Molecular Pharmacology Fast Forward. Published on November 13, 2013 as doi:10.1124/mol.113.085860
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A structural explanation of the effects of dissociated glucocorticoids on glucocorticoid receptor transactivation

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Molecular Pharmacology Fast Forward. Published on November 13, 2013 as DOI: 10.1124/mol.113.085860
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Running title: Molecular determinants of dGC effects

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Number of text pages: 35

Number of tables: 2

Number of figure: 8

Number of references: 43

Number of words:

Entire manuscript: 8587

Abstract: 218

Introduction: 596

Discussion: 1579

ABBREVIATIONS

Dexamethasone, Dex: 9α-fluoro-11β,17α,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione; RU38486, RU486: 11β-[4-(Dimethylamino)phenyl]-17β-hydroxy-17α-(1-propynyl)-estra-4,9-dien-3-one; RU24782: 9α-fluoro-11β-hydroxy-16α-methylpregna-21-thiomethyl-1,4-diene-3,20-dione; RU247858: 9α-fluoro-11β-hydroxy-16α-methylpregna-21-cyanide-1,4-diene-3,20-dione; DRIP: Vitamin D Receptor-interacting protein; dGC, dissociated glucocorticoid; GC, glucocorticoid; GILZ, Glucocorticoid-Induced Leucine Zipper; GR, glucocorticoid receptor; GRE, glucocorticoid response element; GST, glutathione-S-transferase; LBD, ligand-binding domain; SMRT: Silencing Mediator of Retinoid and Thyroid Receptors; SRC-1, steroid receptor coactivator 1.

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ABSTRACT

There is a therapeutic need for glucocorticoid receptor (GR) ligands that distinguish between the transrepression and transactivation activity of the GR, the later thought to be responsible of side effects. These ligands are known as “dissociated glucocorticoids” (dGCs). The first published dGCs, RU24782 and RU24858, do not have the 17α-hydroxyl group that characterizes dexamethasone (Dex), and they differ from one another by having C21-thiomethyl and C21–cyanide moieties, respectively. Our aim was therefore to establish the structural basis of their activity. Both RU24782 and RU24858 induced a transactivation activity highly dependent on the GR expression level but always lower than dexamethasone. They also display less ability than dexamethasone to trigger SRC-1 recruitment and histone H3 acetylation. Docking studies, validated by mutagenesis experiments, revealed that dGCs are not anchored by Gln642, in contrast to Dex which is hydrogen bonded to this residue via its 17α-hydroxyl group. This contact is essential for SRC-1 recruitment and subsequent dexamethasone-induced GR transactivation, but not transrepression. The ability of dGCs to make contacts with Ile747, for both RU24858 and RU24782 and with Asn564 for RU24858 are not strong enough to maintain GR in a conformation able to efficiently recruit SRC-1, unless SRC-1 is overexpressed. Overall, our findings provide some structural guidelines for the synthesis of potential new dissociated glucocorticoids with a better therapeutic ratio.

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INTRODUCTION

Synthetic glucocorticoids (GCs) have been used for decades to treat inflammatory and autoimmune diseases, leukemias, and lymphomas, and in organ transplantation. However, their beneficial effects are limited by corticoid resistance (Schmidt *et al.*, 2004) and by undesirable side effects that emerge during long-term treatment, and which include diabetes, osteoporosis, hypertension and skin thinning (Schacke *et al.*, 2002). A full understanding of the mechanism of action of GCs appears to be crucial for designing new molecules.

At the molecular level, GCs bind to the glucocorticoid receptor (GR), a member of the nuclear receptor superfamily. The GR comprises three main functional domains: a N-terminal domain, which comprises a ligand-independent activation function known as AF-1 (activation function 1), a central and highly-conserved DNA-binding domain (DBD), and a C-terminal ligand-binding domain (LBD), which binds the hormone and harbors a ligand-dependent activation function known as AF-2 (activation function 2) (Oakley and Cidlowski, 2011). In the absence of ligand, GR is located in the cytoplasm in the form of a large complex with heat shock proteins and chaperones (Pratt *et al.*, 1999). Hormone binding induces a conformational change in the GR, leading to an activated hormone/receptor complex. Transcriptional activation (transactivation) results from translocation of the ligand/GR complex into the nucleus, followed by its dimerization and binding to specific Glucocorticoid Response Elements (GREs) on target gene promoters, and subsequent interaction with the transcriptional machinery (Beato *et al.*, 1996). GR-mediated repression of gene expression (transrepression) results from direct or indirect interference with several transcription factors involved in inflammation, such as Nuclear Factor κ B (NF-κB) and Activator Protein 1 (AP-1) at both the cytoplasmic and nuclear levels (De Bosscher *et al.*, 2003).

Transrepression is thought to be responsible for the therapeutic action of glucocorticoids, whereas transactivation is believed to trigger the undesirable side effects that limit long-term treatment (Belvisi *et al.*, 2001a). Indeed, a mutant GR, devoid of both the dimerization property and the transcriptional activity (GR^{dim}) retained both transrepressive and therapeutic activity (Reichardt *et al.*,

1998; Reichardt *et al.*, 2001). These discoveries prompted the search for ligands that could selectively distinguish between the transrepression and transactivation actions of the GR. Vayssiere *et al.* were the first to report some promising molecules, which they termed “dissociated glucocorticoids” (dGCs) (Vayssiere *et al.*, 1997). In their study, they identified two compounds characterized by a high affinity for the GR, RU24782 and RU24858 (Fig. 1), and which displayed limited transactivation potency but strong transrepression activity. Nevertheless, the molecular mechanism underlying the dissociated profile of these two molecules was still not known, and would clearly be relevant to design new, more potent and GR-selective compounds, since these two molecules still display some transactivation activities *in vivo* (Belvisi *et al.*, 2001b; Schacke *et al.*, 2005; Tanigawa *et al.*, 2002; Eberhardt *et al.*, 2005; Chivers *et al.*, 2006).

The crystal structure of the ligand-binding domain (LBD) of GR complexed with dexamethasone (Dex) has been solved, making it possible to characterize the anchoring mode of the ligand within the ligand-binding cavity (Bledsoe *et al.*, 2002). Furthermore, *in-vitro* studies have identified the contacts within the Dex-GR complex that are critical for stabilizing the complex in its active conformation (Bledsoe *et al.*, 2002; Lind *et al.*, 2000). Our aim was to establish the structural basis of the transactivation activities of RU24782 and RU24858. We have demonstrated that the lower GR transactivation activity of RU24782 and RU24858 compared to dexamethasone can be explained by the absence of stabilizing contact between these dissociated ligands and Gln642 in the GR LBD. This leads to a defect in the GR-mediated recruitment of transcriptional coactivators such as SRC-1.

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MATERIALS & METHODS

Reagents

Dexamethasone was purchased from Sigma (St. Louis, MO). RU24782 (RU782), RU24858 (RU858) and RU38486 (RU486) were a gift from Aventis (Romainville, France). 10^{-2} M stock solutions were prepared in absolute ethanol. TPA (12-*O*-Tetradecanoylphorbol-13-acetate) and concanavalin A were purchased from Sigma. [^{35}S]-Methionine was purchased from Amersham Pharmacia Biotech.

Expression and reporter plasmids

The reporter plasmid pNFκB-Luc responding to NFκB activation was purchased from BD Biosciences and pFC31-Luc, which responds to glucocorticoids through the GRE of mouse mammary tumor virus promoter, was a kind gift from Fabrice Gouilleux, and has been described elsewhere (Gouilleux *et al.*, 1991). GST-tagged SRC-1 (Flajollet *et al.*, 2006) and pCMX-SMRT (Carpentier *et al.*, 2008) were kind gifts from Philippe Lefebvre (INSERM U1011, Lille, France). pSRC-1 plasmid was a gift from Bernard Laine (INSERM U837, Lille, France). The pchGR plasmid, which contains the entire human GRα coding sequence, has been described elsewhere (Hellal-Levy *et al.*, 1999). Plasmids of mutant GR (GR_{N564A}, GR_{Q642A}, GR_{I747A} and GR_{I747G}) were obtained from pchGR by site-directed mutagenesis. The desired mutations were identified by direct sequencing.

Cell culture and transfections

HeLa, HEK-293T and COS cells were cultured in DMEM medium (Life Technologies, France), supplemented with 10% heat-inactivated and charcoal-stripped fetal calf serum (Biowhittaker, France)

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plus 100 IU/ml penicillin, 100 µg/ml streptomycin (GibcoBRL) at 37°C in a humidified atmosphere containing 5% CO_2.

Transfections of HeLa and COS cells were performed using polyethyleneimine (ExGen 500, Euromedex) according to the manufacturer's instructions, as previously described (Benkoussa *et al.*, 2002). Briefly, cells were transfected in 6-well plates with 1 µg of the pFC31-Luc or pNFκB-Luc reporter plasmid. COS cells were transfected with 0.3 µg wild-type or mutated human GR expression plasmid. Cells were plated in 96-well plates after transfection, and were exposed to the glucocorticoids for 16 hours.

Transfection of HEK-293T cells was carried out using the calcium phosphate precipitation method. Cells were transfected with 0.3 µg of one of the receptor expression vectors (pchGR$_{WT}$, pchGR$_{N564A}$, pchGR$_{Q642A}$, pchGR$_{I747A}$ or pchGR$_{I747G}$), 7 µg pFC31-Luc, 1.5 µg pcDNA3 as carrier, and 1 µg pcβgal for internal transfection control. Sixteen hours after the transfection, pooled cells were replated in 24-well plates. After six hours, the steroids were added, and the cells were incubated for a further 16h. The cell extracts were assayed for luciferase and galactosidase activities (Fagart *et al.*, 2010).

The luciferase assay was performed with Bright-Glo Luciferase assay system (Promega) according to the Manufacturer's instructions, and the luciferase activity was measured using a LumiCount plate reader (Packard).

Confocal microscopy

HeLa cells were cultured in 6-well plates and transfected with 1 µg of the EGFP-hGR or EGFP plasmid. After 24 hours, the cells were exposed to glucocorticoids for 1 hour. The cells were then washed with PBS, fixed with 2% paraformaldehyde for 20 minutes, washed with PBS, permeabilized with 0.2% Triton X-100 for 5 minutes, and then washed again with PBS. Nuclei were stained with TOPRO-3 (Molecular probes). The slides were examined under a confocal laser scanning microscope CLSM Leica DMRBE TCS-NT (Leica Microsystèmes, Rueil-Malmaison, France).

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Time-lapse analysis

HeLa cells were cultured in 40-mm tissue culture dishes, and transfected with 1 µg of the EGFP-hGR plasmid. After 24 hours, cells were treated with glucocorticoids in complete medium, and imaged using a time-lapse microscope Leica DMIRE2 (Leica Microsystèmes, Rueil-Malmaison, France) at 37°C in the presence of 5% CO_2.

Chromatin Immunoprecipitation assays (ChIP)

ChIP experiments were carried out as described previously (Flajollet *et al.*, 2006) using non-specific IgG, a mix of two anti-GR antibodies PA1-511A (Pierce) and M-20 (sc-1004X, Santa Cruz Biotechnology), anti-SRC-1 (M-341, sc-8995X, Santa Cruz Biotechnology) or anti-acetylated histone H3 antibody (06-599, Upstate Biotech). The GILZ promoter sequence containing 4 GREs was amplified from -1895 to -1716 with the following primers : 5'- GAT ACC AGT TAA GCT CCT GA -3' and 5'- AGG TGG GAG ACA ATA ATG AT-3' as previously described (Chen *et al.*, 2006). ChIP analysis was performed in triplicate using distinct DNA preparations.

GST Pull-Down assay

Escherichia coli strain BL21 was transformed with the GST vectors. GST fusion proteins were adsorbed on glutathione-sepharose beads as previously described (Flajollet *et al.*, 2006). [^{35}S]-labeled human GR was synthesized from the pchGR plasmid by using a Quick T7 TnT kit (Promega) for 1 h at 30°C. Labeled GR was incubated for 2 hours at 4°C on a rotating wheel with the GST proteins bound to glutathione-sepharose beads in binding buffer (20 mM Tris-HCl, pH7.9, 100 mM KCl, 0.05% NP40, 1 mM dithiothreitol, 20% glycerol, 1 mg/ml bovine serum albumin) supplemented with protease inhibitors in the absence or presence of ligands. The beads were then washed 4 times in washing buffer (20 mM Tris-HCl, pH7.4, 100 mM KCl, 0.1% NP40, 1 mM dithiothreitol, 20% glycerol) supplemented with protease inhibitors, and boiled in 2 x SDS-PAGE loading buffer. Proteins

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were then separated by SDS-PAGE on an 8% gel, and the [^{35}S]-labeled hGR was quantified with a PhosphorImager (Molecular Dynamics). Values were averaged from three independent experiments.

Ligand docking studies

RU782 and RU858 were constructed using the InsightII package (Accelrys, Cambridge, UK), and their total energy minimized using the cvff forcefield. Ligands where then manually docked within the crystal structure of the GR-LBD associated with dexamethasone (PDB ID: 1P93).

Analysis of Data

We used a previously-described method in which the activity of a GC is expressed as a percentage of the maximum activity of a full agonist, e.g. dexamethasone. The agonist activity of a corticoid was calculated as follows: 100 x ((the activity with 1 μM of the corticoid) – (the basal level obtained with ethanol)) / ((the activity with 1 μM Dex) – (the basal level obtained with ethanol)) as previously described (Szapary *et al.*, 2008). The same approached was also used to calculate the transrepressive activity.

Statistical analysis

Results are expressed as the mean ± SD. Data were analyzed by t test. $p < 0.05$ was considered significant using Prism (Graphpad, San Diego).

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RESULTS

Transactivation activities of the dissociated glucocorticoids RU24782 (RU782) and RU24858 (RU858)

RU782 and RU858 were described as dGCs and display a high binding affinity, similar to the one of Dex (Vayssiere *et al.*, 1997). A first set of experiments was conducted to confirm the previously-reported anti-inflammatory and transrepressive activities of RU782 and RU858 in activated splenocytes and HeLa cells, respectively. Both compounds effectively inhibited cytokine production in splenocytes, and displayed 65% efficacy versus dexamethasone in repressing NFκB in HeLa cells at 10^{-6} M (data not shown). In this study, we focused on their transactivation activity in an attempt to identify the molecular determinants of the activities of these dGCs. Transactivation assays performed in HeLa cells with a GRE-driven luciferase reporter plasmid revealed that dexamethasone produced strong gene activation (EC_{50} = $1.37x10^{-8}$ M) (Fig. 2A). In contrast, both RU782 and RU858 displayed very weak efficacy, as revealed by activities of 17% and 30% of Dex activity at 10^{-6} M, respectively. Similarly, the expression of the endogenous gene GILZ was strongly induced by Dex whereas RU858 or RU782 displayed a limited activity (Fig. 2B). Altogether, these results indicate that RU782 and RU858 display a limited transactivation activity in HeLa cells.

It has been described that the activity of dGCs depends on cellular models (Schacke *et al.*, 2005; De Bosscher, 2010). Thus we checked whether a variation in GR expression level could lead to differences in dGCs activity. GR overexpression in HeLa cells potentiated the activities of the two compounds (Fig. 2C) in comparison with HeLa with endogenous GR (Fig. 2A). This demonstrated that the transactivation activity of dGCs is linked to GR concentration. We also wondered whether the cellular context might alter dissociated GC activities. Therefore, we performed transactivation assays in COS and HEK-293T cells transiently expressing GR (Fig. 2D and E) in conditions used to overexpress GR in HeLa cells. RU782 also displayed a weak transactivation activity in COS cells (22% of Dex activity at 10^{-6} M). The activities of RU858 were higher in COS and HEK-293T than in

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HeLa cells, but still lower than dexamethasone. In HEK-293T, both RU782 and RU858 were full agonists, demonstrating that their activities are highly dependent on the cellular context.

We further tested the ability of these ligands to antagonize dexamethasone-induced transactivation activity. No antagonistic effect was observed with RU858 (Fig.3). On the contrary, RU782 was able to inhibit Dex-induced transcriptional activity, with an IC_{50} value of 38.5 nM (Fig. 3 and table 1 for IC_{50} values). As expected, RU486 was the most potent antagonist. The same experiment was carried out in COS cell line, in which human GR was transiently transfected and the same results were obtained despite weaker antagonistic potency for RU782 (higher IC_{50} values, table 1).

Taken together, these data indicate that the two molecules display a lower potency than Dex in all tested conditions but that their transactivation activity is highly dependent on the cellular context, especially on the GR content. Moreover, only RU782 displayed antagonistic activities. These results prompted us to find out an explanation for these properties. We therefore wondered whether these features were due either to ligand-induced GR nuclear shuttling or coactivator recruitment.

Dissociated glucocorticoids induce slower GR nuclear translocation

We investigated whether dGCs trigger GR nuclear transfer as Dex does. The GR subcellular localization was determined using both confocal microscopy and time-lapse analysis in HeLa cells transiently transfected with the hGR-EGFP construct (Fig. 4). Untreated cells showed only hGR-associated fluorescence in the cytoplasm (Fig. 4A). Exposure to 1 or 100 nM Dex for one hour induced the complete nuclear translocation of GR (Fig. 4, A and B). The two dGCs behaved slightly differently. At a low concentration (1 nM), RU782 induced almost complete nuclear translocation of GR, whereas GR translocation was limited in response to RU858 (Fig. 4B). However, at a higher concentration of either RU782 or RU858 (100 nM), complete nuclear translocation of GR was observed, suggesting that its lower transcriptional activity is not attributable to defective nuclear translocation. We also performed time-lapse experiments to compare the kinetics of GR nuclear translocation in response to ligand binding (Fig. 4C). Dex treatment (100 nM) induced almost

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complete GR nuclear transfer within 5 minutes. In contrast, 20 minutes were required for RU858 and RU782 to drive GR into the nucleus. Thus, the lower dGCs-induced transactivation activity cannot be explained solely by a GR inability to enter the nucleus, despite their slower mobility.

Analysis of coactivator recruitment by GR in response to dissociated GC binding

Interactions between GR and transcriptional coactivators are an essential step in the regulation of transcription. This led us to wonder whether GR was able to recruit transcriptional coactivators in response to dGC binding. To do this, we performed GST-pull down assays with a GST peptide fused to Steroid Receptor Coactivator-1 (SRC-1), a member of the p160 family of coactivators (Leo and Chen, 2000) or fused to DRIP205/Med1, a sub-unit of the Mediator complex (Rachez *et al.*, 1999). Dex was able to promote SRC-1 recruitment by GR in a dose-dependent manner (Fig. 5A). Only a weak interaction between GR and SRC-1 or DRIP205 was obtained in the presence of saturating concentrations of RU782 or RU858. We then performed the same experiment with the corepressor SMRT (Silencing Mediator of Retinoid and Thyroid Receptors). We observed a constitutive binding between GR and SMRT and this interaction was strengthened by RU486 but not by Dex and RU782 (Fig. 5B), demonstrating that properties of dGCs do not seem to be linked to a differential recruitment of transcriptional corepressors. Altogether, these data point out a correlation between the dGC-induced GR transcriptional activity and the GR ability to recruit coactivators.

One of the key functions of transcriptional coactivators is histone acetylation which opens the chromatin and makes promoters accessible to the transcription machinery. We therefore used chromatin immunoprecipitation assay to check the histone H3 acetylation status at the GILZ promoter after treatment with dexamethasone or dGCs (Fig. 5C). As expected, GILZ induction by dexamethasone was associated with a potent histone H3 acetylation (Fig. 5C). In contrast, RU858 induced only limited histone H3 acetylation, while no acetylated H3 was detected in the presence of RU782, depicting a defect in coactivator recruitment. We then performed chromatin immunoprecipitation to assess GR binding and SRC-1 recruitment on GILZ promoter (Fig. 5D). Dex treatment induced a strong interaction between GR and the response element and a strong SRC-1

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recruitment, while RU486 effect was half the one Dex on GR binding and negative on SRC-1 recruitment. As expected, both RU782 and RU858 induced a potent GR binding to GILZ promoter but a restricted SRC-1 recruitment (Fig. 5D). Taken together, these results provide evidence that the limited transcriptional activities of RU782 and RU858 are related to a reduction of the ability of GR to recruit transcriptional coactivators.

We then wondered whether the expression level of SRC-1 coactivator or SMRT corepressor can modulate dissociated GC transactivation activity in the HeLa cell model. As shown in Fig. 6A, SRC-1 overexpression potentiated the transcriptional activity of both Dex and dissociated GC. RU858 and RU782 behaved like potent agonists in SRC-1 overexpressing cells, displaying 65 and 62 % of Dex activity, respectively as compared to 30 and 17% in control cells. An increase in residual agonist activity of RU486 was also obtained (Fig. 6A). This increase in transcriptional efficiency was dependent on SRC-1 concentration (Fig. 6B, left panel), highlighting that SRC-1 overexpression can remedy the weak interaction between dGC-GR complexes and coactivators. At the opposite, overexpression of the corepressor SMRT decreased both Dex and dGC transcriptional potencies (Fig. 6A) and reduced dGC efficiency relative to Dex (Fig. 6B, right panel). Moreover, SMRT overexpression counterbalanced the increased dGCs activity induced by GR or SRC-1 transient overexpression (Fig. 6C). Taken together, these results clearly show that the limited GR transcriptional activities induced by RU782 and RU858 are due to a limited capacity of GR to recruit coactivators and provide evidence that the cofactor expression levels influence the agonistic properties of dGCs.

Docking of dissociated glucocorticoids within the GR ligand-binding domain

The crystal structure of the GR LBD complexed with dexamethasone has been solved. The ligand binding mode has been described, and the contacts involved in its interaction with GR identified. We performed docking studies of the two dGCs within the ligand binding pocket of GR in order to determine how they are accommodated (Fig. 7). The constructed and energy-minimized compounds were manually docked within the crystal structure of the GR LBD (PDB ID: 1P93). The positioning of RU782 and RU858 was the same as that of dexamethasone. Their common C3 ketone

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function is anchored by Gln570 and Arg611 (not shown in the figure). The C11-hydroxyl group, which is also common to the three molecules, is hydrogen-bonded to Asn564 (Fig. 7, A-C). The main differences between Dex and the dGCs concern the anchoring of their D ring. The 17α-hydroxyl group of Dex is anchored by Gln642 (Fig. 7A), and constitutes an essential contact for transactivation (Bledsoe et al., 2002; Lind et al., 2000). In contrast, in RU782 and RU858 the 17α-hydroxyl function is lacking, preventing any contact with Gln642 (Fig. 7, B and C). The Dex 21-hydroxyl group establishes a strong hydrogen bond with Asn564 (Fig. 7A). The polar 21-cyanide group of RU858 is in the vicinity of Asn564; however, it is unfavorably oriented to establish a hydrogen bond with Asn564, although some electrostatic interaction may occur (Fig. 7C). In contrast, the hydrophobic 21-thiomethyl group of RU782 is unable to contact Asn564 (Fig. 7B). Interestingly, the 21-substituents of Dex, RU782, and RU858 are all in the vicinity of Ile747, a residue of the loop between the H11 and H12 helices (Fig. 7, A-C). The polar 21- substituents of Dex and RU858 are located 4.0 and 3.4 Å, respectively from Ile747, thus forming stabilizing van der Waals contacts with this residue. In the 3D model, the short distance between the 21-thiomethyl group of RU782 and Ile747 (2.1 Å) suggests that displacement of the H11-H12 loop is required for ligand accommodation. Taken together these observations clearly show that the D-ring of dGCs and of Dex establish different contacts with the Asn564, Gln642 and Ile747 residues.

Interaction with Gln642 is essential for dexamethasone-induced GR transcriptional activity

To evaluate the respective contribution of the contacts between the two dGCs and the Asn564, Gln642 and Ile747 residues in their activity, we substituted Ala for Asn564, Gln642 and Ile747, and Gly for Ile747 in the wild type GR and measured the transactivation activity of the corresponding mutant GRs (GR_{N564A}, GR_{Q642A}, GR_{I747A} and GR_{I747G}) in HEK-293T cells devoid of endogenous GR (Fig. 8A and Table 2 for EC_{50}). In this model, Dex and the two dGCs displayed a high efficacy to activate the overexpressed GR (Fig. 8A and Table 2 for EC_{50}).

The N564A mutation modified GR activity in response to Dex and dGCs in different ways. This mutation completely abolished the GR activity in response to RU858 (Fig. 8A). In contrast,

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GR_{N564A} was still activated by Dex and RU782 (Fig 8A). Nevertheless, the GR_{N564A}-activating potencies of Dex and RU782 were lower than with the wild type GR. Interestingly, the activity of GR_{N564A} observed with RU782 was higher than with Dex. Altogether, these results clearly show that the contacts between Asn564 and the 11- and 21-substituents of RU858 are a prerequisite for GR transactivation activity. They also show that the contacts between Asn564 and the 11- and 21-hydroxyl groups of Dex, and the 11-hydroxyl of RU782, contribute to stabilizing GR in its active state, but that additional stabilizing contacts are also involved, especially in the case of RU782.

Transactivation assays performed with GR_{I747G} and GR_{I747A} revealed that the activity of Dex and dGCs were severely decreased, with a more pronounced effect observed for GR_{I747G} than for GR_{I747A} (Fig. 8A; Table 2). Interestingly, the very low efficacy of RU782 to activate GR_{I747A} and GR_{I747G} highlights a major role of the strong contact with Ile747 in RU782-induced GR activation. These results suggest that Ile747 is involved in the GR transactivation activity induced by Dex and by the two dGCs.

We then analyzed the effect of the Q642A mutation on the GR activity. We observed that the potency of dexamethasone is one order of magnitude lower than that with wild type GR (Fig 8A; EC_{50} = 33.2 $x10^{-10}$ M versus 2.7 $x10^{-10}$ M for the wild-type GR), a finding that is in good agreement with the stabilizing contact between the 17α-hydroxyl group of dexamethasone and Gln642. In sharp contrast, the potency of the dGCs was either unchanged (RU858) or even slightly increased (RU782) with GR_{Q642A} (EC_{50} = 54.4 $x10^{-10}$ M and 4.87 $x10^{-10}$ M, respectively, versus 110 $x10^{-10}$ M and 29.8 $x10^{-10}$ M for the wild-type GR). These findings demonstrate that the weak transactivation activity of dGCs can be explained by the lack of a stabilizing contact with Gln642.

To further investigate the role of the contact between Gln642 and the 17α-hydroxyl of Dex in its high potency, we performed GST pull-down assay with GR_{Q642A}. Interestingly, the Q642A mutation abolished SRC-1 recruitment (Fig. 8B), demonstrating that this contact is essential for Dex-induced coactivator recruitment.

We therefore used this GR mutant in COS cells to measure both transactivation and transrepression activities (Fig. 8C and D). Similarly to results obtained in HEK-293T (Fig. 8A), Q642A mutation highly impaired Dex transcriptional activity (Fig. 8C). RU858 activity was less

affected. At the opposite, RU782 transcriptional activity was unaffected by this mutation. At the transrepression level, Q642A mutation did not show any consequence on GR transrepressive activity whatever the ligand used (Fig. 8D), suggesting Q642 is only essential for GR transactivation activity.

Taken together, our data demonstrate that ligand interaction with Gln642 residue is essential to stabilize the complex in a conformation that allows coactivator recruitment and GR transcriptional activity to occur. Thus, the moderate transcriptional activity of dGCs can be directly attributed to the lack of interaction between these ligands and the Gln642 residue, which is due to the absence of 17α-hydroxyl group in their structure.

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DISCUSSION

The search for dissociated glucocorticoids began with several studies demonstrating that the therapeutic properties of GCs were due to their ability to trigger GR transrepression. Vayssiere *et al.* were the first to describe such compounds. In their study, two compounds RU782 and RU858 seemed to dissociate the transrepression and transactivation activities of the GR, both *in vitro* and *in vivo*, suggesting their potential therapeutic interest. However, these two dGCs did not always display dissociated activity. Our study confirms GR transrepressive activity in response to both RU782 and RU858, and shows that their transactivation activity is highly dependent on the expression levels of the GR (Fig. 2C and 6C). We also characterize their binding mode to GR and identify the molecular factors responsible for their low transactivation activity compared to dexamethasone.

On the basis of crystallographic studies, it has been proposed that the LBD of the nuclear receptors is dynamic, and exhibits some of the properties of a molten globule in the absence of a ligand. These studies also highlighted that ligand binding compacts the LBD by establishing numerous polar and hydrophobic contacts. Some of these are involved in the stability of the ligand-receptor complex, and others are required to stabilize the complex in its active state, facilitating the recruitment of transcriptional coactivators (Nagy and Schwabe, 2004). From our docking and mutagenesis studies, we have identified differences between dGCs and Dex in the way they interact with the GR that are responsible for their differing activities. The most striking difference between ligands is the inability of the two dGCs to establish a contact with Gln642, a residue that is hydrogen bonded to the 17α-hydroxyl group of Dex. In support of this, dGC-induced GR transactivation activity is unaffected by the Q642A mutation, whereas that of Dex is decreased as is its ability to recruit SRC1. Two residues, Asn564 and Ile747, also contribute to the stability of complexes involving dGCs and Dex. Nevertheless, the respective contributions of these residues differ for each GC, due to the nature of their C21-substituents. In the case of Dex, both Asn564 and Ile747 help to stabilize the complex, since GR activity is reduced by both the N564A and I747A mutations. This is due to van der Waals contact between Ile747 and the C21-hydroxyl group, and the hydrogen bond between Asn564 and the C21-hydroxyl group. In the case of RU782, the absence of contact between Asn564 and the C21-

Molecular Pharmacology Fast Forward. Published on November 13, 2013 as DOI: 10.1124/mol.113.085860
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thiomethyl group renders the van der Waals contact with Ile747 decisive. This is supported by the dramatic reduction of GR activity in both the I747A and I747G mutations. The role of Ile747 in stabilizing the complex is weaker in RU858 than in RU782, but this is partially offset by the electrostatic contact between Asn564 and the C21-cyanide group.

Our study suggests that the GR complexed with dGC adopts a conformation which is stable enough to allow cross-talk to occur between GR and pro-inflammatory pathways. GR bound to dGC may be able to interfere directly or indirectly with several transcription factors involved in inflammation, such as Nuclear Factor κ B (NFκB) and Activator Protein 1 (AP-1). Indeed, we observed interference with NFκB in HeLa cells (data not shown) and in COS cells (Fig. 8D) in the presence of dGC. Direct interference with NF-κB or AP-1 occurs by protein-protein interactions involving the GR DBD. It is mediated by monomeric GR, and does not necessitate GR dimerization and DNA binding, whereas transactivation does (Reichardt *et al.*, 1998; Reichardt *et al.*, 2001; Heck *et al.*, 1994; Caldenhoven *et al.*, 1995; De Bosscher *et al.*, 2003; Schule *et al.*, 1990; Yang-Yen *et al.*, 1990). Recently, it has been demonstrated that direct GR binding on negative response element in pro-inflammatory gene promoters may also participate in GR transrepression (Surjit *et al.*, 2011). Interestingly, GR, when bound to RU858, one of the dGCs tested in this study, was shown to be able to bind to these negative GRE and to repress NF-κB activity. All together, these cross-talks impede the interaction between transcription factors and DNA, cofactor proteins or the general transcription machinery in order to repress inflammatory gene expression (for review, see De Bosscher *et al.*, 2003). GR may also block the activation cascade upstream of these transcription factors by inhibiting various kinases and phosphatases. Rapid nongenomic events may also be involved in this transrepressive activity (Buttgereit and Scheffold, 2002).

Our study revealed two steps in the cascade of events leading to GR-mediated transcription that are impaired upon dGC binding as compared to Dex. First, the dynamics of the nuclear translocation of dGC-GR complexes are limited compared to Dex-GR, especially in the case of RU858 (Fig. 4). Second, dGC-GR complexes only weakly recruit coactivators such as SRC-1 and DRIP205 (Fig. 5). The main conformational change induced by dexamethasone binding to GR is the repositioning of

helix H12 towards the ligand pocket, allowing interaction to occur with transcriptional coactivators (Bledsoe *et al.*, 2002; Kauppi *et al.*, 2003). From our results it can be proposed that the conformation induced by dGC binding is not stable enough to allow the required interaction to occur between the AF-2 sub-domain and coactivators. This may be due to the absence of stabilizing interaction with Q642, essential for coactivator recruitment upon Dex binding. In presence of dGCs, contacts with I747 and N564 may not be sufficient to stably maintain the H12 helix in a suitable position allowing cofactor recruitment and transcriptional activity. In the case of RU782, docking experiments suggested that the very short contact between Ile747 and the methyl sulfide of RU782 may result in a displacement of the loop connecting the H11 and H12, impairing coactivator recruitment. This resembles RU486 which possesses a bulky side chain at position 11β projected toward the protein surface to displace the AF-2 helix from its active conformation (Kauppi *et al.*, 2003), and can explain the antagonistic activity of RU782.

We also demonstrated that dGC transcriptional potencies depend on the expression level of GR and the balance between SRC-1 and SMRT. Actually, it was previously reported that GR expression level and the balance between the cellular level of coactivators and corepressors can modify the residual agonist activity of antiglucocorticoids (Szapary *et al.*, 1996; Szapary *et al.*, 1999). Szapary *et al*. have proposed an equilibrium model in which the transcriptional properties of glucocorticoids are regulated by the ratio of coactivators/corepressors bound to GR, depending on the affinity of ligand-bound GR for coactivators or corepressors (Szapary *et al.*, 1999). It has been shown that both agonist- and antagonist-bound GR interact with coactivators and corepressors, but with different binding affinities. While agonist-bound GR preferentially interacts with coactivators , antagonist-bound GR favors interaction with corepressors (Wang *et al.*, 2004; Szapary *et al.*, 1999). Based on our GST-pull down experiments, dGC/GR complex only weakly interacts with the coactivator SRC-1 but this can be overcome by SRC-1 overexpression. These results suggest that the conformation induced by dGC binding is not stable enough to allow a suitable interaction between AF-2 and coactivators, but any increase in GR or SRC-1 expression level can modify the equilibrium and stabilize the dGC/GR complex, changing dGC into potent agonists (Fig. 6). On the opposite, overexpression of the corepressor SMRT decreased dGC transcriptional potencies. Therefore, transactivation activities of

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dGC are closely linked to intracellular levels of GR, coactivators and corepressors and are cell type- and tissue-dependent, and difficult to anticipate. This can explain why Eberhardt *et al*. have observed that both RU858 and RU782 behave like agonists in rat mesangial cells (Eberhardt *et al.*, 2005). Finally, as we observed with HeLa and COS cells, Tanigawa *et al*. have demonstrated that RU858 displayed a weak transactivating activity in HeLa cells (like Vayssiere *et al.* and our study), contrary to CV-1 cells transfected with GR (Tanigawa *et al.*, 2002). On the other hand, Humphrey *et al.* have confirmed that dGCs have a poor effect on receptor-activator of NFκB ligand (RANKL) involved in bone resorption (Humphrey *et al.*, 2006). Since dGCs behavior appears to be cell-specific, these ligands could be considered as SGRM (*Selective Glucocorticoid Receptor modulator*) i.e. corticoids which display different capacities depending on cellular- and tissue-context.

In the light of our results, it is clear that finding a fully dissociated corticoid, displaying potent transrepressive activity without any transactivation in any kind of tissues is going to be a major challenge. In addition, transactivation also contributes to the mechanisms of immunosuppression by activating anti-inflammatory genes, such as IκB, GILZ and MKP-1 (De Bosscher and Haegeman, 2009). For instance, the GILZ and MKP-1 genes have been shown to be induced by RU858 in A549 cells, although less potently than by Dex (Chivers *et al.*, 2006). This emphasizes the need to find out more about the tissue- and promoter-dependent nature of the various different GR-mediated transactivation mechanisms, since many different factors may influence dGC potency. In the future, it will be necessary to focus on identifying and targeting of factors that allow GR-mediated expression of genes involved in immune response regulation (GILZ, MKP-1, IκB) to occur, but limit GR activity on genes responsible for GC side effects, in order to improve the therapeutic ratio of dissociated compounds.

In conclusion, we report here that the dissociation between the transrepressive and transactivation activities of dissociated GCs can be explained at the level of the GR-mediated recruitment of transcriptional coactivators. We have identified some of the molecular features that

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determine dGC activities, and provide structural guidelines for the synthesis of new dissociated corticoids that are likely to have a better therapeutic ratio.

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ACKNOWLEDGMENTS

XD was supported by the Institut pour la Recherche sur le Cancer de Lille.

AUTHORSHIP CONTRIBUTIONS

Participated in research design: Dezitter, Fagart, Formstecher, Rafestin-Oblin, Idziorek

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Contributed new reagents or analytic tools: Fagart

Performed data analysis: Dezitter, Fagart, Fay, Rafestin-Oblin, Idziorek

Wrote or contributed to the writing of the manuscript: Dezitter, Fagart, Rafestin-Oblin, Idziorek

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Molecular Pharmacology Fast Forward. Published on November 13, 2013 as DOI: 10.1124/mol.113.085860
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FOOTNOTES

Disclosure statement: The authors have nothing to disclose

Molecular Pharmacology Fast Forward. Published on November 13, 2013 as DOI: 10.1124/mol.113.085860
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FIGURE LEGENDS

Figure 1: Structures of dexamethasone, dissociated glucocorticoids and the antagonist RU36486.

Figure 2: RU782 and RU858 transactivation activities

A - HeLa cells transiently transfected with pFC31-Luc reporter plasmid were incubated for 16 hours without or with glucocorticoids at different concentrations. The resulting luciferase activity was then measured. Transactivation obtained with 1 µM dexamethasone was set to 100 % and the transactivation activity of indicated corticoids was expressed as a percentage of maximal induction obtained with 1 µM dexamethasone. The results are means ± SD of three independent experiments. On the right panel, the transactivation activities of GC at 1 µM were expressed as percent of maximal induction by 1 µM Dex. The results are means ± SD of three independent experiments.

B - HeLa cells were incubated for 4 hours without or with glucocorticoids at different concentrations. GILZ expression was quantified by RT-PCR. GILZ/GAPDH expression ratios were calculated and expressed relative to control set to 1.

C, D and E – HeLa (C), COS (D) and HEK-293T (E) cells were transiently transfected with pFC31-Luc reporter plasmid and 0.3 µg pchGR. Cells were incubated for 16 hours without or with glucocorticoids at increasing concentrations. The resulting luciferase activity was then measured. Transactivation activity was expressed as in A. The results are means ± SD of three independent experiments.

Figure 3: Antagonist activity of dissociated glucocorticoids

HeLa cells, transiently transfected with pFC31-Luc reporter plasmid, and COS cells, transiently transfected with pchGR and pFC31-Luc, were incubated for 16 hours with 10 nM dexamethasone with or without indicated corticoids at various concentrations. The resulting luciferase activity was then

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measured. Dex-induced luciferase activity in the absence of other glucocorticoids was set to 100%. The results are means ± SD of three independent experiments.

Figure 4: Effect of dissociated glucocorticoids on GR subcellular localization.

A - HeLa cells transiently transfected with pEGFP or pEGFP-GR were incubated in the absence (CTR) or presence of dexamethasone (DEX) at 100 nM for 1 hour. Nuclei were stained by TOPRO-3 (red).

B - HeLa cells transiently transfected with pEGFP-GR were incubated in the absence (CTR) or presence of 1 or 100 nM of indicated glucocorticoids for 1 hour.

C - HeLa cells transiently transfected with pEGFP-GR were treated with indicated glucocorticoids at 100 nM and imaged by a time lapse microscope before treatment (T0) and after 5, 20 and 60 min.

Figure 5: Dissociated glucocorticoids limit transcriptional coactivator recruitment by the GR.

A - GST pull-down assays were performed with *in vitro* translated and [^{35}S]-labeled hGR incubated with GST-fused SRC1 (left panel) or DRIP205 (right panel) in the absence (vehicle ethanol, EtOH) or presence of different glucocorticoids, at indicated concentrations. Each band was quantified and plotted on the graph. The basal interaction with ethanol was scaled up to 1, and all ligand-dependent interactions are expressed relative to the basal level. The results are means ± SD of three independent experiments.

B - GST pull-down assays were performed with *in vitro* translated and [^{35}S]-labeled hGR incubated with GST-fused SMRT in the absence (vehicle ethanol) or presence of indicated glucocorticoids. The results are means ± SD of three independent experiments.

C - HeLa cells were treated with indicated glucocorticoids at 1 µM for 1 hour. Chromatin immunoprecipitation assay were performed with nonspecific IgG or anti-acetylated histone H3. Immunoprecipitated DNA fragments were analyzed by semiquantitative PCR for the presence of GILZ promoter. The results are representative of three independent experiments.

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D - HeLa cells were treated with indicated glucocorticoids at 1 µM for 1 hour. Chromatin immunoprecipitation assay were performed with nonspecific IgG or anti-GR or anti-SRC-1 antibodies, respectively. Immunoprecipitated DNA fragments were analyzed by real-time PCR for the presence of GILZ promoter. The results are expressed as a percent of CTR and are representative of three independent experiments.

Figure 6: Mutual antagonism between SRC-1 and SMRT on dGC transactivation activity.

A - HeLa cells were transiently cotransfected with pFC31-Luc reporter plasmid and an empty plasmid (CTR), SRC1 expression plasmid (300 ng) or SMRT expression plasmid (50 ng). Cells were then incubated for 15 hours with indicated glucocorticoids at different concentrations. GC transactivation activities were expressed as percent of maximal induction by 1 µM Dex in CTR cells. The results are representative of three independent experiments.

B - HeLa cells were transiently cotransfected with pFC31-Luc reporter plasmid and indicated amounts of SRC1 or SMRT expression plasmid. Cells were then incubated for 15 hours with indicated glucocorticoids at 1 µM. The transactivation activities of dGC at 1 µM were expressed as percent of maximal induction by 1 µM Dex. The results are representative of three independent experiments.

C - HeLa cells were transiently cotransfected with pFC31-Luc reporter, pchGR (50 ng), pSRC-1 (50 ng) and different amounts of pCMX-SMRT, as indicated. Cells were then incubated for 15 hours with indicated glucocorticoids at 1 µM. The resulting luciferase activity was then measured and expressed as in B. The results are representative of three independent experiments.

Figure 7: Docking of dissociated glucocorticoid in GR ligand-binding domain

Glucocorticoid docking within the GR-LBD: Stereoviews showing the positioning of Dex (A), RU24782 (B) and RU24858 (C) within the binding pocket of GR. The H3, H5, H7 and H12 helices of GR LBD are displayed as grey ribbons. The carbon atoms of Dex, RU24782 and RU24858 are colored in grey, pink and yellow, respectively. Hydrogen bonds and short van der Waals contacts between the ligands and the residues are depicted as red and black dots, respectively. The figures panels were

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generated using the Dino package (DINO: Visualizing Structural Biology (2002) http://www.dino3d.org).

Figure 8: Q642A mutation affects dexamethasone transcriptional activity but has a low impact on dissociated glucocorticoids transactivation activities.

A – HEK-293T cells were transiently transfected with pFC31-Luc reporter plasmid and wild-type human GR, GR_{Q642A}, GR_{I747A}, GR_{I747G} or GR_{N564A} expression plasmids (0.5 µg). Cells were then incubated for 16 hours with glucocorticoids at indicated concentrations. The resulting luciferase activity was then measured. For each ligand, the transactivation activity of indicated GR mutant was expressed as a percentage of the maximal induction obtained with the wild-type GR. The results are means ± SD of three independent experiments.

B - GST pull-down assays were performed with *in vitro* translated and [^{35}S]-labeled wild-type GR or GR_{Q642A} incubated with GST-fused SRC-1 in the absence (CTR) or presence of dexamethasone at 1 µM. The results are representative of three independent experiments.

C - COS cells were transiently transfected with pFC31-Luc reporter plasmid and wild-type human GR or GR_{Q642A} expression plasmids (0.3 µg). Cells were then incubated for 16 hours with different glucocorticoids at indicated concentrations. The transactivation activities of GC were expressed as percent of maximal induction by 1 µM Dex. The results are representative of three independent experiments.

D - GC transrepressive activities on NFκB target gene were monitored in COS cells transiently transfected with pNFκB reporter plasmid and wild-type human GR or GR_{Q642A} expression plasmids (0.3 µg). Cells were treated with 10^{-6}M TPA (Surjit *et al.*, 2011; Chun *et al.*, 2007; Chun *et al.*, 2007) without or with glucocorticoids at different concentrations for 16 hours. The TPA-induced luciferase activity was set to 100 %. The results are representative of three independent experiments.

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Table 1: Antagonistic activities of dGC in HeLa and COS cells (IC_{50} are expressed in nM)

Steroids	Antagonistic Activity IC_{50} (nM)	
	HeLa	COS
RU858	ND	ND
RU782	38,5	690
RU486	7,6	6,1

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Table 2: Effects of GR mutations on GC transcriptional activity in HEK-293T cells (EC_{50} are expressed in 10^{-10} M)

EC_{50} ($x10^{10}M$)	DEX	RU782	RU858
WT	2.71	29.8	110
Q642A	33.2	4.87	54.4
I747A	102	ND	ND
I747G	486	ND	ND
N564	ND	222	ND

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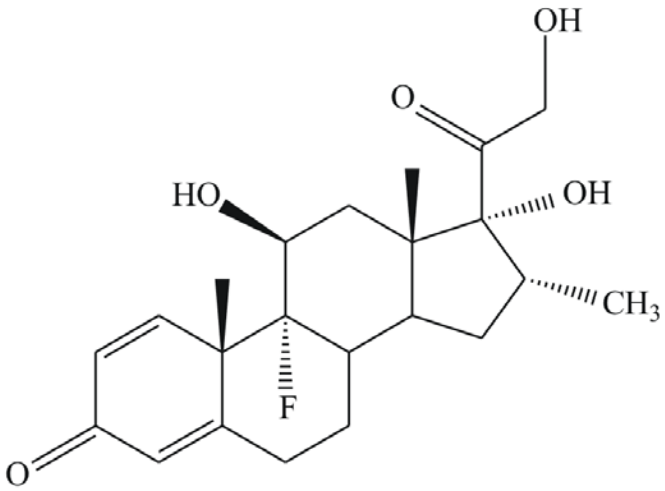
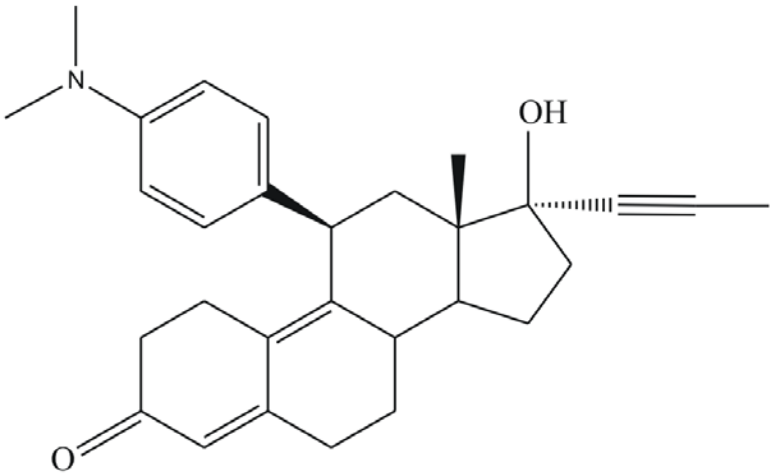
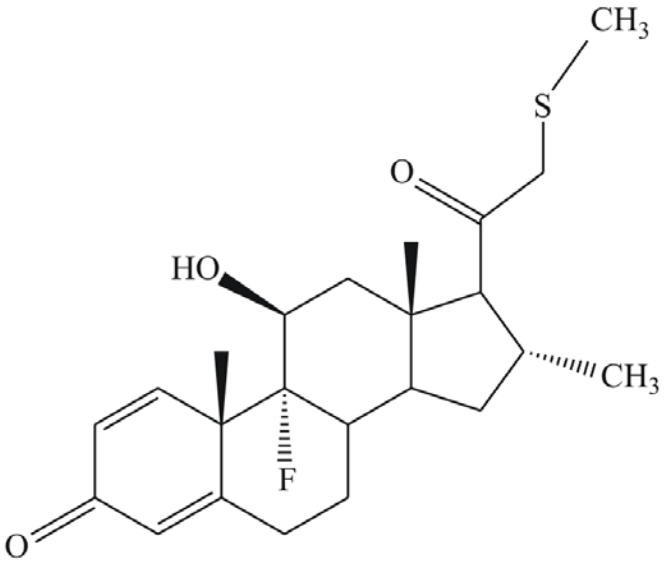
Figure 1



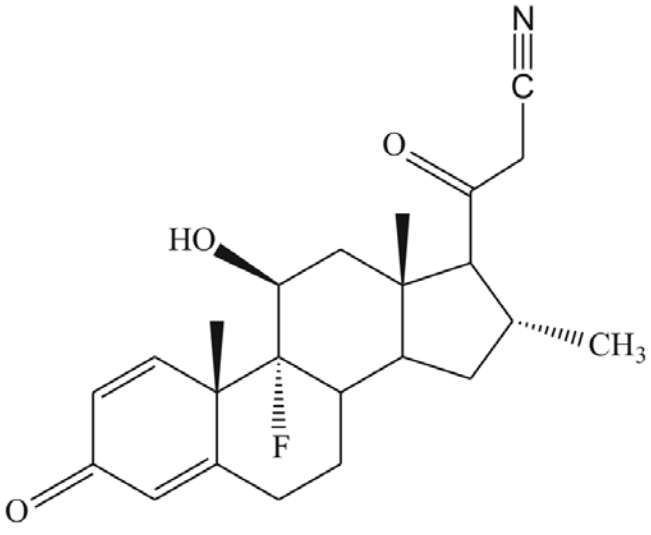
Dexamethasone



RU36486



RU24782



RU24858

Molecular Pharmacology Fast Forward. Published on November 13, 2013 as DOI: 10.1124/mol.113.085860
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Figure 2

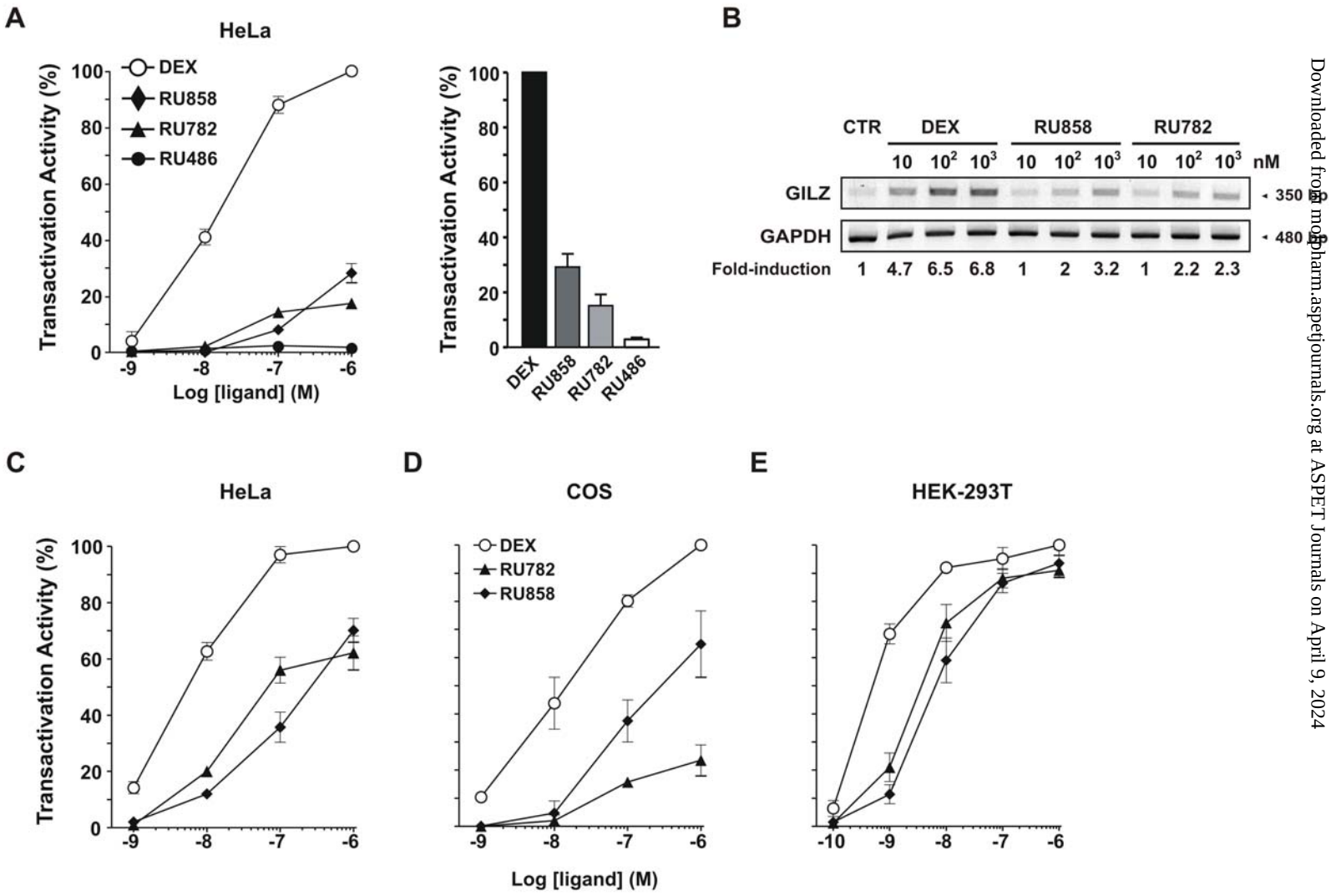


Figure 3

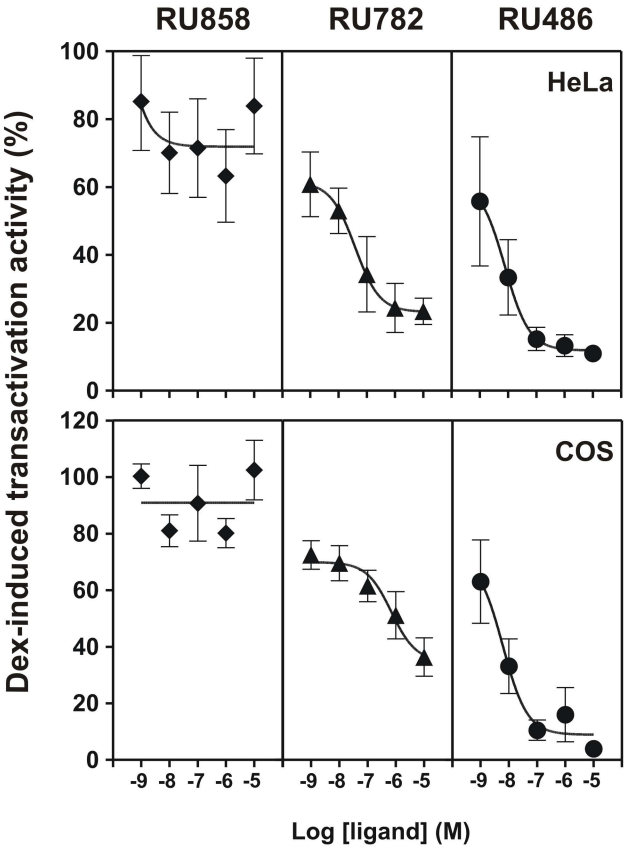

RU858
RU782
RU486
HeLa
COS
Dex-induced transactivation activity (%)
Log [ligand] (M)
0
20
40
60
80
100
120
-9
-8
-7
-6
-5

Figure 4

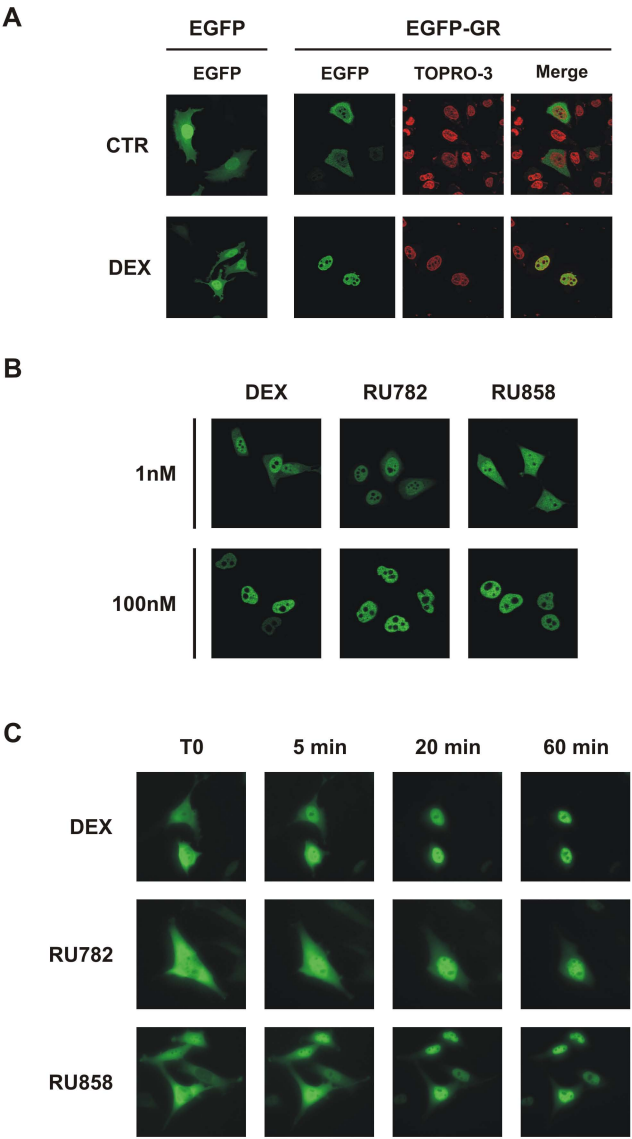

A
EGFP
EGFP-GR
EGFP
EGFP
TOPRO-3
Merge
CTR
DEX
B
DEX
RU782
RU858
1nM
100nM
C
T0
5 min
20 min
60 min
DEX
RU782
RU858

Figure 5

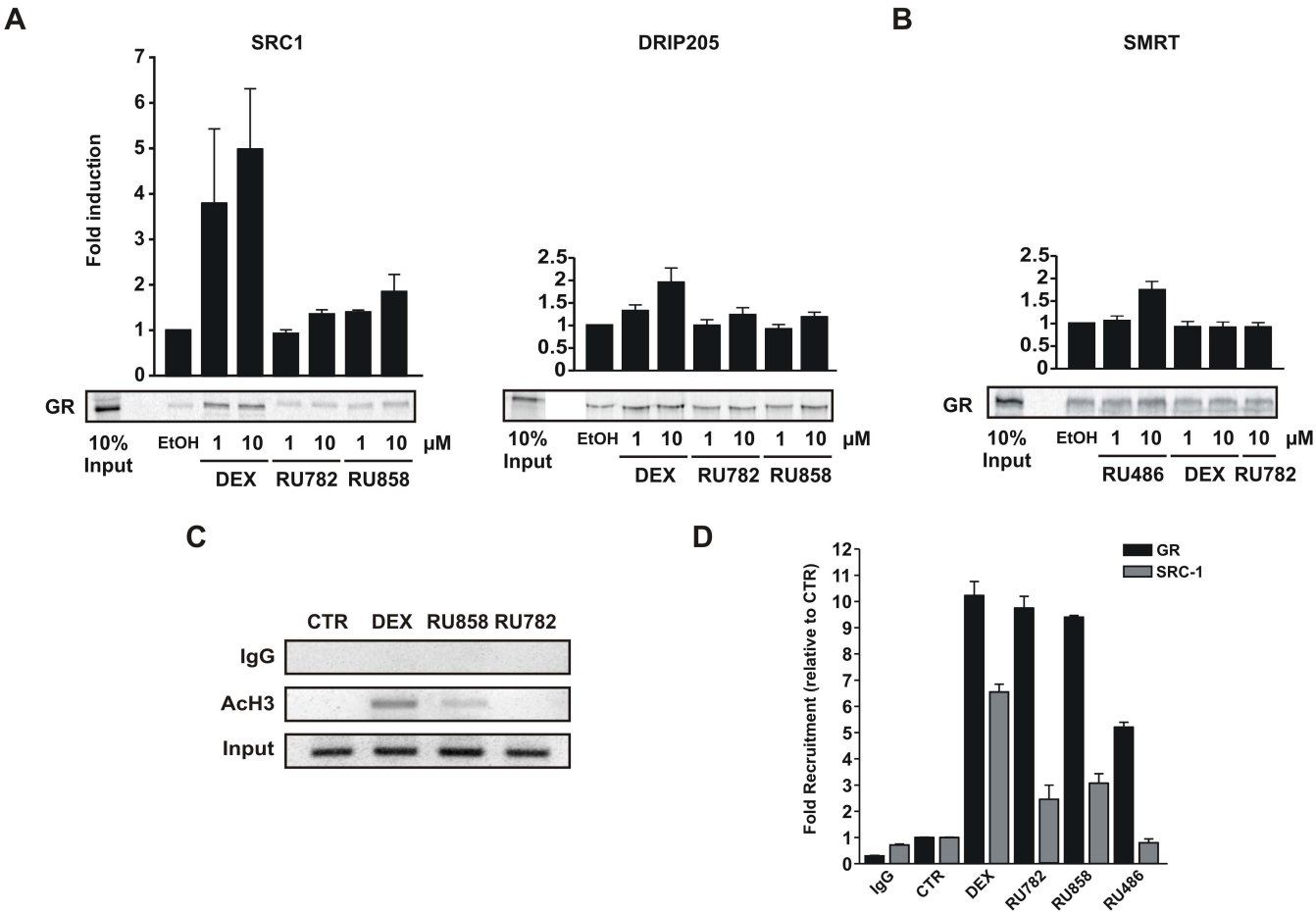

A
SRC1
DRIP205
Fold induction
GR
10% Input
EtOH
1
10
μM
DEX
RU782
RU858
B
SMRT
RU486
C
CTR
DEX
RU858
RU782
IgG
AcH3
Input
D
GR
SRC-1
Fold Recruitment (relative to CTR)
IgG
CTR
DEX
RU782
RU858
RU486

Figure 6

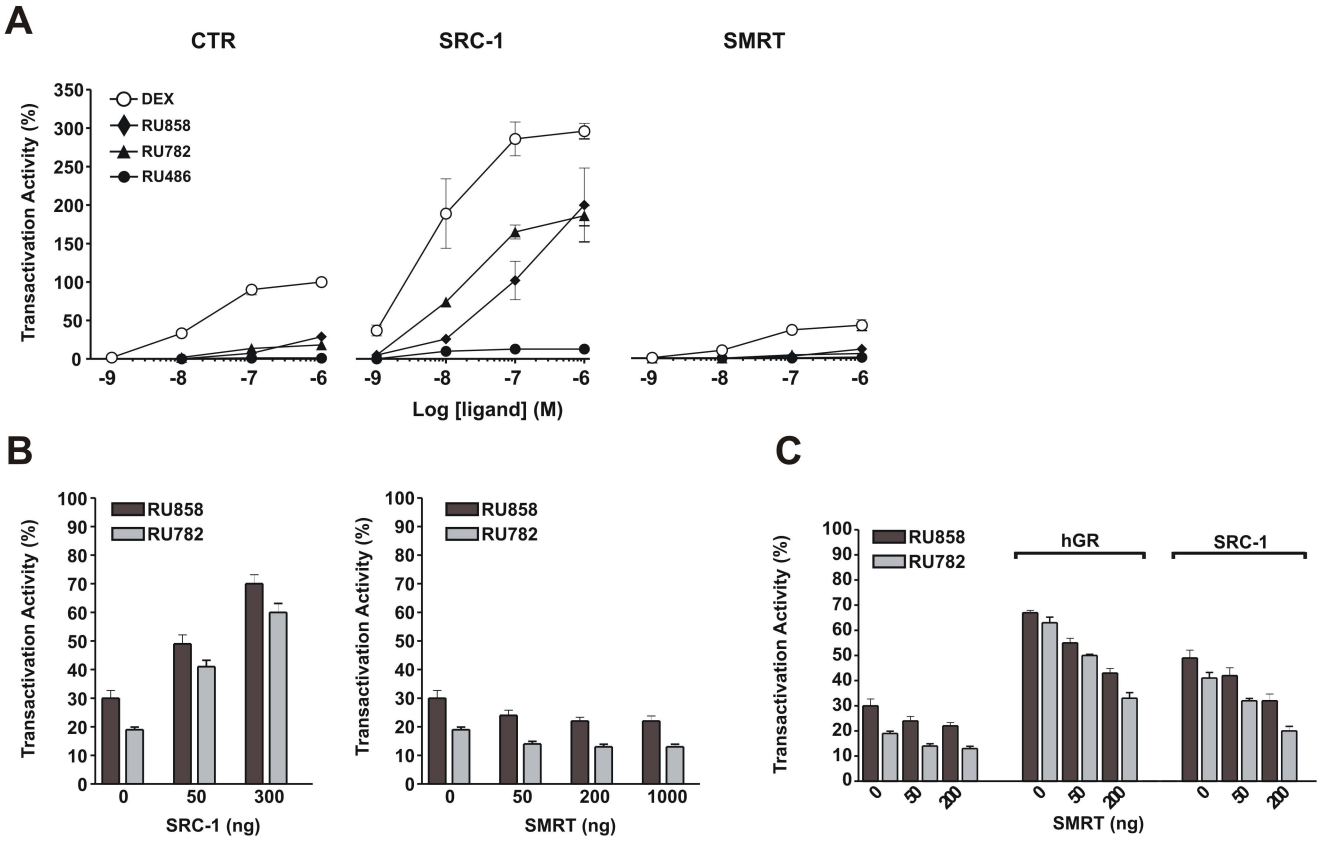
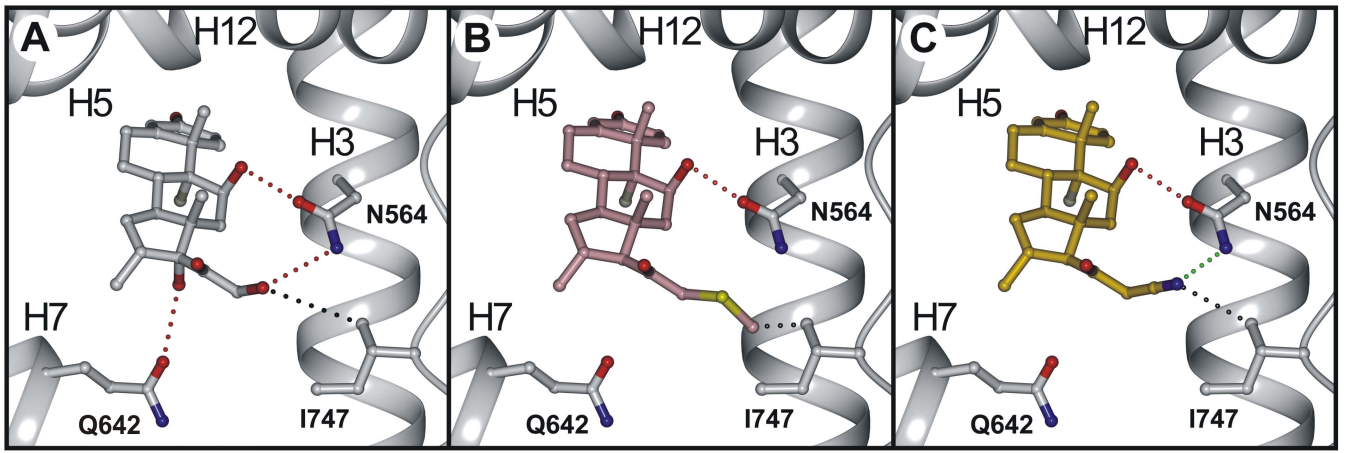
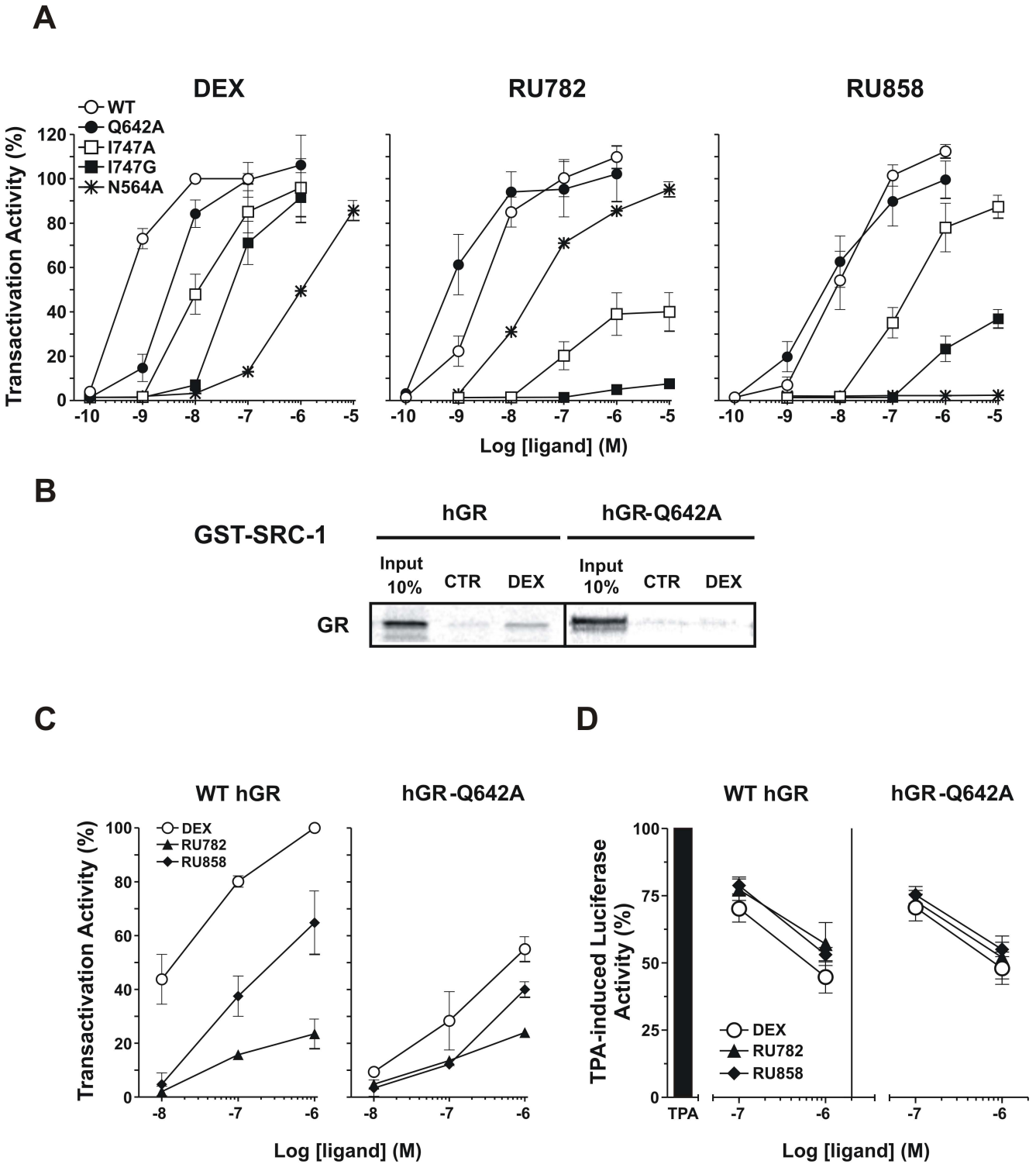

A
CTR
SRC-1
SMRT
DEX
RU858
RU782
RU486
Transactivation Activity (%)
Log [ligand] (M)
B
RU858
RU782
Transactivation Activity (%)
SRC-1 (ng)
SMRT (ng)
C
hGR
SRC-1
SMRT (ng)

Figure 7



DEX RU782 RU858

Figure 8


A
DEX
RU782
RU858
WT
Q642A
I747A
I747G
N564A
Transactivation Activity (%)
Log [ligand] (M)
B
GST-SRC-1
hGR
hGR-Q642A
Input 10%
CTR
DEX
GR
C
WT hGR
hGR-Q642A
DEX
RU782
RU858
Transactivation Activity (%)
Log [ligand] (M)
D
WT hGR
hGR-Q642A
TPA-induced Luciferase Activity (%)
TPA
DEX
RU782
RU858
Log [ligand] (M)