Selective Regulation of Gene Expression Using Rationally-Modified Retinoic Acid Receptors

John T. Koh,* Marc Putnam, Marjana Tomic-Canic,† and Christine M. McDaniel

Department of Chemistry and Biochemistry
University of Delaware, Newark, Delaware 19716

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Methods for remotely regulating gene transcription have become an extremely powerful tool in the study of gene function.[1−3] New methods for regulating gene expression by small bioavailable molecules hold great promise as approaches to regulating new gene therapies and for the precise control of eucaryotic bioreactors.[4,5] A necessity for such applications is the ability to selectively activate target genes without activating endogenous transcription pathways. In this work rational molecular design is used to re-engineer the human retinoic acid receptor gamma (RARγ) to respond to novel synthetic ligands.

The superfamily of nuclear hormone receptors (NHRs) represents a unique family of transcriptional regulators that possess both DNA binding and ligand-dependent transactivation functions that enable these receptors to single-handedly provide a transactivation signal to specific genes upon selective binding of small molecule ligands.[6−8]

The ligand binding domains (LBDs) of the NHRs are believed to have the same general structure and differ primarily in the residues that are involved in ligand recognition. This observation suggests that the LBD of NHRs might be modified to selectively provide a transactivation response from synthetic ligands that otherwise do not activate the "wild-type" (Wt) receptor. Indeed, mutations to the NHRs that altered ligand binding specificities have recently been reported.[9,10] The high-resolution crystal structures of *all-trans*-retinoic acid (tRA) complexed with RARγ provide an opportunity to use rational design to study molecular recognition and to provide insights into genetic diseases associated with mutations to the LBD of NHRs.[11,12]

Here, we report two new ligand−receptor pairs designed by modeling modified forms of both the ligand and the receptor using the cocrystal structure of the ligand binding domain of human RARγ and tRA.[13] We have identified two residues, Ser289 and Arg 278, that modify the physical size and the electrostatic environment within the ligand binding site (Figure 1). A series of potentially complementary ligands based on tRA that alter both the expected charge of the ligand and steric size have also been synthesized (Figure 2).

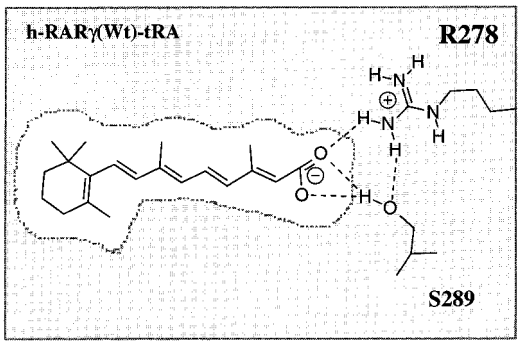

Figure 1. Representation of binding site residues altered to provide new ligand specificities.

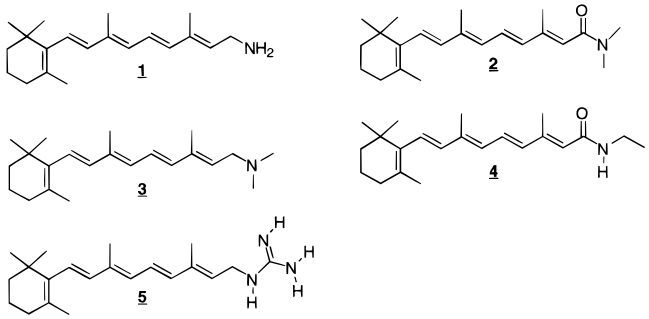

Figure 2. All-*trans*-retinoic acid and synthetic ligands.

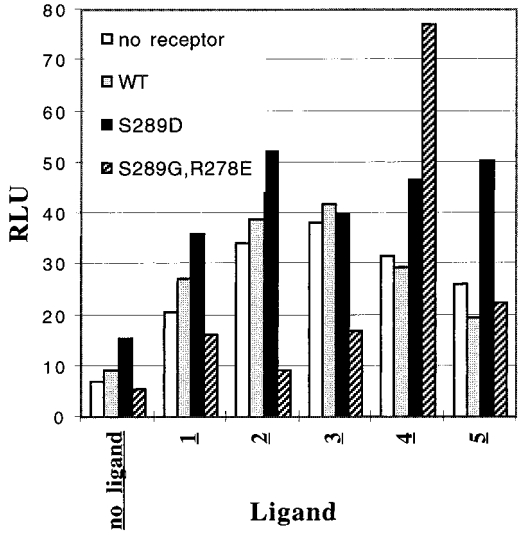

Figure 3. Transcriptional activation of modified receptors by synthetic ligands (1μM).

Molecular modeling suggested that modification of Ser289 → Gly, would provide additional room in the binding cavity that could then accommodate a sterically larger ligand such as **4**, the *N*-ethyl amide of retinoic acid. The substitution of Arg278 → Glu was expected to further enhance the binding of this neutral receptor to this otherwise highly basic receptor site.

Whereas the known NHRs have been shown to bind either neutral or acidic ligands,[8] the modified receptor RARγ(S289D) was envisioned to uniquely respond to cationic ligands such as **3**

† Department of Dermatology, N.Y.U. Medical Center, New York, NY 10016.

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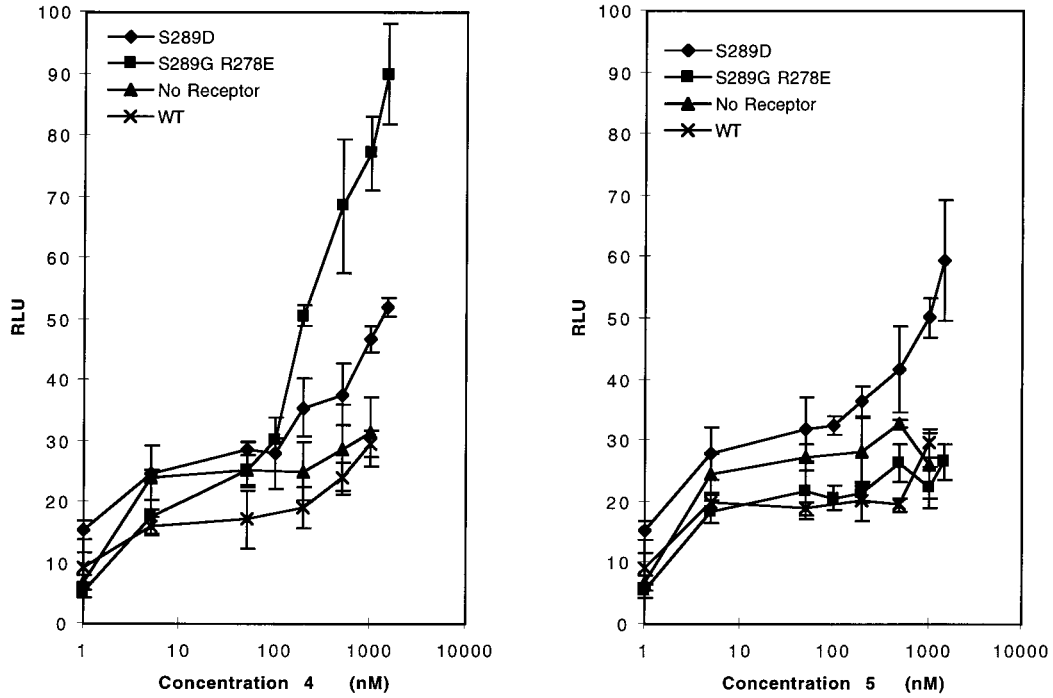

Figure 4. Transactivation response toward (a) ligand **4** and (b) ligand **5** by *HeLa* cells expressing Wt and modified receptors.

and **4**. Such design strategies could be important for eliminating cross-talk between engineered and endogenous receptors.

HeLa cells, transiently cotransfected with the luciferase reporter gene (DR4-luc) and Renilla luciferase control plasmid pRL-CMV along with either pSGRARγ(Wt), the double mutant, pSG5RARγ-(S289G/R278E), or pSG5RARγ(S289D) were grown in the presence of 1 μM ligand.[14] The amount of transcriptional response is determined by means of a dual-luciferase assay (Promega) and is reported in relative light units (RLU) (Figure 3).

As can be seen in Figure 3, the synthetic ligands **1**−**5** are all capable of eliciting a transactivation response to one or both of the modified receptors. However, whereas ligands **1**−**3** activate transcription in cells expressing the wild-type or the mutant receptors to a similar extent, the ligands **4** and **5** can be used to preferentially activate cells expressing the modified receptors. The ligand **5** can provide a 3-fold induction of the luciferase reporter in cells expressing RARγ(S289D) compared to the same cells grown in the absence of ligand. Although the relative induction is somewhat modest, it is remarkable that the apparent polarity of the ligand−receptor complex has been reversed. More dramatic are the effects observed when cells expressing RARγ(S289G,-R278E) are treated with the ligand **4** which shows a 14.4-fold induction of reporter gene expression, whereas RARγ(Wt) shows only a 3.2-fold induction with the same concentration of **4**. To further examine the ligand concentration dependence of these effects, HeLa cells expressing either RARγ(Wt), RARγ(S289D), or RARγ(S289G/R278E) were grown under a range of ligand concentrations. The data reflect the expected dose-dependent behavior for ligand-dependent transactivation (Figure 4a and b).[15]

The two modified receptors can be activated with significant selectivity over the wild-type receptor with the synthetic ligands **4** and **5** at sub-micromolar concentrations. Clearly, the observed behavior reflects a combination of many complex events including cellular uptake and localization, ligand binding, and ligand-dependent allosterism. Future studies that directly measure ligand binding in vitro will provide a method to dissect the complex series of events that lead to trancriptional activation. Coupled with strategies to modify the DNA binding specificity of these receptors, these ligand−receptor pairs could represent an important step toward the precise temporal regulation of multiple gene expression that could have broad applications in science, engineering, and medicine.

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Supporting Information Available: Experimental details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(14) HPLC analysis confirms that the amide ligands 2 and 4 were contain less than 0.1% retinoic, which should not provide a significant response at the ligand concentrations tested.

(15) Assays were not run at higher ligand concentrations because high ligand concentrations, 10 and 20 μM ligand, affected cell viability.