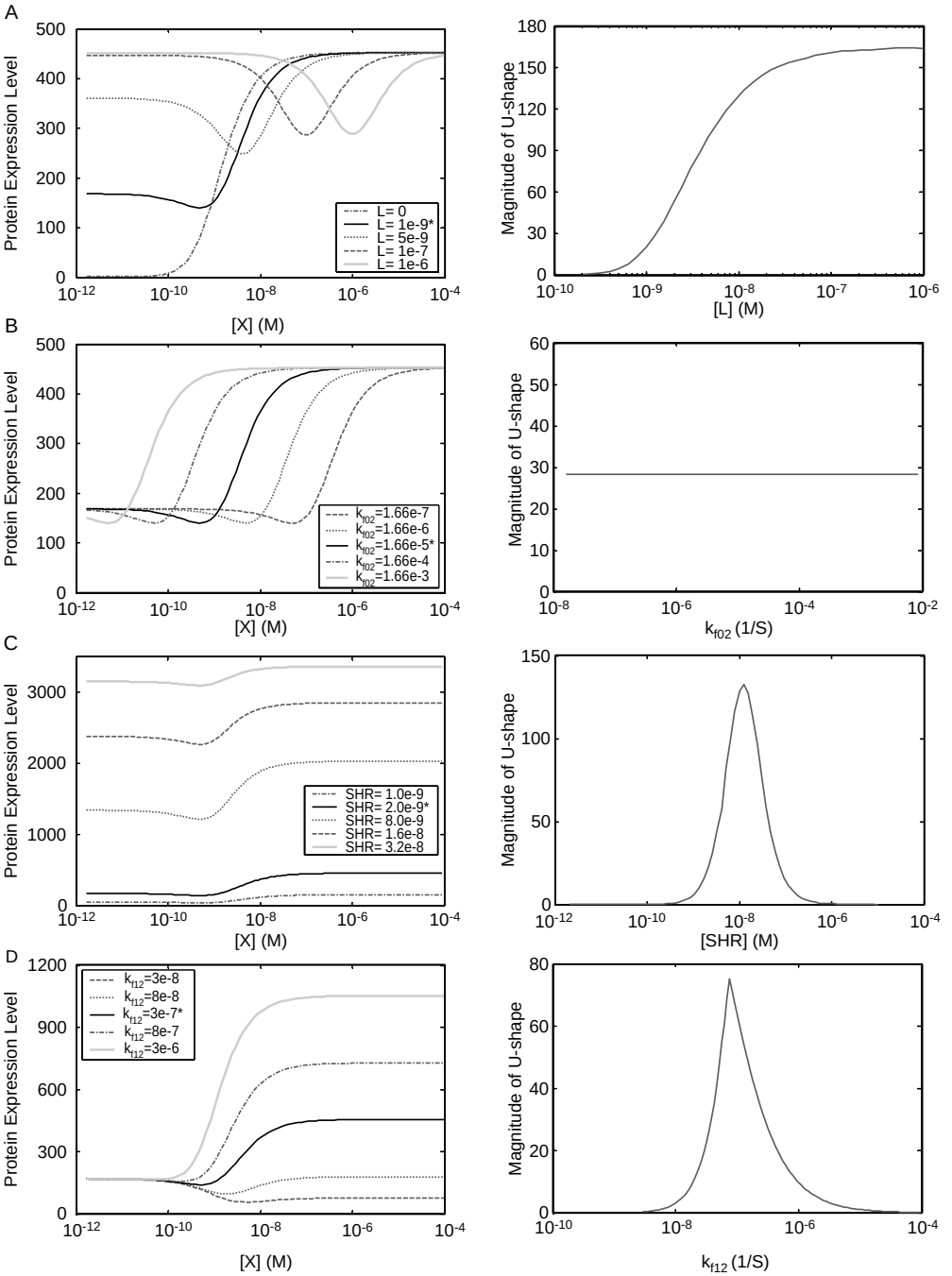

Figure 2 U-shaped steady-state dose–response in gene expression and magnitude of U-shape when exogenous ligand X is a pure agonist and the heterodimer LRRX is absent. (A) Effect of the level of endogenous ligand L. (B) Effect of the binding affinity between X and SHR (implemented by varying the association rate constant k_{f02}). (C) Effect of SHR concentrations. (D) Effect of the binding affinity between XRs to form homodimer XRRX (implemented by varying the association rate constant k_{f12}). Note: parameter values marked by asterisk are default settings (same denotation for other figures).

U-shape, which then diminishes as SHR increases further. Next, we examined how the ability of receptor monomer XR to form homodimer XRRX affects the dose–response curve. With a low association rate constant (k_{f12}) for homodimerization, exogenous ligand X behaves almost as a pure antagonist (Fig. 2D). In contrast, with a high k_{f12} value, X produces a complete agonistic effect. With intermediate k_{f12} values, however, the dose–response curve remains U-shaped. Similar to the effect of varying SHR abundance, the magnitude of U-shape also has a biphasic appearance (Fig. 2D, right panel). Lastly, we investigated how the ability of receptor dimer XRRX to bind HRE (k_{f22}), XRRXH to recruit CoA (k_{f32}), and CoAXRRXH to activate $GENE_i$-to-$GENE_a$ transition (k_{f52}), affects the shape of the dose–response curve. Variations in these parameters give rise to similar curvature changes as varying k_{f12} (results not shown).

With the above analyses, it appears that the U-shaped profile of dose–response persists in most of the situations explored. Varying parameter values at different stages of the signaling pathway seems to affect, in most cases, only the magnitude and/or position of the U-shape, rather than completely eradicate it. To identify the origin of the U-shaped dose–response, we then focused on the step of homodimerization between receptor monomers. This is an inherently nonlinear process, with a quadratic term describing the forward association rate, as indicated in Eqs (3) and (4),

$$\text{fflux}_{11} = k_{f11}[\text{LR}]^2, \tag{3}$$

$$\text{fflux}_{12} = k_{f12}[\text{XR}]^2. \tag{4}$$

Linearizing the dimerization processes by converting Eqs (3) and (4) into (5) and (6) respectively

$$\text{fflux}_{11} = k'_{f11}[\text{LR}], \tag{5}$$

$$\text{fflux}_{12} = k'_{f12}[\text{XR}] \tag{6}$$

(where k'_{f11} and k'_{f12} were set to maintain the same basal gene expression level), we found that the U-shape was completely eliminated, and under no circumstances did it recur by varying parameter values in any of the signaling steps (Fig. 3). These results indicated that the U-shaped response must originate from receptor homodimerization, and it may be understood as follows. When X is competing against L for SHR at a low dose, the loss of homodimer LRRL from this competition cannot be fully compensated by newly formed XRRX due to the nonlinearity inherent in homodimerization. This inability to replenish lost LRRL with XRRX results in an initial depression in gene expression. At a higher dose of X, more XRRX will be formed, which is eventually high enough to compensate for all the losses of LRRL, thus reversing the downtrend in gene expression.

In the presence of mixed-ligand heterodimer LRRX

In this case, we considered situations in which LRRX acts as either a transcriptional activator by recruiting CoA, an active repressor by recruiting CoR, an passive repressor by not binding to HRE or recruiting any coregulators, or a partial activator by recruiting both CoA and CoR, though not simultaneously.

LRRX as an activator. Compared with the situation devoid of heterodimer formation (i.e. $k_{f13}=0$), emergence of LRRX as a transcriptional activator attenuates the magnitude of U-shape. As k_{f13}, the association rate constant between LR and XR, increases, more LRRX heterodimers are formed to activate gene expression, pushing the nadir of the U-shaped dose–response curve upward and thereby reducing the magnitude of U-shape (Fig. 4A, top panel). When k_{f13} reaches a value comparable to the equivalent parameters in the homodimerization processes (i.e. k_{f11} and k_{f12}), the U-shape essentially disappears and the response only increases monotonically. The dose–response curve remains monotonic within a range of k_{f13}, as indicated by the extended horizontal line at zero in Fig. 4A (middle panel). As k_{f13} increases further, the dose–response curve leaves the monotonic bounds and appears non-monotonic again. Instead of a U-shape, an inverted U-shape emerges in this case, and its magnitude, as represented by negative values, increases sharply with small increments of k_{f13}. Notably, the overall shape of the dose–response curve is the sum of contributions from both homodimers LRRL and XRRX, and the heterodimer LRRX (Fig. 4A bottom panel). At a high k_{f13} value, an inverted U-shaped dose–response curve results because LRRX, which itself is inverted U-shaped in appearance, has a dominant influence. Varying the association rate constant for LRRXH to recruit CoA (k_{f33}) has a modulatory effect similar to k_{f13} on the steady-state dose–response (Fig. 4B). However, when k_{f33} is too low, the magnitude of the U-shape has a tendency to increase as LRRX essentially degenerates to a passive repressor. Variations in the association rate constant for LRRX to bind HRE (k_{f23}) and for CoALRRXH to activate $GENE_i$-to-$GENE_a$ transition (k_{f53}) have an effect similar to varying k_{f33} (results not shown). Overall, the inverted U-shaped curve originates from the formation of LRRX, which play a dominant role in gene expression when they are in high abundance or are highly transcriptionally active.

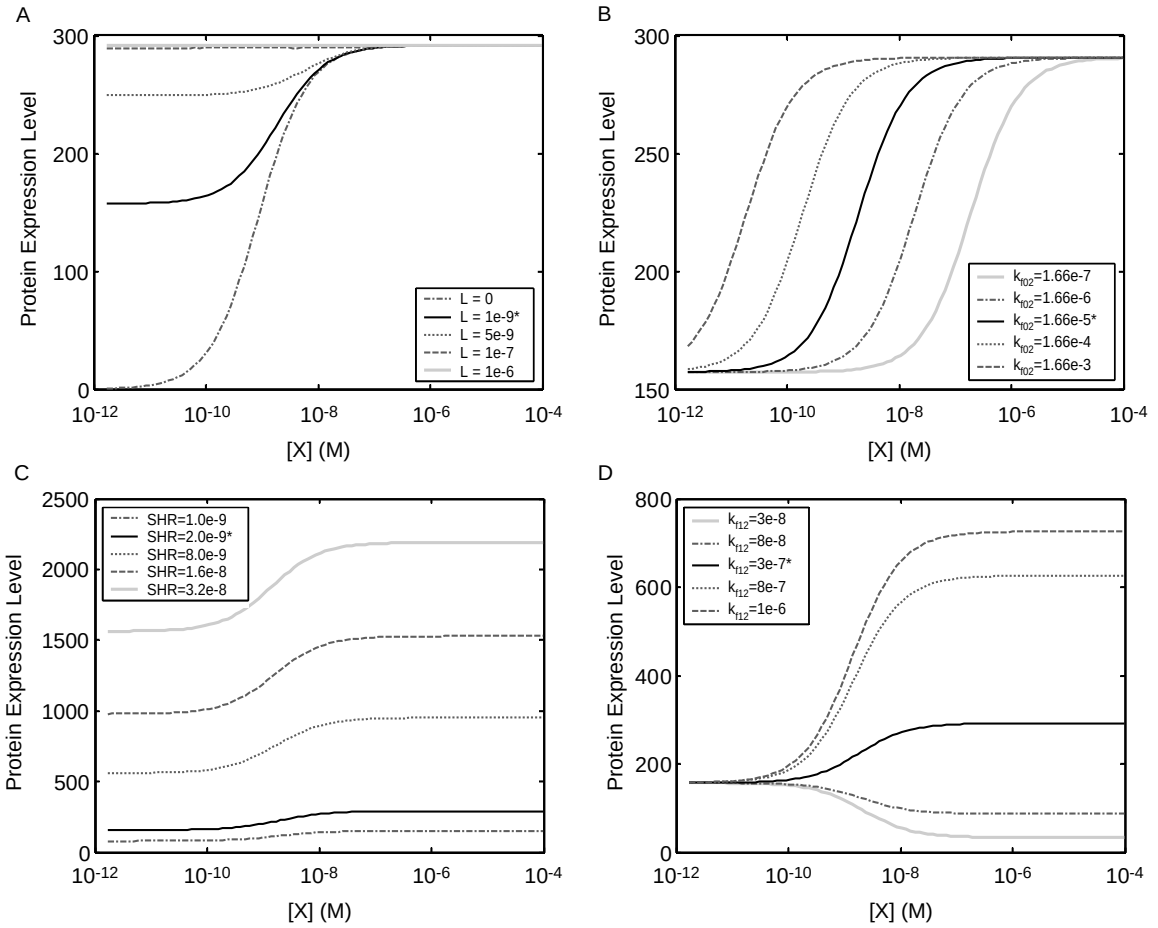

Figure 3 Linearization of the homodimerization process eliminates the U-shaped dose–response when exogenous ligand X is a pure agonist and the heterodimer LRRX is absent. Refer to Eqs (5) and (6) for method of linearization. (A) Effect of the level of endogenous ligand L. (B) Effect of the binding affinity between X and SHR (implemented by varying the association rate constant k_{f02}). (C) Effect of SHR concentrations. (D) Effect of the binding affinity between XRs to form homodimer XRRX (implemented by varying the association rate constant k_{f12}).

LRRX as a repressor. If the mixed-ligand heterodimer LRRX does not recruit CoA, it functions as a transcriptional repressor, either active or passive, depending on whether CoR can be recruited. If LRRX does not bind to the response element HRE or, after binding, does not recruit CoR, then LRRX would behave as a passive repressor by making LR and XR less available for formation of homodimers, or by making HRE less available for homodimers which activate gene expression. If LRRX recruits CoR after binding to HRE, then LRRX acts as an active repressor by promoting transition from $GENE_a$ to $GENE_i$. Simulations revealed that regardless of being passive or active, the existence of LRRX as a repressor further deepens the U-shaped dose–response curve observed in the absence of LRRX formation (Fig. 5). When LRRX is mimicked as a passive repressor, which cannot bind to HRE (i.e. $k_{f23}=0$), increasing k_{f13}, the association rate constant between LR and XR, progressively enhances the magnitude of the U-shape, with the nadir of the U-shape eventually dropping to zero expression level (Fig. 5A). Similarly, mimicking LRRX as a passive repressor that can bind to HRE but unable to recruit CoR (i.e. $k_{f43}=0$) also produces a U-shape-deepening effect (results not shown). When LRRX acts as an active repressor, increasing k_{f43} (which represents the ability of LRRXH to recruit CoR) augments the magnitude of the U-shape, as well (Fig. 5B).

LRRX as a partial activator. When LRRX is able to recruit both CoA and CoR, thus acting as a partial activator by itself, its effect on regulating the direction of gene expression in the presence of endogenous ligand L depends on the relative strength of activation and repression. If LRRX recruits CoA more strongly than CoR, the shape of dose–response curves will range from blunted U-shape to inverted U-shape (results not

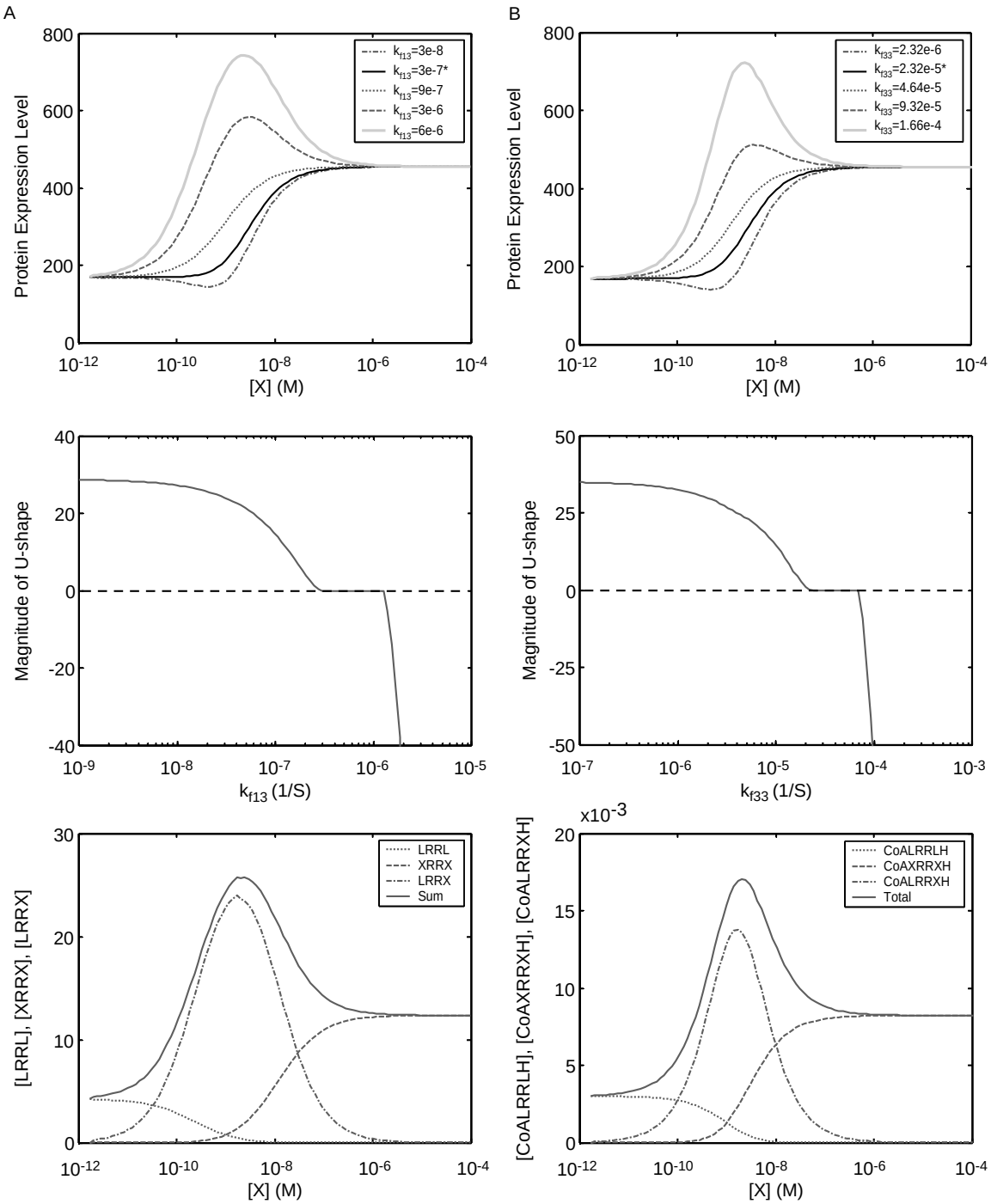

Figure 4 Mixed-ligand heterodimer LRRX acting as an activator reduces and inverts the U-shaped dose–response when exogenous ligand X is a pure agonist. (A) Effect of the binding affinity between LR and XR to form heterodimer LRRX (implemented by varying the association rate constant k_{f13}). Results in the bottom panel were obtained with k_{f13} set at 3×10^{-6}. (B) Effect of the ability of LRRX to recruit CoA (implemented by varying the association rate constant k_{f33}). Results in the bottom panel were obtained with k_{f33} set at 2.32×10^{-4}.

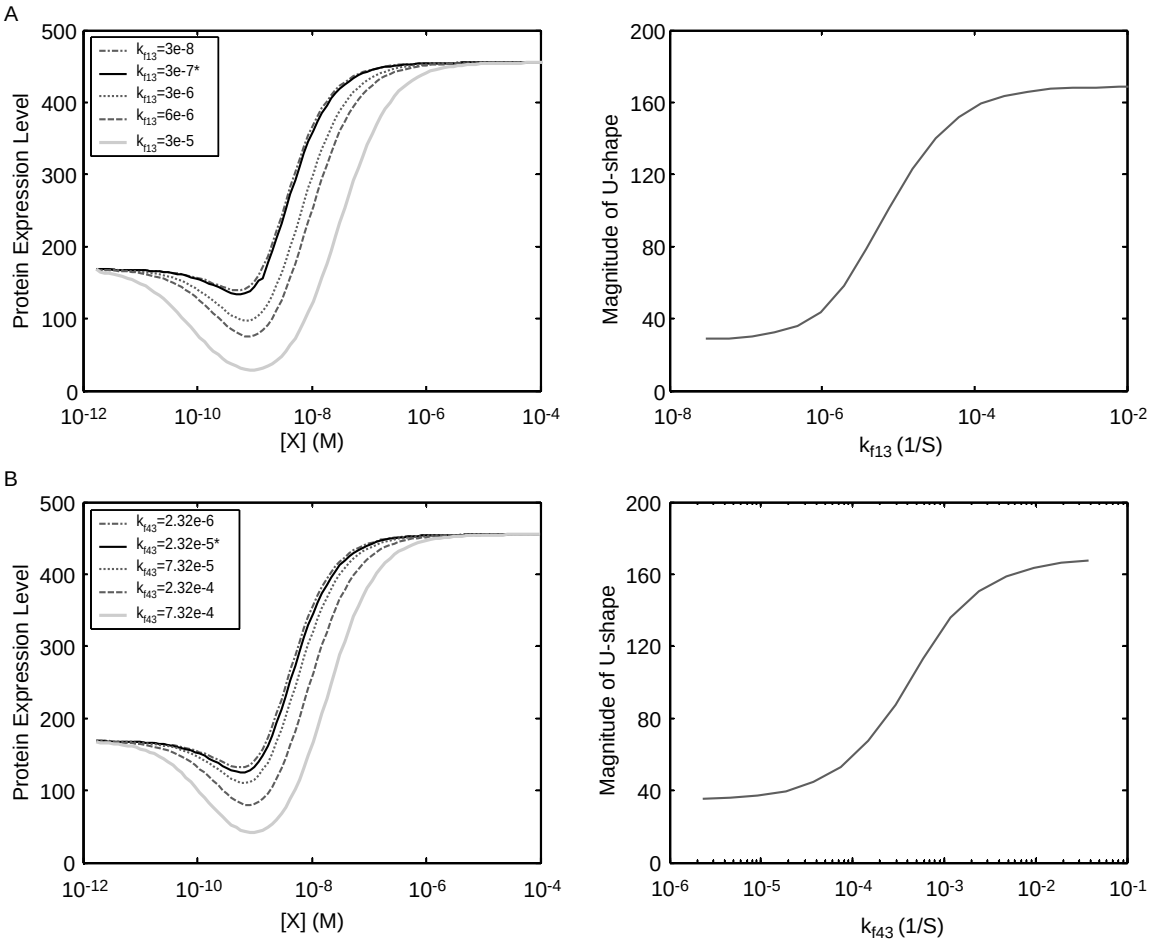

Figure 5 Mixed-ligand heterodimer LRRX acting as a repressor deepens the U-shaped dose–response when exogenous ligand X is a pure agonist. (A) Effect of the binding affinity between LR and XR to form heterodimer LRRX (implemented by varying the association rate constant k_{f13}). In this case, LRRX was simulated as a passive repressor by setting k_{f23} to zero. (B) Effect of the ability of LRRX to recruit CoR (implemented by varying the association rate constant k_{f43}).

shown), similar to the effect of LRRX acting as a pure activator. Conversely, if LRRX recruits CoR more strongly than CoA, a deepened U-shaped dose–response curve arises (results not shown), similar to the effect of LRRX acting as a repressor.

Exogenous ligand X as an antagonist

In the absence of mixed-ligand heterodimer LRRX

As an antagonist, X may repress gene expression either passively or actively. With passive repression, X competes against L for receptors or response elements; with active repression, X recruits CoR to promote deactivation of actively transcribing genes. Simulations revealed that as an antagonist, regardless of being passive or active, X produces monotonically decreasing responses in gene expression (Fig. 6). Increasing the binding affinity between X and SHR by adjusting k_{f02} shifts the dose–response curve to the left in parallel (Fig. 6A). In comparison, increasing the association rate constants in downstream steps (i.e. k_{f12}, k_{f22}, k_{f42}) not only shifts the dose–response curve to the left, but also steepens the monotonically decreasing slope (Fig. 6B–D). In no circumstances was a non-monotonic dose–response curve, either U- or inverted U-shaped, observed.

In the presence of mixed-ligand heterodimer LRRX

LRRX as an activator. By acting as an activator, LRRX alleviates the antagonistic action of X (Fig. 7). Increasing k_{f13}, the association rate constant between LR and XR, initially shifts the monotonically decreasing dose–response curve to the right without changing its monotonic nature (Fig. 7A). As k_{f13} increases further, the activity of LRRX starts to dominate the shape of the dose–response curve, resulting in an inverted U-shape,

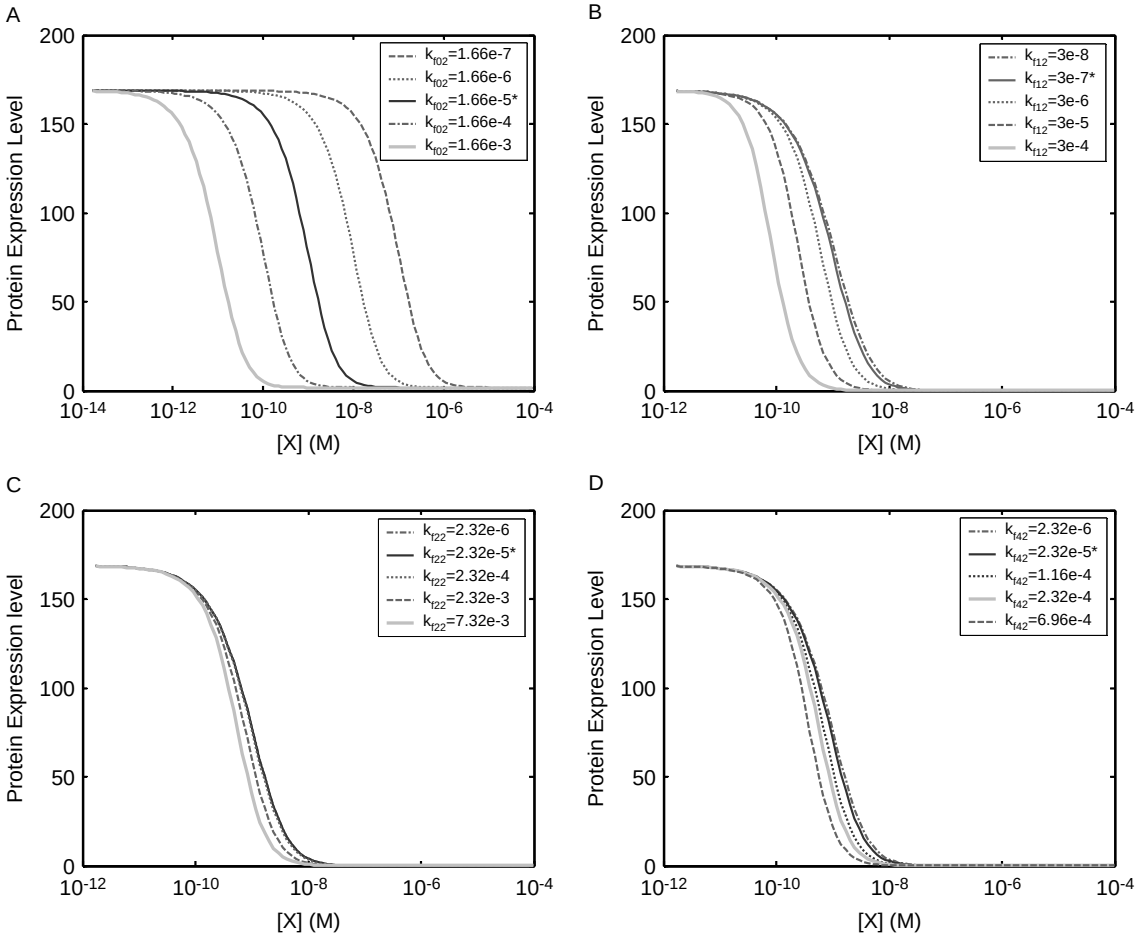

Figure 6 Monotonically decreasing dose–response in gene expression when exogenous ligand X is a pure antagonist and the heterodimer LRRX is absent. (A) Effect of the binding affinity between X and SHR (implemented by varying the association rate constant k_{f02}). In this case, X was simulated as a passive antagonist by setting k_{f12} to zero. (B) Effect of the binding affinity between XRs to form homodimer XRRX (implemented by varying the association rate constant k_{f12}). In this case, X was simulated as a passive antagonist by setting k_{f22} to zero. (C) Effect of the binding affinity between XRRX and HRE (implemented by varying the association rate constant k_{f22}). In this case, X was simulated as a passive antagonist by setting k_{f42} to zero. (D) Effect of the ability of XRRX to recruit CoR (implemented by varying the association rate constant k_{f42}).

the magnitude of which (in negative values) increases sharply for small increment of k_{f13}. Similar to the effect of varying k_{f13}, emergence of the inverted U-shape was also obtained by varying the following constants: k_{f23}, the association rate constant between LRRX and HRE (results not shown); k_{f33}, the association rate constant for LRRXH to recruit CoA (Fig. 7B); and k_{f53}, the constant for CoALRRXH to activate $GENE_i$-to-$GENE_a$ transition (results not shown). In all the cases, high doses of X eventually repress the gene expression completely after an expression peak. This behavior is in contrast to the situation when X is a pure agonist, which induces high expression at high doses instead of depressing it to zero (Fig. 7 versus Fig. 4).

LRRX as a repressor. By acting as a repressor, either passive or active, LRRX further strengthens the antagonistic action of X (Fig. 8). Increasing k_{f13}, k_{f23} (results not shown), k_{f43}, and k_{b53} (results not shown), which enhances LRRX as a repressor at different signaling stages, results in leftward shifting of the monotonically decreasing dose–response curve. But unlike the case where X functions as an antagonist in the absence of heterodimer LRRX, the slope of the dose–response curves tends to decrease in steepness as they shift to the left (Fig. 8 versus Fig. 6).

LRRX as a partial activator. When LRRX is able to recruit both CoA and CoR, thus acting as a partial activator by itself, its effect on regulating the direction of gene expression depends on the relative strength of activation and repression. If LRRX recruits CoA more strongly than CoR, the shape of dose–response curves

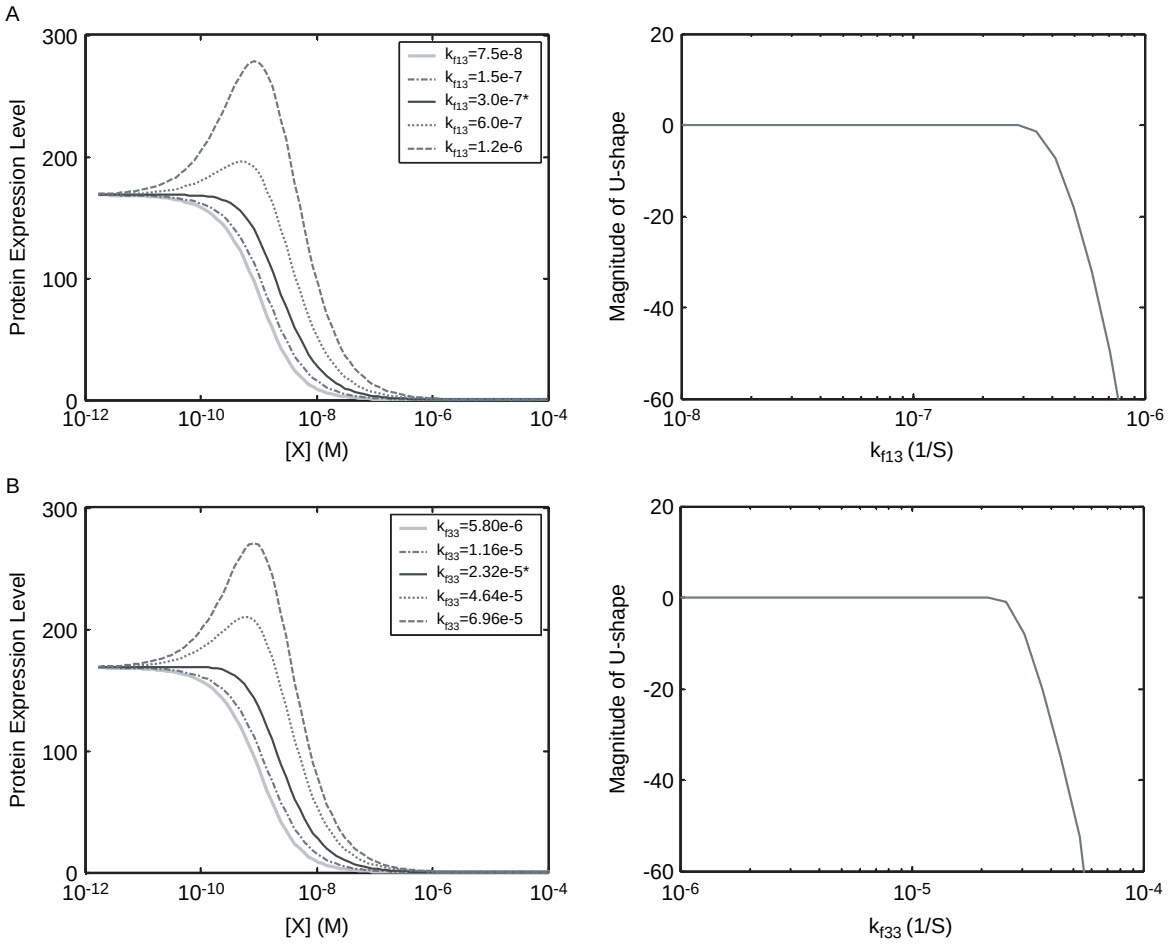

Figure 7 Mixed-ligand heterodimer LRRX acting as an activator shifts rightward and converts the monotonically decreasing dose–response to inverted U-shape, when exogenous ligand X is a pure antagonist. (A) Effect of the binding affinity between LR and XR to form heterodimer LRRX (implemented by varying the association rate constant k_{f13}). (B) Effect of the ability of LRRX to recruit CoA (implemented by varying the association rate constant k_{f33}).

will range from monotonically decreasing to inverted U-shaped (results not shown), similar to the effect of LRRX acting as a pure activator. Conversely, if LRRX recruits CoR more strongly than CoA, only a monotonically decreasing dose–response curve can be observed (results not shown), similar to the effect of LRRX acting as a repressor.

Exogenous ligand X as a partial agonist

If exogenous ligand X is a partial agonist, then by our definition both CoA and CoR can be recruited, albeit not simultaneously, by XRRX occupying the response element HRE. A series of simulations revealed that when heterodimer LRRX is absent or acts as a repressor, U-shaped dose–response curves can arise (Fig. 9A and B). When LRRX acts as a pure or partial activator, both U-shaped and inverted U-shaped responses can be observed, depending on its abundance and strength of activity (Fig. 9C and D). The ratio between intracellular CoR and CoA has been proposed to explain the differential effects in different target tissues of many exogenous steroid mimics acting through SHRs (Smith *et al.* 1997, Smith & O'Malley 2004). Our simulation indicated that the CoR/CoA ratio does indeed affect the dose–response behavior in a very sensitive manner (Fig. 9). An increase in CoR/CoA ratio tends to render the action of X completely antagonistic, whereas a decrease in the ratio makes X behave more like an agonist. Therefore, the relative abundance of CoR and CoA is a key modulator of SHR-mediated gene expression.

In summary, we have simulated the steady-state gene expression in an endogenous hormonal background with varying assumptions about the exogenous

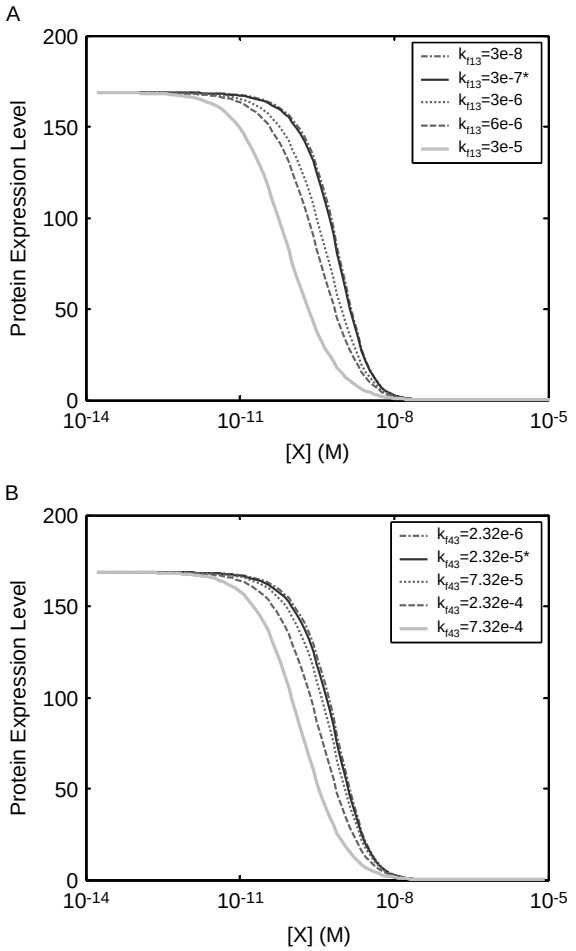

Figure 8 Mixed-ligand heterodimer LRRX acting as a repressor shifts leftward the monotonically decreasing dose–response when exogenous ligand X is a pure antagonist. (A) Effect of the binding affinity between LR and XR to form heterodimer LRRX (implemented by varying the association rate constant k_{f13}). In this case, LRRX was simulated as a passive repressor by setting k_{f23} to zero. (B) Effect of the ability of LRRX to recruit CoR (implemented by varying the association rate constant k_{f43}).

steroid ligand. The shape of dose–response curves can vary from monotonically increasing or decreasing to non-monotonically U-shaped or inverted U-shaped, depending on the transcriptional nature of the exogenous ligand and the intermediate complexes formed with the endogenous ligand. Conditions under which non-monotonic dose–responses could occur are summarized in Table 1. U-shaped dose–response curves may arise when exogenous ligand X is either a pure or partial agonist, regardless of the presence of mixed-ligand heterodimer LRRX; while inverted U-shape arises only when LRRX functions as a pure or partial activator, regardless of whether X by itself is an agonist or antagonist.

Discussion

The biological responses invoked by an exogenous chemical may be non-monotonic (Calabrese & Baldwin 2001*b*, 2003). With respect to SHR-mediated action, a variety of mechanisms have been proposed to explain the non-monotonic effects of certain EACs and the bidirectional effects of SRMs in different target tissues (Smith *et al.* 1997, McInerney *et al.* 1998, Kohn & Melnick 2002, Conolly & Lutz 2004, Smith & O'Malley 2004). Using numerical simulation of a relatively standard model of steroid hormone action, the present study demonstrated that non-monotonic steady-state response may be a property intrinsic to SHR-mediated gene expression under a variety of conditions.

An essential step in the genomic action of steroid hormones is the dimerization of liganded receptor monomers (Kumar & Chambon 1988, Wrange *et al.* 1989). The requirement for SHRs to function as a dimer lies at least in the fact that a steroid HRE invariably comprises two half-sites of either direct or inverted repeats, and each monomer can only recognize one half-site weakly (Nordeen *et al.* 1990, Langley *et al.* 1995, Kuntz & Shapiro 1997). Therefore, SHRs need to function as a dimer, with each monomer binding to one half-site to gain enough overall affinity for the promoter (Kuntz & Shapiro 1997). Homodimerization of liganded receptors is a nonlinear process because of the quadratic term defining the association between receptor monomers (Eqs (3) and (4)). In the absence of mixed-ligand heterodimer formation and at low doses of exogenous ligand X, newly formed XRRX cannot completely compensate for the loss of LRRL from receptor competition. As a result, even if X is a pure or partial agonist with enough activity, X would initially reduce gene expression from the basal level instead of adding to it. At higher doses, the amount/activity of XRRX formed is able to completely replace and surpass lost LRRL, thereby reversing the downtrend in gene expression and producing a U-shaped dose–response curve. The essentiality of homodimerization to the occurrence of U-shape was demonstrated by linearization of this process, which produced monotonic dose–responses in all circumstances. Although in our model receptor monomers form dimers prior to binding to HRE, a similar nonlinear response would also be expected if monomers were able to bind HRE sequentially and in a positively cooperative manner, forming the homodimer on the promoter.

As illustrated in Fig. 2A, the magnitude of the U-shape is positively correlated with endogenous hormone levels. The U-shape becomes less pronounced or would be too subtle to be identified if the endogenous hormone is at levels below its K_d for the receptors (K_d is 1 nM in the model). Given that endogenous hormones are usually

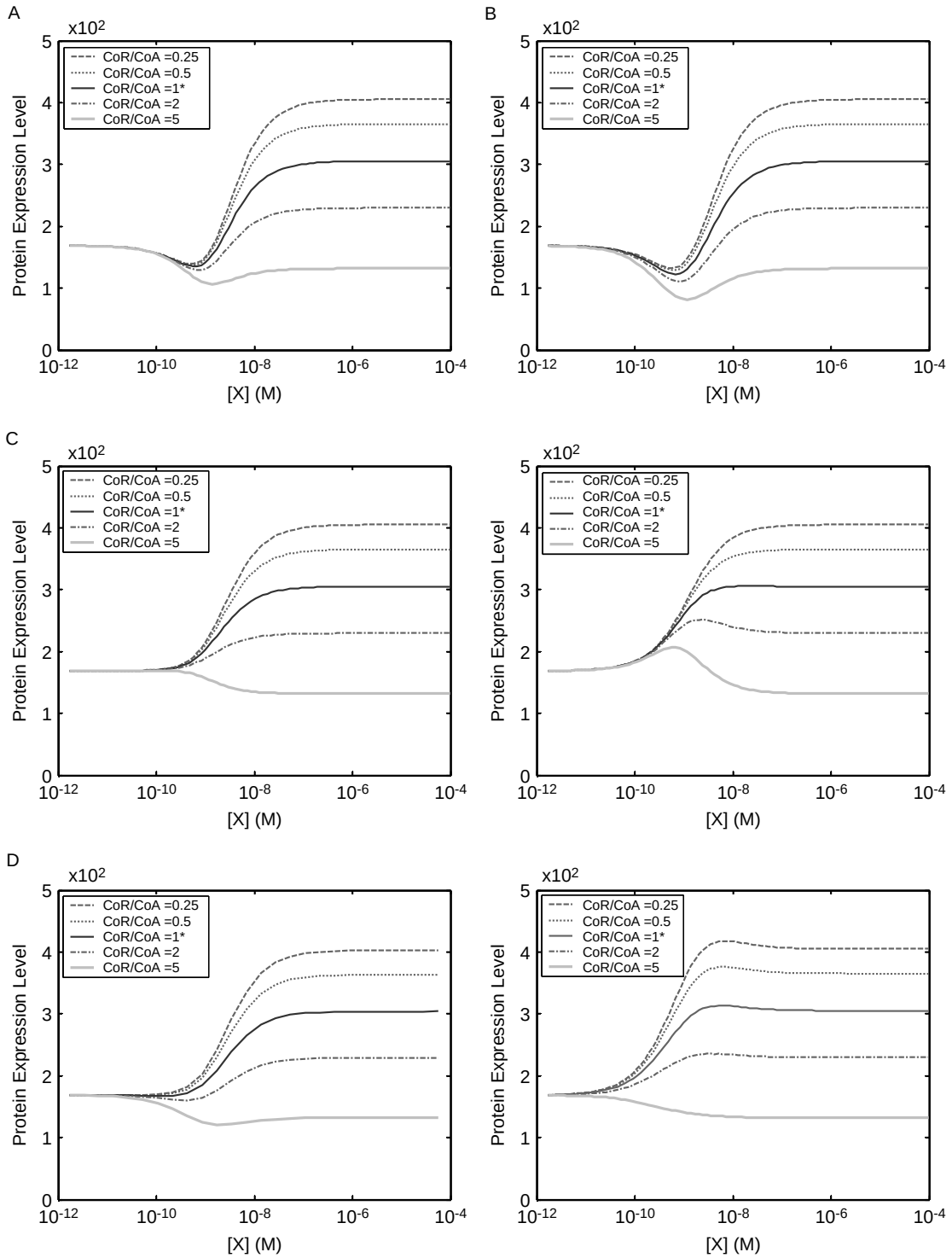

Figure 9 Effect of the CoR/CoA ratio on dose–responses when exogenous ligand X is a partial agonist. To achieve different CoR/CoA ratios while keeping the basal expression level sustained by L unchanged, only the initial concentration of CoR was varied. (A) In the absence of heterodimer LRRX. (B) LRRX acting as an active repressor. (C) LRRX acting as a pure activator. In this case, k_{f13} was set to 50% of the default value in the left panel, and twofold in the right panel. (D) LRRX acting as a partial activator. In this case, k_{f13} was set to 50% of the default value in the left panel and fourfold in the right panel.

Table 1 Conditions in which U- or inverted U-shaped dose–response curves may appear

		LRRX		
		Absent	Pure/ partial activator	Repressor
X	Pure/partial agonist	U	U Inverted U	U
	Antagonist	None	Inverted U	None

at relatively low physiological levels, this may explain, at least in part, why SHR-mediated dose–responses are observed more often as monotonic rather than as U-shaped. Importantly, our results further showed that formation of mixed-ligand heterodimer LRRX can modulate the magnitude of U-shape observed with agonistic X. As the activity of LRRX becomes more influential, the overall shape of the dose–response curve for gene expression is increasingly amenable to the profile of LRRX, which itself is inverted U-shaped (Fig. 4, bottom panels). If it is an activator, LRRX would first lessen the original U-shape of the dose–response curve, and then push it upward into an inverted U-shape; conversely, if LRRX is a repressor, it would further deepen the original U-shape. In comparison, when the exogenous ligand X is an antagonist, no U-shape is observed, and only an inverted U-shape can be obtained when LRRX functions as a strong activator. Despite their significant role discussed here, it remains to be investigated whether LRRX indeed exist in cells *in vitro* and *in vivo*. But apparently, if they cannot be formed at all, cells would have a tendency to exhibit U-shaped responses when the exogenous ligand is an agonist.

Non-monotonic dose–responses in steroid or nuclear receptor signaling have been previously investigated through numerical simulations (Kohn & Portier 1993, Kohn & Melnick 2002, Conolly & Lutz 2004). Kohn and Portier had proposed that positive cooperative binding between liganded receptor and DNA may result in a U-shaped dose–response curve (Kohn & Portier 1993). The origin of U-shape from the nonlinear dimerization process, as we noted in the present study, has a similar mathematical basis to their findings. Generation of U-shaped dose–responses with the latter mechanism relies, however, on the existence of more than one copy of the HRE in the promoter, which may not always be the case. With respect to the role of LRRX in U-shaped responses, Conolly and Lutz have relied on regarding them as transcriptionally inactive (Conolly & Lutz 2004) to explain the U-shaped response observed with AR agonist hydroxyflutamide (Maness *et al.* 1998). This transcriptionally inactive heterodimer is equivalent to the case of LRRX acting as a passive repressor in our model, which deepens the U-shape (Fig. 5A). With respect to inverted U-shape, Kohn & Melnick (2002) suggested that one condition for this to occur is when there are excessive unoccupied receptors and recruitment of CoA by xenobiotic ligands is weaker than by endogenous ligands. However, in their model, receptor dimerization was not considered. In comparison, occurrence of inverted U-shaped curves in our model relies on the formation of LRRX functioning as activators. Additionally, as noted in the Method, an inverted U-shape can also arise if SHRs exist in such a high abundance that the excessive receptors may titrate away free CoA, provided DNA-independent association between these two species is allowed. This auto-inhibitory phenomenon due to self-squelching has been demonstrated *in vitro* (Bocquel *et al.* 1989, Webb *et al.* 1992).

SRMs represent a group of compounds whose activity, i.e. agonistic or antagonistic, varies in a cellular and tissue context-dependent manner. For example, tamoxifen and raloxifene are selective ER modulators, which are antiestrogenic in the breast but estrogenic in the bone (Dutertre & Smith 2000, Francucci *et al.* 2005). Asoprisnil, a selective PR modulator, has an antiproliferative effect on primate endometrium, but cannot induce labor in animal models of pregnancy and parturition (Chwalisz *et al.* 2005). Current understanding of the molecular mechanism for tissue-selective action of SRMs hinges on the notion that SRMs can induce different conformational changes to their cognate SHRs, particularly in the C-terminal LBD, which in turn determine whether CoA or CoR will be recruited (Brzozowski *et al.* 1997, Shiau *et al.* 1998). Conformational changes and differential coregulator recruitment also appear to be regulated by the phosphorylation status of SHRs and coregulators (Atanaskova *et al.* 2002, Michalides *et al.* 2004). For SRMs that are capable of recruiting, to some degree, both CoA and CoR, the relative abundance between these two types of opposing coregulators is a key to the direction of their genomic actions (Smith *et al.* 1997, Smith & O'Malley 2004, Wang *et al.* 2004). In keeping with this concept, our simulation demonstrated that with a high CoR/CoA ratio, the activity of exogenous ligand X is primarily antagonistic, whereas with a low CoR/CoA ratio, the activity is primarily agonistic (Fig. 9). Moreover, the present study revealed that the differential effect of SRMs could result from factors other than the CoR/CoA ratio. For instance, an exogenous ligand may have different affinities for the same type of receptor in different cell types or tissues. This affinity difference may cause, in a certain dose range, antagonistic activity in cells with lower affinity (resulting from the U-shape) and agonistic activity in cells with higher affinity (Fig. 2B). In the presence of a mixed-ligand heterodimer, the ability of LXXR to recruit CoA or CoR, which may depend on the phosphorylation status of the receptor and coactivator

(Michalides *et al.* 2004), can as well explain tissue selectivity within a certain dose range (Figs 4B and 5B).

EACs represent a large set of environmental pollutants and naturally occurring chemicals, such as phytoestrogen that can interfere with the endocrine system. Many EACs act through SHRs to exert their endocrine disrupting effects (Amaral Mendes 2002, Markey *et al.* 2002). Establishing and understanding the dose–response curves of EACs of interest constitutes an integral part of toxicological research and health risk assessment for these chemicals. The responses at low doses are particularly relevant to human health and may contribute to the etiology of a variety of endocrine-related diseases (Dewailly *et al.* 1994, Lebel *et al.* 1998, Snedeker 2001). Of interest, some EACs display biphasic effects within large dose ranges (Kemppainen & Wilson 1996, vom Saal *et al.* 1997, Maness *et al.* 1998, Calabrese 2001*a*,*b*, Putz *et al.* 2001*a*,*b*, Almstrup *et al.* 2002, Terouanne *et al.* 2003, Kohlerova & Skarda 2004). In parallel to this, the concept of hormesis has increasingly gained advocacy in recent years as a dose–response scheme for xenobiotics (Calabrese & Baldwin 2001*a*, Calabrese 2005). A hormetic dose–response curve, either U- or inverted U-shaped, challenges the default linear model, which has been in practice for decades. A general explanation for hormetic phenomena has been that at low levels of disruption, biological systems can launch compensatory responses that may overcorrect the initially perturbed state, while at higher doses the system may reach its maximum capacity for compensation, thus it is unable to counteract the perturbations (Calabrese 2004). Although quite common, this mechanism may not be the only explanation that can account for hormetic dose–response curves (Conolly & Lutz 2004, Weltje *et al.* 2005). The present study provides an additional yet distinct mechanism that may operate to produce U- and inverted U-shaped dose–responses to steroid mimics. Although perturbation of gene expression studied here only represents one of the initial responses in a series of molecular events leading to the adverse effects of EACs, the potential non-monotonic responses suggest that linear extrapolation for the low-dose effect of certain EACs may not be appropriate to evaluate their biological risks.

Experimentally obtained SHR-mediated dose–responses usually describe the averaged behavior of a population of cells. The ODE-based deterministic model presented in the present study was also designed to examine averaged responses. However, it is important to note that gene expression in individual cells is expected to be stochastic and may fluctuate to a great extent even at steady state (Thattai & van Oudenaarden 2001, Elowitz *et al.* 2002, Ozbudak *et al.* 2002, Swain *et al.* 2002, Blake *et al.* 2003, Paulsson 2004, Raser & O'Shea 2004, 2005). Variations between cells in the abundance of receptors, coregulators, and other regulatory factors contribute further to the noise in gene expression as extrinsic sources. As a result, cells may respond differently to the same dose of an exogenous ligand. In certain cases, these heterogeneous responses may be critical to an individual cell's fate decision as whether to proliferate, differentiate, or undergo apoptosis. An important implication of this effect is in breast cancer treatment with antiestrogen. Even though the majority of the cancer cells may respond to antiestrogenic therapy well, the heterogeneity arising from noisy gene expression may render a small fraction of the cancer cells insensitive to antiestrogen. These surviving cells may be responsible, at least in part, for the possibility of relapse of the disease after the termination of antiestrogenic therapy. Clearly, if individual cell behavior is important, a stochastic simulation approach is preferred. Nevertheless, the frequency of these cellular incidents in a population of isogenic cells in response to varying doses of exogenous ligands is otherwise deterministic, and may still be usefully modeled with an ODE-based approach. Another scenario is concerned with SHR controlling the gene expression of a secretory peptide hormone. Although in this situation individual cells may synthesize the hormone at very different levels due to gene expression noise, the overall output from the cell population, which is more relevant to the fitness of the organism as a whole, should be deterministic with respect to the dose of the exogenous ligand.

In conclusion, the present computational study revealed a novel mechanism, likely inherent in SHR-mediated steroid signaling, to explain the non-monotonic dose–responses and bidirectional effects observed with many steroid mimics. Our results may contribute to the understanding of how SRMs work and improve risk assessment for EACs.

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