Article

Resveratrol Potentiates Vitamin D and Nuclear Receptor Signaling[†]

Angelika Dampf-Stone[1,*], Shane Batie[1,2,*], Marya Sabir[1,*], Elizabeth T. Jacobs[3,4], Jamie H. Lee[1], G. Kerr Whitfield[2], Mark R. Haussler[2] and Peter W. Jurutka[1,2]

[1]*School of Mathematical and Natural Sciences, Arizona State University, Glendale, AZ 85306*
[2]*Department of Basic Medical Sciences, The University of Arizona, College of Medicine, Phoenix, AZ, 85004*
[3]*University of Arizona Cancer Center, Tucson AZ, 85724*
[4]*Mel and Enid Zuckerman College of Public Health, University of Arizona, Tucson, AZ, 85724*

*****These authors contributed equally to this work.

Corresponding Author: Peter W. Jurutka, School of Mathematical and Natural Sciences, Arizona State University, 4701 W. Thunderbird Rd., Glendale, AZ 85306. Phone: 602-543-6087; Fax: 602-543-6074; E-mail: pjurutka@asu.edu

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ABSTRACT

The 1,25-dihydroxyvitamin D_3 (1,25D) hormone is derived from vitamin D generated in skin or obtained from the diet, and binds to and activates the vitamin D receptor (VDR) in target tissues including kidney, colon/small intestine, and bone/muscle. We tested resveratrol for its ability to modulate VDR signaling, using vitamin D responsive element (VDRE) and mammalian two-hybrid (M2H) transcriptional system technology. Via VDRE-based assays in kidney, colon and myoblast cells, VDR-mediated transcription was activated by resveratrol, and a cooperative effect on transactivation was observed with resveratrol plus 1,25D. The M2H assay revealed a modest, resveratrol-induced dimerization of VDR with its retinoid X receptor (RXR) heteropartner. Cells treated with both resveratrol and 1,25D displayed synergistic stimulation of VDR-RXR heterodimerization, while resveratrol antagonized rexinoid-mediated RXR-RXR homodimerization. Increased transactivation in response to resveratrol was also observed with a subset of other nuclear receptors and their respective cognate responsive elements. Evaluation of wild-type versus a ligand-binding domain mutant VDR revealed that hormone-responsiveness to 1,25D was severely depressed, while the response to resveratrol was only moderately attenuated. Moreover, radiolabeled 1,25D-displacement assays demonstrated an increase in VDR-bound 1,25D in the presence of resveratrol. Thus, resveratrol may affect VDR and other nuclear receptors indirectly, likely via the ability of resveratrol to: 1) potentiate 1,25D binding to VDR, 2) activate RXR, and/or 3) stimulate SIRT1, an enzyme known to deacetylate nuclear receptors. The results of this study elucidate a possible pathway for crosstalk between two nutritionally derived lipids, vitamin D and resveratrol, both of which converge on VDR signaling.

INTRODUCTION

1,25-dihydroxyvitamin D_3 (1,25D), the active hormonal metabolite derived from vitamin D, elicits classical regulation of calcium and phosphate homeostasis [Haussler et al., 2013], and modulates a variety of additional pathways such as cell growth and division, regulation of immune responses and antimicrobial defense, xenobiotic detoxification, carcinogenesis, insulin regulation, and various cardiovascular actions [Haussler et al., 2011a; Haussler et al., 2013]. The role of 1,25D in cellular growth regulation is demonstrated by its ability to arrest cells in the G1/G0 phase of the cell cycle, and by up-regulating p21, a powerful tumor suppressor gene. In this manner, 1,25D can control cell division and proliferation [Bartik et al., 2010]. The effects of 1,25D are almost exclusively mediated by the vitamin D receptor (VDR) which belongs to the superfamily of steroid and nuclear receptors. The 1,25D hormone must bind to and activate VDR, which functions as a transcription factor, in order to modulate transcriptional activity of 1,25D target genes. The binding of 1,25D causes conformational changes in VDR that allow the receptor to recruit its co-receptor, the retinoid X receptor (RXR). A 1,25D-liganded VDR–RXR heterocomplex then binds to vitamin D responsive elements (VDREs), which typically consist of an imperfect direct repeat of six nucleotide bases separated by a three base pair spacer [Haussler et al., 2011b; Haussler et al., 2013]. VDREs are generally found in the promoter region of 1,25D-regulated genes, but in some cases multiple copies of the VDRE are positioned not only in the proximal promoter but dispersed up to several thousand or more kilobases 5' or 3' of the transcription start site in vitamin D-controlled genes. The VDR-RXR duplex, now bound to a VDRE, then recruits either coactivators to induce transcription, or co-repressors to silence gene activity. The coactivators such as steroid receptor coactivator-1 (SRC-1) display histone acetyl transferase activity in addition to aiding in assembly of other components of the RNA polymerase-promoter complex [Haussler et al., 2013].

Because liganded VDR has been shown to play a critical role in a broad array of important pathways, it is of particular interest to evaluate novel, putative 1,25D analogs and/or VDR modulators. Recently, several nutritionally-derived compounds have been shown to act as VDR agonists in lieu of 1,25D to influence receptor activity. For example, the carcinogenic secondary bile acid lithocholate (LCA) may be detoxified by VDR-mediated induction of cytochrome P450-3A4 (CYP3A4), rendering this an important pathway for the prevention of colon cancer [Bartik et al., 2010]. Intriguingly, LCA has been shown to act as a low-affinity VDR ligand [Makishima et al., 2002] that is able to perform some of the same traditional functions of 1,25D in vitamin D-deficient rats [Jurutka et al., 2005; Nehring et al., 2007; Matsubara et al., 2008; Masuno et al., 2013]. Thus, LCA can, via direct binding to VDR, execute self-detoxification [Makishima et al., 2002; Bartik et al., 2010]. In addition to inducing CYP3A4, VDR liganded with either LCA or 1,25D also derepresses SULT2A [Uppal et al., 2007], the colonic enzyme that 3α-sulfates LCA to facilitate its extrusion by an ABC transporter and elimination in the feces. The observation that VDR detoxifies colonic LCA may explain the apparent protective effect of vitamin D against colon cancer [Garland et al., 1999; Jacobs et al., 2011; Jacobs et al., 2013; Hibler et al., 2014b], particularly among individuals consuming a high fat-Western diet. Another nutritionally-derived ligand of VDR is curcumin (CM). CM is able to bind VDR, induce recruitment of its co-receptor RXR and co-activator SRC-1, and induce transcription of several VDR-target genes [Bartik et al., 2010; Batie et al., 2013]. Finally, other studies have demonstrated that ω-3 polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) compete with tritiated 1,25D for binding to VDR, albeit with affinities for the receptor much lower than that of the 1,25D ligand [Haussler et al., 2010]. These previous results supporting the capacity of alternative ligands to

regulate VDR activity led us to hypothesize that there may be additional unique lipophilic ligands and/or modulators for VDR. In the present study, we evaluate resveratrol as one such candidate. Resveratrol is abundant in many foods, including grapes, cranberries, blueberries and peanuts [Raederstorff et al., 2013]. Resveratrol is a natural polyphenolic compound of considerable interest due to its putative anti-aging properties and proposed ability to promote an increase in lifespan [Marchal et al., 2013]. Studies have demonstrated that resveratrol increases longevity, health, and survival of model organisms [Park et al., 2012] and also displays anti-inflammatory properties [Menzies et al., 2013]. Moreover, resveratrol has been well documented for its phytoestrogenic and antioxidant properties, as well as anticancer and neuroprotective effects [Raederstorff et al., 2013]. In addition, clinical research reports a wide assortment of cardiovascular benefits such as improvements in cholesterol profiles, decreased atherosclerosis, enhanced glucose homeostasis, and lowered blood pressure [Raederstorff et al., 2013]. Interestingly, many of these observed bioeffects of resveratrol overlap with reported benefits from high circulating levels of vitamin D. Thus, given the ability of 1,25D to elicit a myriad of bioeffects via transcriptional regulation, evaluating resveratrol in the context of VDR signaling is of particular interest to help elucidate the molecular pathways employed by these two dietary lipids in optimizing healthspan and aging.

The current study evaluates resveratrol as a potential novel ligand and/or regulator of VDR action through either direct or indirect mechanisms. We assess the ability of resveratrol to induce VDR-RXR heterodimerization in M2H assays, and to stimulate transcriptional activity in VDRE-based assays. Furthermore, we report a potent cooperative effect of 1,25D and resveratrol on VDR activity. The synergistic effect between 1,25D and resveratrol, and the non-

competitive nature of interaction between 1,25D and resveratrol in terms of VDR binding, reveals a novel mechanism whereby VDR signaling is potentiated by resveratrol action.

MATERIALS AND METHODS

DNA CLONING AND REPORTER VECTORS

The DNA elements cloned into the luciferase reporter vector included the vitamin D responsive elements (VDREs) designated XDR3, CYP24, ROC, and PER6; the estrogen responsive element (ERE), retinoic acid responsive element (RARE), glucocorticoid responsive element (GRE), pregnane X-receptor responsive element (PXRE), and liver X-responsive element (LXRE).

The VDRE designated XDR3 is taken from the distal direct repeat element in the human cytochrome P450 (CYP)3A4 gene [Batie et al., 2013]. A tandem repeat of this VDRE was inserted into pLuc-MCS reporter vector with the following sequence: CAGAGGGTCAGCAAGTTCATTCACAGAGGGTCAGCAAGTTCATTCA, with the half elements underlined. The reporter construct designated CYP24 contained 5.5 kb of the promoter region of the human CYP24A1 gene (kindly provided by Drs. S. Christakos and J. W. Pike, New Jersey Medical School and University of Wisconsin, respectively) cloned into a firefly luciferase plasmid [Batie et al., 2013]. This portion of the human CYP24 gene possesses two antisense DR3 VDREs (AGGTGAGCGAGGGCG and AGTTCACCGGGTGTG). The sequence of the rat osteocalcin (ROC) VDRE was GGGTGAATGAGGACA, with the half elements underlined [Jurutka et al., 2005]. Four tandem copies were linked to a luciferase reporter construct. The proximal everted repeat-6 (PER6) VDRE from the human CYP3A4 gene promoter region, also linked as a tandem repeat to a luciferase reporter construct, had the sequence TGAACTCAAAGGAGGTCA, with the half elements underlined [Jurutka et al.,

2005]. A GRE derived from the rat tyrosine aminotransferase gene was employed [Jantzen et al., 1987]; the sequence cloned into pLuc-MCS was AGCTAGAACATCCTGTACAGCAGAGCTAGAACATCCTGTACAGCAG. The RARE is an optimized element [Batie et al., 2013] and is responsive to the RAR ligand all-trans retinoic acid. The sequence of the double RARE is AAAGGTCACCGAAAGGTCACCATCCCGGGAAAAGGTCACCGAAAGGTCACC, with the half elements underlined. The PXRE was the same as the PER6 VDRE from CYP3A4 [Hustert et al., 2001]. The ERE was from the *Xenopus laevis* vitellogenin A2 gene; the sequence placed into the reporter construct was GATCAGGTCACTGTGACCTGACTGATCAGGTCACTGTGACCTGACT [Gerber-Huber et al., 1987; Murdoch et al., 1990]. The DR4 LXRE sequence was GGTTTAAATAAGTTCA [Willy et al., 1995], with the half elements underlined. Three copies were linked to a luciferase reporter construct.

CELL CULTURE

Human colorectal adenocarcinoma cells (Caco-2, HCT116), human embryonic kidney cells (HEK293) and mouse myoblast cells (C2C12) cells were grown in a humidified atmosphere at 37°C and 5% carbon dioxide. All cell lines in this study originated from the ATCC (Manassas, VA, USA). Caco-2, HCT116, HEK293 and C2C12 cells were plated at a density of 80,000-100,000 cells/well. Cells were plated approximately 24 h prior to transfection in Dulbecco's modified eagle medium (DMEM) for HCT116 cells, and minimal essential media (MEM) for Caco-2, HEK293 and C2C12 cells. Caco-2 cells were supplemented with 20% fetal bovine serum (FBS); HCT116, HEK293 and C2C12 cells were supplemented with 10% FBS. Caco-2

and HEK293 cells also received 1 mM sodium pyruvate (Invitrogen), and all cell lines were given 0.1 mM nonessential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin.

TRANSFECTION OF CULTURED MAMMALIAN CELLS AND TRANSCRIPTIONAL ACTIVATION ASSAYS

Cells were transfected in Falcon 24-well plates (Beckton Dickinson, Franklin Lakes, NJ) using Express-In transfection reagent supplied by Thermo Scientific (Waltham, MA). The transfection procedure was based on the manufacturer's protocol. Briefly, each well received 2 μl/well Express-In and the respective DNA plasmids specified in the figure legends, along with 20 ng of pRL-null (constitutively expressing low levels of *Renilla reniformis* luciferase) to monitor transfection efficacy. The luciferase-containing vectors (250 ng/well) were derived from the pLuc-MCS plasmid (Stratagene, La Jolla, CA, USA) containing an oligonucleotide (cloned between the HindIII and BglII sites) with multiple copies of the appropriate nuclear receptor responsive element upstream of the firefly (Photinus pyralis) luciferase gene (see explanation of constructs above). Twenty-four hours post-transfection, the cells were dosed with either ethanol vehicle, the respective known nuclear receptor ligand, and/or resveratrol, as described in the figure legends. Dosing/treatment times ranged from 22 to 24 hrs. Following incubation with ligands, cells were lysed in passive lysis buffer (Promega, Madison, WI) and the amount of reporter gene product (luciferase) was quantified using the Dual-Luciferase Reporter Assay System based on the manufacturer's protocol (Promega). Luminescence resulting from the inducible firefly luciferase was divided by luminescence from the constitutively expressed *Renilla* luciferase in order to normalize for transfection efficacy, cell death, and cellular toxicity from ligand exposure. The mean ratio of firefly/*Renilla* luciferase was determined for each

treatment group and the standard deviation was calculated (expressed as error bars). Each experimental treatment group was replicated in at least three, but usually six wells. All data are reported as the average of all wells within the treatment group, and denotes one representative experiment of three or more independent replicates.

MAMMALIAN 2-HYBRID ASSAYS

HEK293, HCT116, and Caco-2 cells were transfected and dosed using the same procedures described for transcriptional activation assays (above). The pCMV-BD bait plasmids included VDR and RXR; and the pCMV-AD prey transfection vectors contained either RXR, steroid receptor coactivator 1 (SRC-1), or D-receptor interacting protein-205 ($DRIP_{205}$) [Jurutka et al., 2005]. Each well received 50 ng of bait and 50 ng of prey vectors, 20 ng of pRL-null, and 200 ng of a firefly luciferase reporter construct (pFR-luc). Luciferase activity was quantitatively analyzed as described above.

COMPETITIVE LIGAND BINDING ASSAYS

VDR-deficient COS-7 cells (2.5×10^6 cells per 150-mm plate) were incubated overnight and then transfected using Express-In reagent (Waltham, MA). To 8.5 mL of serum-free medium were added 2 µg of pSG5-hVDR, 2 µg of pSG5-hRXRa and 17.5 µg of pTZ18U plasmid as carrier DNA. This mixture was combined with an equal volume of serum-free medium containing 150 µL of Express-In reagent, incubated for 30 min, diluted to a total of 20 mL with serum-free medium, and added to cells for a 3 h incubation, then 20 mL of medium containing 20% FBS was added and the plate was incubated for 48 h. Cells were harvested by trypsinization and the cell pellet was resuspended in 1.0 ml of $KTEZ_{0.3}$+2 buffer (10 mM Tris-HCl, pH 7.5, 1 mM

EDTA, 0.3 mM zinc acetate, 0.3 M KCl) plus 5 mM dithiothreitol and mini EDTA free protease inhibitors (Roche Applied Science, Indianapolis IN). Resuspended cells were sonicated, ultracentrifuged, and the clarified supernatant was stored at -80 C. For the competition assay, 1α,25-dihydroxy[26,27-methyl-^{3}H]cholecalciferol ([^{3}H]1,25D, 155 Ci/mmol, Perkin Elmer, Waltham MA) was diluted to 54 Ci/mmol by drying down 36.4 µL of [^{3}H]1,25D with nitrogen and redissolving in 395.6 µl ethanol along with 4.25 µL 1 µM unlabeled 1,25D. Each assay tube received 5 µL diluted [^{3}H]1,25D in a 209 µl total volume (final concentration 0.4 nM) containing 4 µL cell lysate, 196 µL KTEZ$_{0.3}$ +2 and 4 µL of appropriately diluted ligands. After a 15 h incubation on ice, unbound 1,25D was removed by addition of 80 µL of dextran-coated charcoal (Sigma-Aldrich, St. Louis, MO, USA) for 15 min, followed by a 2 min microcentrifugation. Supernatant (200 µL) was combined with 4 mL of ScintiSafe 30% (Fisher Scientific, Pittsburgh, PA) and counted in a Beckman LS 5801 scintillation counter. Data were analyzed with Prism 4 software (GraphPad Software, San Diego, CA, USA).

RESULTS

RESVERATROL STIMULATES VDRE-MEDIATED TRANSCRIPTION

We hypothesized that VDR is one of the direct mediators of resveratrol bioactions. Several experiments were performed to test this hypothesis, including the use of transcription assays employing a VDRE-firefly luciferase reporter plasmid, mammalian two-hybrid assays, and competition binding assays to evaluate the direct association of resveratrol and VDR, in vitro. Figures 1-2 and Table 1 depict luciferase-based transcriptional assay experiments in which Caco-2 human colon cancer cells, mouse myoblast cells (C2C12), human embryonic kidney cells (HEK293), and HCT116 human colon cancer cells (HCT116), respectively, were transfected with a reporter plasmid containing an array of VDREs (CYP24, XDR3, ROC, or PER6; see Methods for detailed description of each VDRE) and treated with resveratrol and/or 1,25-dihydroxyvitamin D_3 (1,25D). In Caco-2 cells with the CYP24 VDRE (Fig. 1A and Table 1), treatment with $2.5x10^{-5}$ M resveratrol alone (Fig. 1A, left panel) or 10^{-9} M 1,25D alone (right panel) resulted in a 1.7-fold and 370-fold increase in luciferase activity over the ethanol vehicle control, respectively. When 10^{-9} M 1,25D and $2.5x10^{-5}$ M resveratrol were combined, the treated cells displayed a 1194-fold increase of transcriptional activity over treatment with ethanol, which represents a 3.2-fold stimulation over treatment with 10^{-9} M 1,25D alone (Fig. 1A, right panel). Similarly, Caco-2 cells transfected with XDR3, ROC, and PER6 (Fig. 1B-D) and treated with resveratrol alone (left panels) revealed a 2.8-fold, 3.2-fold, and 2.4-fold increase over ethanol vehicle, respectively. Treatment with 10^{-9} M 1,25D (Fig. 1B-D, right panels) resulted in a 131-fold (XDR3), 263-fold (ROC), and 83-fold (PER6) increase over ethanol vehicle, respectively, while exposure to 1,25D+resveratrol stimulated transcription by 556-fold, 991-fold and 247-fold

over ethanol controls with XDR3, ROC, and PER6, respectively (Fig. 1B-D), values that represent a 4.2-fold, 3.8-fold, or 3.0-fold increase over the corresponding 1,25D-only group.

A similar analysis was performed in mouse myoblast cells (C2C12) (Fig. 2 and Table 1). In these experiments, as well as in identical assays conducted in human embryonic kidney (HEK293) and in human colorectal adenocarcinoma cells (HCT116) cells (Table 1), we employed the same four VDRE-luciferase reporter constructs as those in Figure 1, and we also included an examination of dose-dependence in the same experimental procedure by using 10^{-9} M (1 nM) and 10^{-10} M (0.1 nM) 1,25-dihydroxyvitamin D in the absence and presence of 2.5×10^{-5} M resveratrol. The results of the various dosing regimens in C2C12 cells (Fig. 2) and in HEK293 and HCT116 cells are summarized in Table 1. Taken together, these experiments probing the effect of resveratrol on VDR-VDRE-mediated transcription in four different cell lines, and utilizing four distinct VDREs, reveal that resveratrol alone can weakly activate VDR under most conditions. Moreover, there is an effect of resveratrol to potentiate 1,25D-stimulated transactivation. This potentiation by resveratrol is consistently and reproducibly observed, but can vary in magnitude depending on the cellular background and on the nature of the vitamin D responsive element.

VDR HETERODIMERIZES WITH RXR AND INTERACTS WITH NUCLEAR RECEPTOR COACTIVATORS IN THE PRESENCE OF RESVERATROL

The next approach to evaluating resveratrol as a potential ligand and/or modulator for VDR was assessment of its ability to promote recruitment of the VDR coreceptor, namely RXR, in the context of a mammalian two-hybrid assay (M2H). Formation of the VDR–RXR heterodimer is an obligatory step for initiation of VDR-mediated transcription [Haussler et al., 1998]. The data (Fig. 3A) indicate that resveratrol stimulates VDR-RXR dimerization. Recruitment of RXR by

VDR in the presence of resveratrol is measured by luciferase output in the M2H. Under the conditions of the M2H assay, there was a 1.4-fold increase in RXR-VDR heterodimerization in the presence of $3.3x10^{-5}$ M resveratrol in Caco-2 cells, a 2.2-fold increase in HCT116 cells, and 2.7-fold stimulation in HEK293 cells compared to the ethanol vehicle control (Fig. 3A). We also tested the effect of 1,25D on heterodimer formation in the presence and absence of resveratrol (Fig. 3B). Compared to the ethanol control, in Caco-2 cells, the combination of 10^{-8} M 1,25D and $3.3x10^{-5}$ M resveratrol results in a 295-fold increase in the recruitment of RXR by VDR, compared to a 170-fold increase generated by 1,25D alone (although the increase with resveratrol is not statistically significant, Fig. 3B, far *left* bars). However, a statistically significant 81-fold increase in heterodimer activity is observed with 1,25D+resveratrol versus only a 37-fold increase with 1,25D alone in HCT116 cells ($p<0.05$), and likewise a 122-fold versus a 57-fold increase was measured in HEK293 cells (Fig. 3B, $p<0.05$).

To further evaluate resveratrol as a possible ligand that stimulates VDR-mediated transcription of target genes, the capacity of resveratrol to facilitate recruitment of the SRC-1 and $DRIP_{205}$ comodulators was measured in the mammalian two-hybrid system (Fig. 3C and D). Ligand-activated VDR binds the coactivator SRC-1 for chromatin remodeling, and also associates with D-receptor interacting protein-205 ($DRIP_{205}$) to position a mediator complex for RNA polymerase II. The ability of resveratrol alone to stimulate VDR-SRC-1 interaction was weak (1.9-fold versus ethanol, Fig. 3C, compare bars 1 and 3) compared to the 78-fold increase (versus ethanol) of this association in the presence of 1,25D alone (Fig. 3C, compare bars 1 and 2). However, in the presence of both 10^{-9} M 1,25D and $3.3x10^{-5}$ M resveratrol, there was a significant (33%) potentiation of the interaction between VDR and SRC-1 (104-fold versus 78-fold compared to the ethanol only control; Fig. 3C, compare bars 2 and 4, $p<0.05$). Similar

results were obtained for the VDR-DRIP$_{205}$ interaction in the presence of resveratrol alone (2.8-fold increase versus ethanol, Fig. 3D, compare bars 1 and 3), but in the presence of both 1,25D and resveratrol there did not appear to be an additional increase in the interaction of these proteins over that detected with 1,25D alone (Fig. 3D, compare bars 2 and 4). These results indicate that the specific interaction of VDR with SRC-1 can be further enhanced by VDR modulators such as resveratrol that may function to conform or modify VDR, or the VDR-RXR heterocomplex, in a way that favors interaction with SRC-1. However, this enhancement by resveratrol of 1,25D -mediated recruitment is not observed with DRIP205, suggesting that this effect is specific to certain VDR cofactors and not others.

POINT MUTATION IN THE HORMONE-BINDING DOMAIN OF VDR REVEALS DIFFERENTIAL EFFECTS ON 1,25D AND RESVERATROL ACTION.

To investigate the role of the hormone binding domain, and potentially elucidate a mechanism of direct binding of resveratrol to VDR, identical ligand treatments were applied to "wild-type" VDR and to R274L VDR. R274L is a well-documented mutation in the hormone binding domain of VDR that results in a significant loss of 1,25D binding to the receptor and causes tissue resistance to vitamin D in human patients [Kristjansson et al., 1993]. WT and R274L VDR were transfected in HEK293 cells along with a PER6 VDRE-linked reporter vector. After treatment of the cells with either 1,25D alone, resveratrol alone, or the two compounds in combination, the results indicate a 57% drop in resveratrol-only activity between WT and mutant R274L VDR (Fig. 4A, compare bars 3 and 4) compared to a 98% reduction in 1,25D-only (Fig. 4B, compare bars 3 and 4) or a 96% reduction in 1,25D+resveratrol activity (Fig. 4B, compare bars 5 and 6), suggesting the possibility of another pathway for resveratrol action besides direct occupation of the VDR ligand-binding pocket.

RESVERATROL AND 1,25D DO NOT COMPETE FOR BINDING TO VDR

To test directly the possibility that resveratrol is not a VDR ligand, but rather affects VDRE-mediated transcription indirectly, *in vitro* competition binding assays were performed (Fig. 4C). Radiolabeled 1,25D was used to determine if resveratrol could compete with 1,25D for binding to the vitamin D binding domain in VDR. DHA, a known low-affinity lipid VDR ligand, was used as positive control and dexamethasone (Dex), a non-VDR lipophilic ligand for the related glucocorticoid receptor, was utilized as a negative control. When cell lysates containing VDR were exposed to resveratrol, bound radiolabeled 1,25D actually *increased*, while DHA competed with 1,25D, as expected for a low-affinity VDR ligand, and Dex had no effect. This surprising result suggests that resveratrol may "stabilize" the vitamin D-VDR complex, as opposed to competing with 1,25D for the binding pocket as initially predicted.

ASSESSMENT OF RESVERATROL BINDING TO RXR

One potential mechanism to stabilize 1,25D-VDR binding, indirectly, might involve resveratrol association with the RXR heterodimeric partner instead of binding directly to VDR. Under certain conditions such as interaction of VDR with a negative VDRE [Haussler et al., 2011a], or perhaps if RXR is bound to a non-traditional RXR ligand such as resveratrol, the RXR protein can exert an allosteric influence to conform VDR into a "ligand-locked" state which could manifest as a potentiation of 1,25D-VDR signaling. To further probe the possibility that resveratrol binds to RXR, mammalian-2-hybrid assays were employed to determine the ability of resveratrol to drive, or to disrupt, RXR-RXR homodimerization. Fig. 5A reveals the ability of resveratrol to generate only a very mild 2.3-fold (HCT116) and 2.5-fold (HEK293) increase in the production of RXR-RXR homodimers versus the ethanol vehicle control, and no stimulation

of RXR-RXR dimers in Caco-2 cells. In contrast, resveratrol is significantly more effective at *inhibiting* the potent RXR-RXR dimerization directed by the RXR-selective rexinoid, bexarotene (Bex; Fig. 5B), especially in HEK293 cells where high-affinity binding of bexarotene to RXR is attenuated 43% by resveratrol (Fig. 5B, compare bars 5 and 6, 375-fold versus 215-fold activation by Bex, $p<0.05$). Taken together, these results suggest that resveratrol may either bind directly to RXR, or modulate RXR dimerization, preferentially driving heterodimer- rather than homodimer-selective pathways. This effect may occur both in permissive heterodimeric complexes such as RXR-LXR, and even in generally non-permissive receptors such as VDR.

THE ACTIVITY OF SEVERAL HETERODIMERIZING BUT NOT A HOMODIMERIZING RECEPTOR ARE POTENTIATED BY RESVERATROL

In order to test the hypothesis immediately above, we measured activation of the retinoic acid response element (RARE), the pregnane X-receptor response element (PXRE), and the liver X-response element (LXRE) by their cognate ligands and/or resveratrol using a luciferase reporter system (Fig. 6). These responsive elements are activated by nuclear receptors for which the obligate pathway, like the pathway of the VDR, includes recruitment of the coreceptor RXR to form a heterodimer complex necessary for transcriptional induction. We also assessed an estrogen responsive element (ERE) and glucocorticoid responsive element (GRE), as these elements dock receptors which form liganded homodimers devoid of RXR. When cells were exposed only to $3.3x10^{-5}$ M resveratrol, the effects on transcriptional activity ranged from 1.0-fold (GRE) to a 7.9-fold stimulation (ERE) in HCT116 cells (Fig. 6A) and extending from 1.5-fold (GRE) to a 15.4-fold stimulation (PXRE) in HEK293 cells (Fig. 6B). Thus, only GRE-mediated activity was not statistically significantly induced above one-fold. To determine synergistic activity, the overexpressed receptors in intact cells were treated with their respective

ligands (10^{-7} M), and with a combination of the cognate ligand and resveratrol (Fig. 6C and D). In HCT116 cells, transcriptional activity induced by the ligands alone ranged from 2.8-fold to 93-fold, whereas the combination of the ligand with resveratrol shows activity ranging from 5.8-fold to 244-fold compared to the vehicle control (ethanol). Importantly, the nuclear receptors that dimerize with RXR consistently displayed a statistically significant increase in transactivation in the presence of resveratrol (ranging from a 100% potentiation with RAR to a 386% potentiation with VDR). GR, which is a homodimerizing steroid receptor, did not exhibit statistically significant potentiation with resveratrol, even across a wide range of receptor ligand concentrations (Fig. 6C and data not shown). Similar results were obtained in HEK-293 cells, with resveratrol-mediated synergism ranging from 154% (ER) to 1195% (PXR). The VDR and a PER6 VDRE were also included in this set of experiments (Fig. 6C and 6D) for direct comparison of resveratrol potentiation in VDR-directed transcription compared to the other RXR heterodimeric and the homodimeric receptor complexes. Taken together, these experiments, that evaluate the effect of resveratrol on transcriptional signaling mediated by six different nuclear receptors in two distinct cell lines, reveal a consistent and reproducible potentiation of ligand-dependent transactivation by resveratrol, especially in the RXR-heterodimerizing subfamily of receptors, suggesting that resveratrol may mediate some of its actions in nuclear receptor signaling via the RXR master heteropartner.

Whereas the homodimerizing GR did not exhibit an effect in transactivation in response to resveratrol alone, or in combination with a GR ligand, the likewise homodimerizing ER displayed increased activity with either resveratrol alone or in combination with its estradiol ligand. Thus, whereas all the RXR-heterodimerizing receptors tested were susceptible to modulation by resveratrol, RXR heterocomplex formation is not the sole determinant of

resveratrol responsiveness, suggesting that other mechanistic factors may endow a nuclear receptor with this property. Interestingly, ER has been shown to be activated by resveratrol [Gehm et al., 1997], although the biological effects of resveratrol on ER remain controversial because both estrogenic and anti-estrogenic properties have been reported [Chakraborty et al., 2013]. In addition, ER is also deacetylated by SIRT1 [Kim et al., 2006], and SIRT1 is activated by resveratrol [Baur, 2010]. Moreover, inhibition of SIRT1 suppresses estrogen receptor signaling [Yao et al., 2010]; thus, resveratrol would be predicted to potentiate ER activity, as it does in the present study (Fig. 6), perhaps via SIRT1 activation. Collectively, these observations further intimate a more complex multi-layered coordination of nuclear receptor regulation by resveratrol that appears to involve RXR-dependent and independent pathways.

DISCUSSION

The potential for resveratrol to modulate vitamin D receptor signaling has recently been postulated by our group [Jurutka et al., 2013] and others [Guo et al., 2014]. There is an overall structural symmetry and parallel configuration of resveratrol and known VDR ligands, which could suggest that resveratrol might serve as a low-affinity VDR ligand with the ability to activate VDR. In fact, our early preliminary studies [Batie et al., 2011] revealed that resveratrol activates VDR-mediated transcription in human embryonic kidney cells employing a VDRE from the human xenobiotic detoxification gene, CYP3A4. In the present study, we directly probed resveratrol as a potential regulator of vitamin D signaling and evaluated both the cellular context and the VDRE platform for their influence on the ability of resveratrol to modulate VDR signal transduction. Experiments in four different cell lines, representing vitamin D target tissues, were consistent in demonstrating an increase in transcriptional activity induced by combining vitamin D with resveratrol. Additive stimulation of VDR was an improbable explanation for this uniform observation because most of the ligand binding sites of VDR were likely to be occupied by the high affinity 1,25D ligand at the concentrations of 1,25D employed in some of the assays. Moreover, the fold-effects in the 1,25D+resveratrol dosing group displayed synergism when compared to 1,25D activation or resveratrol activation alone. Most importantly, when the VDR binding pocket was mutated, a dramatic attenuation in 1,25D-induced transcription was observed, while only a modest reduction in resveratrol-induced transcription was detected. These results all support the hypothesis that resveratrol is a modulator of 1,25D-VDR-mediated transactivation, rather than binding the VDR directly. Further support for this hypothesis is the observation that a 1,25D ligand displacement assay revealed the presence of resveratrol actually amplified binding of 1,25D to VDR. We propose

that one potential molecular mechanism for this phenomenon involves increased RXR recruitment and/or stabilization of the VDR-RXR complex induced by resveratrol binding to RXR. The vitamin D pathway requires association of VDR and RXR to form a heterodimer which can then recruit additional comodulators including SRC-1 and D-receptor interacting protein-205 ($DRIP_{205}$) to stimulate transcription. SRC-1 serves as a catalyst for chromatin remodeling; and $DRIP_{205}$ is required to position a mediator complex for RNA polymerase II to begin transcription. Interestingly, SRC-1 binding to VDR was also increased by resveratrol treatment, but $DRIP_{205}$ recruitment did not increase. This could be further evidence that resveratrol aids in configuring the RXR-VDR heterodimer in such a way as to attract SRC-1 (but not $DRIP_{205}$) more strongly than 1,25D alone. The level of RXR-VDR heterodimerization is higher in cells dosed with resveratrol+1,25D compared to 1,25D treatment alone, supporting the hypothesis of increased RXR recruitment induced by resveratrol. With increased binding of RXR to VDR, we contend that RXR, when bound to a non-traditional RXR ligand such as resveratrol, may exert an allosteric influence on VDR to assume a conformation with an increased affinity for the vitamin D ligand, thus potentiating 1,25D signaling. Further investigation of resveratrol-influenced RXR dimerization revealed only very modest RXR-RXR homodimerization induced by resveratrol alone, and in fact resveratrol-mediated inhibition of bexarotene-directed RXR-RXR homodimerization, suggesting that resveratrol preferably induces heterodimerization over homodimerization. Interestingly, resveratrol normalizes the DNA-binding activity for RAR and RXR in diabetic rats [Singh et al., 2012], further suggesting that this polyphenol may exert its influence via RXRs and its heterodimerization status. Finally, we tested an array of nuclear receptors that either homodimerize or heterodimerize with RXR as part of the signaling pathway initiated by their cognate ligands. The results depict a consistent synergistic effect with

resveratrol+ligand, but predominantly in the heterodimerizing receptors, providing further evidence that resveratrol may potentiate signaling pathways by acting on RXR and increasing the level of heterodimerization.

Of particular interest in elucidating the precise mechanism of resveratrol action in VDR signaling is SIRT1, an NAD^+-dependent deacetylase and mammalian ortholog of the reported longevity factor sir2 in yeast that is activated by resveratrol [Denu, 2012]. Increased activity of SIRT1 has been shown to slow the aging process and aid in the prevention of age-related diseases [Park et al., 2012]. Resveratrol is thought to increase SIRT1 activity and although this observation has been debated, more recent work has further established this link [Price et al., 2012]. Future directions for our current work would include the determination of the causal connection between resveratrol, VDR activity, and increased SIRT1 activation, perhaps involving deacetylation of VDR, as has been shown for other nuclear receptors [Popov et al., 2007]. Resveratrol has also been demonstrated to target multiple cellular pathways including phosphodiesterases for cAMP degradation (thereby increasing NAD^+ levels), which leads to anti-aging actions via increased levels of SIRT1 activity [Tennen et al., 2012]. The cAMP pathway is also central to calorie restriction (CR) as the dominant strategy for anti-aging and the mitigation of age-related metabolic diseases. Park and colleagues demonstrated the possibility that one set of direct resveratrol targets is the PDE enzymes, which consequently initiates the AMPK-SIRT1 pathway leading to increased SIRT1 activity [Park et al., 2012]. Determining the specific, and likely multiple, cellular targets of resveratrol will allow a more complete understanding of the potential for crosstalk between various signaling pathways that may interact with or converge on VDR signal transduction. Ultimately, the elucidation of these pathways would enhance our

ability to predict the synergistic benefits of combined vitamin D and resveratrol intake to elicit positive health benefits of these putative anti-aging nutrients.

Specific benefits of resveratrol have been evaluated and they overlap with putative vitamin D actions, including the effect of resveratrol on cardiovascular and cancer health outcomes. Li et al. [Li et al., 2012] recently summarized the cardiovascular effects of resveratrol with emphasis on the molecular targets of the compound. Intriguingly, several targets emerge such as eNOS, cyclooxygenase, and Akt kinase, all of which are likewise regulated by 1,25D. Heart health applications of resveratrol also are promising based on animal models showing reduction of ischemia-reperfusion injuries, mitigation of blood pressure complications, and a slower progression of atherosclerosis [Li et al., 2012]. Polyphenolic compounds including resveratrol also have been shown to reduce the viability of adipocytes, slow or halt adipocyte differentiation, activate lipid degradation and beta-oxidation, and suppress triglyceride accumulation [Wang et al., 2014].

With respect to cancer, various studies have shown resveratrol to associate with the prevention of colon cancer, likely via a SIRT1-mediated mechanism [Kumazaki et al., 2013; Saud et al., 2014]. Several cancer prevention studies involving resveratrol have been reported, including colitis-related cancer [Hofseth et al., 2010], and IGF-1-induced colon cancer, in which resveratrol was shown to inhibit tumor cell division, and increase apoptotic activity [Vanamala et al., 2010]. This effect of resveratrol is achieved by a combination of attenuating the IGF-1R/Wnt pathway while increasing p53 activity [Juan et al., 2012]. Cancer studies involving resveratrol have recently reached clinical trial phases, where oral administration of resveratrol shows promising results for the prevention of colon cancer [Juan et al., 2012]. Moreover, a recent study of natural compounds to assess epigenetic anticancer therapy aimed at inhibition of promoter

methylation of tumor suppressor genes evaluated the expression of the phosphatase and tensin homologue (PTEN) gene in MCF-7 breast cancer cells [Stefanska et al., 2012]. In this report, among the natural compounds tested, only vitamin D_3 and resveratrol were able to mediate an increase (approximately 35% with either ligand) in PTEN tumor suppressor gene expression, which was associated with a 50% reduction of PTEN promoter methylation. Interestingly, the overall effect of vitamin D_3 or resveratrol was similar to the effect exerted by nucleoside analogues which are direct potent inhibitors of DNA methylation, although the combined effect of 1,25D and resveratrol was not assessed [Stefanska et al., 2012]. These observations signal the potential for epigenetic modulation by 1,25D and resveratrol as an additional level of control for exerting the anticancer effects of these "nutraceuticals". Yet another interesting mechanism with clinical applications was reported by Hibler et al. [Hibler et al., 2014a], who showed that certain genetic polymorphisms that generate distinct Gc-globulin isotypes more effectively deliver vitamin D to target cells. This allows populations at risk to be determined, and resveratrol could potentially be utilized as a preventive nutritional supplement to boost VDR activity in patients with Gc isotypes that attenuate vitamin D action.

Because the cancer preventive properties of resveratrol also lessen the impact of UV radiation and oxidative damage, additional clinical application of combined vitamin D and resveratrol may be translated to other types of malignancy such as skin and breast cancer [Wietzke and Welsh, 2003; Ndiaye et al., 2011; Reis et al., 2014]. Moreover, vitamin D is classically associated with its integral function in bone mineral metabolism, and deficiency of vitamin D causes bone diseases such as rickets. The interplay between resveratrol and vitamin D could be exploited to enhance treatment or facilitate preventive measures for diseases of bone mineral homeostasis. In fact, a very recent randomized, double-blinded, placebo-controlled trial

revealed that high-dose resveratrol supplementation positively affects bone, predominantly via stimulating skeletal mineralization [Ornstrup et al., 2014]. Thus, while vitamin D and its analogs continue to be employed for management of disorders of bone, or are being evaluated as cancer chemopreventative and treatment agents [Bartik et al., 2010; Batie et al., 2013; Jacobs et al., 2013], the identification of compounds that can be employed in combination with vitamin D or D analogs is of particular interest to allow for the application of lower doses to minimize undesired toxicity and side effects such as hypercalcemia while potentially providing a broader treatment efficacy.

The observed VDR-dependent transcriptional synergism of 1,25D and resveratrol reported herein, and the non-competitive nature of the interaction between 1,25D and resveratrol in terms of VDR binding, provide novel evidence that VDR signaling is potentiated by resveratrol, and we hypothesize that resveratrol may act via multiple mechanisms, including the ability of resveratrol to: 1) potentiate 1,25D binding to VDR, 2) activate RXR in the context of the VDR-RXR heterocomplex, 3) stimulate SIRT1 to deacetylate VDR, and 4) modulate epigenetic control of VDR target genes. Moreover, we have shown a significant effect of resveratrol on VDR-mediated transcription in four different cell lines, which offers intriguing nutraceutical possibilities for this compound in multiple vitamin D target tissues by suggesting that the myriad of bioactions mediated by vitamin D via VDR signaling may be further augmented through increased dietary consumption of foods rich in resveratrol to elicit optimal health [Hayes, 2011; Jurutka et al., 2013]. While resveratrol and vitamin D have each been researched extensively, virtually all studies investigate the two pathways separately. Thus, the interplay between resveratrol and vitamin D must be further elucidated if the true potential of their clinical applications is to be revealed.

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FIGURE LEGENDS

Figure 1. Assessment of resveratrol and 1,25D to activate VDR in human colon cancer cells (Caco-2). Experiments were performed using 10^{-9} M 1,25-dihydroxyvitamin D_3 (1,25D) and $2.5x10^{-5}$ M resveratrol in complete media with 24 hours of treatment. A: Luciferase-based transcriptional assay using 5.5 kb of the promoter region of the human CYP24 gene that contains multiple VDREs. B: Transfection with a firefly luciferase plasmid containing two copies of the distal vitamin D responsive element, XDR3, from the human cytochrome P450 (CYP)3A4 gene. C: Transcriptional assay utilizing the rat osteocalcin (ROC) VDRE. D: Transcriptional assay using the proximal everted repeat-6 (PER6) VDRE from CYP3A4. Error bars represent standard deviations; the data are representative of 3 independent experiments with 3-6 wells in each treatment group. $P<0.05$ for all treatment groups versus ethanol vehicle.

Figure 2. Evaluation of resveratrol and 1,25D as VDR activators using mouse myoblast cells (C2C12). Experiments performed using 10^{-9} M (++) and 10^{-10} M (+) 1,25-dihydroxyvitamin D (1,25D) and $2.5x10^{-5}$ M resveratrol in complete media with 24 hours of treatment. A: Luciferase-based transcriptional assay using 5.5 kb of the promoter region of the human CYP24 gene that contains multiple VDREs. B: Transfection with a firefly luciferase plasmid containing two copies of the distal vitamin D responsive element, XDR3, from the human cytochrome P450 (CYP)3A4 gene. C: Transcriptional assay utilizing the rat osteocalcin (ROC) VDRE. D: Transcriptional assay using the proximal everted repeat-6 (PER6) VDRE from CYP3A4. Error bars represent standard deviations; the data are representative of 3 independent experiments with 3-6 wells in each treatment group. $P<0.05$ for all groups except those indicated with an asterisk (*) in Table 1.

Figure 3. Ligand-dependent VDR dimerization and binding of cofactors in a mammalian 2-hybrid system (M2H). A: Heterodimerization of VDR and RXR in response to resveratrol was measured using a luciferase reporter gene in the M2H system. Following a 24 hr transfection with VDR-BD bait and RXR-AD prey vectors, Caco2, HCT116, and HEK293 cells were dosed with $3.3x10^{-5}$ M resveratrol and incubated for 24 h. After normalization for transfection efficiency, results were expressed as fold induction over the vehicle (ethanol) control group. B: Dimerization of VDR-BD and RXR-AD in response to 1,25D or 1,25D + resveratrol was assessed using M2H. Results were expressed as fold-induction of cells dosed with 10^{-8} M 1,25-dihydroxyvitamin D and/or $3.3x10^{-5}$ M resveratrol versus ethanol vehicle. C: Ligand-induced VDR binding to SRC-1 was evaluated in HEK293 cells using VDR-BD, SRC-1-AD and the same transfection procedures described above. Treatment groups included ethanol

(vehicle), 10^{-9} M 1,25-dihydroxyvitamin D (1,25D), $3.3x10^{-5}$ M resveratrol, and the combination of 10^{-9} M 1,25D and $3.3x10^{-5}$ M resveratrol. The luciferase reporter plasmid (pFR-Luc) was used to measure transcriptional activity in relative light units (RLU), and results are plotted as the ratio of pFR-Luc to Renilla luciferase x 10,000. D: Activated VDR binds D-receptor interacting protein-205 ($DRIP_{205}$). HEK293 cells were transfected with VDR-BD and $DRIP_{205}$-AD and incubated in the presence of ligands (as in C). The pFR-Luc reporter vector was used to measure transcriptional activity, expressed as the Firefly/Renilla ratio x 10,000 as in C. Error bars represent standard deviation; the data are representative of 3 independent experiments with 3-6 wells in each treatment group.

Figure 4. The effect of single-point mutation in the hormone-binding domain of VDR on 1,25D and resveratrol responsiveness and competitive ligand binding assay. HEK293 cells were transfected with wild-type hVDR (WT) or R274L hVDR (Mutant). R274L hVDR has a single inactivating mutation in the hormone-binding pocket of human VDR and causes tissue resistance to vitamin D in human patients. The ability of each VDR to respond to 1,25D and/or resveratrol was assessed using vitamin D responsive element PER6 for measurement of transcriptional activity. After normalization for transfection efficiency, results were expressed as relative light units (RLU). A: Treatment with $3.3x10^{-5}$ M resveratrol. B: Treatment with 10^{-8} M 1,25D alone or in combination with $3.3x10^{-5}$ M resveratrol. Error bars represent standard deviations; the data are representative of 3 independent experiments with 6 wells in each treatment group. C: Radiolabeled 1,25D was used in a competitive binding assay to evaluate competition between resveratrol and 1,25D for association to the ligand binding domain of VDR. DHA, a known low affinity VDR ligand, was the positive control and dexamethasone, a non-VDR ligand, was a negative control. Unlabeled 1,25D was also included to show the full extant of competition possible in this assay. Cell lysates were preincubated with radioinert ligands, then exposed to [^{3}H]1,25D-VDR, and the amounts of bound radiolabeled 1,25D were quantitated to measure the ability of each ligand to compete with 1,25D for VDR binding. The data are representative of 2 independent experiments with triplicate samples in each treatment group.

Figure 5. Resveratrol binds to RXR in a mammalian 2-hybrid system but does not significantly promote RXR homodimerization. Following a 24 hr transfection with RXR-AD and RXR-BD, Caco2, HCT116, or HEK293 cells were dosed for 24 h as indicated. Ligand-mediated activation of RXR is followed by RXR homodimerization, and is quantitated via a luciferase reporter gene, pFR-luc. The fold activation with ligand(s) is measured relative to the negative control (ethanol vehicle). A: Cells dosed with $3.3x10^{-5}$ M resveratrol. B: Cells dosed with the RXR-selective ligand bexarotene (Bex, 10^{-7} M) alone or in combination with $3.3x10^{-5}$ M resveratrol. Error bars represent standard deviation; the data are representative of 3 independent experiments with 6 wells in each treatment group.

Figure 6. Evaluating transcriptional potentiation mediated by resveratrol using several diverse nuclear receptors and their cognate ligands. Responsive elements in conjunction with a luciferase reporter system were used to measure nuclear receptor activation by the indicated ligand(s). A: HCT116 cells were transfected with the indicated responsive element driving the expression of a luciferase reporter gene along with 50 ng of expression vector encoding the associated nuclear receptor. Following transfection, cells were dosed with $3.3x10^{-5}$ M resveratrol and incubated for 24 h before measuring resveratrol-mediated transcriptional activity in comparison to the vehicle control (ethanol). B: The same method applied to HEK293 cells. C, D: Transcriptional potentiation was analyzed by measuring receptor activity in the presence of the respective ligand for each nuclear receptor compared to activation in the presence of the cognate ligand plus $3.3x10^{-5}$ M resveratrol. Nuclear receptor ligand concentrations used were 10^{-7} M. The transcriptional activity is depicted as a fold-induction over the ethanol vehicle. Cell lines tested were HCT116 (C) and HEK293 (D). Error bars represent standard deviation; the data are representative of 3 independent experiments with 6 wells in each treatment group.

Table 1. Summary of Data to Assess Resveratrol as a VDR Activator in Multiple Cell Lines Employing Diverse VDREs

Cell/VDRE	+RES (fold vs EtOH)	+1,25D (fold vs EtOH)	++1,25D (fold vs EtOH)	+RES/+1,25D (fold vs EtOH)	+RES/++1,25D (fold vs EtOH)	+RES/+1,25D (fold vs +1,25D)	+RES/++1,25D (fold vs ++1,25D)
Caco-2/CYP24	1.7	ND	370.0	ND	1194.4	ND	3.2
Caco-2/XDR3	2.8	ND	131.4	ND	556.1	ND	4.2
Caco-2/ROC	3.2	ND	263.2	ND	991.4	ND	3.8
Caco-2/PER6	2.4	ND	83.0	ND	247.3	ND	3.0
C2C12/CYP24	1.6*	7.9	13.1	22.3	22.5	2.8	1.7
C2C12/XDR3	1.0*	3.2	4.5	4.4	5.6	1.4	1.2*
C2C12/ROC	1.4*	5.5	9.7	16.4	36.9	3.0	3.8
C2C12/PER6	1.3*	3.5	6.2	7.5	10.4	2.2	1.7
HEK293/CYP24	2.9	27.6	63.8	74.5	140.1	2.7	2.2
HEK293/XDR3	2.9	18.6	32.3	106.1	110.7	5.7	3.4
HEK293/ROC	4.5	22.2	205.2	197.4	1099.8	8.9	5.4
HEK293/PER6	3.7	12.4	33.3	88.8	176.8	7.1	5.3
HCT116/CYP24	2.4	2.9	8.5	6.1	13.7	2.1	1.6
HCT116/XDR3	1.3*	2.6	8.5	5.3	13.1	2.0	1.5
HCT116/ROC	2.9	8.5	60.4	58.7	308.3	6.9	5.1
HCT116/PER6	2.5	2.9	10.2	8.3	25.9	2.9	2.5

All values represent significant fold-effects of indicated ligand(s) versus EtOH or 1,25D ($p<0.05$) except as indicated with an asterisk (*). ND, Not determined. EtOH=vehicle; +1,25D=0.1 nM; ++1,25D=1.0 nM; +RES=25 μM

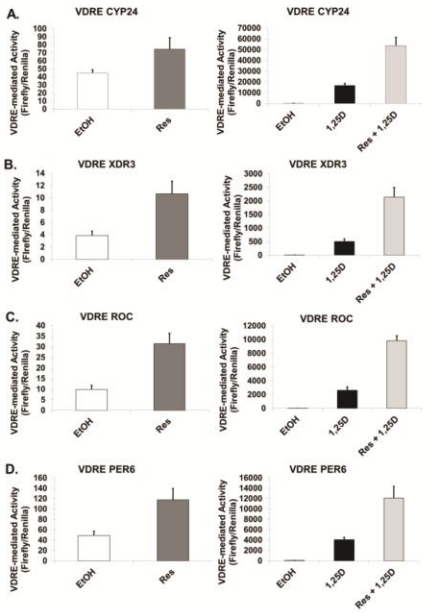

Figure 1

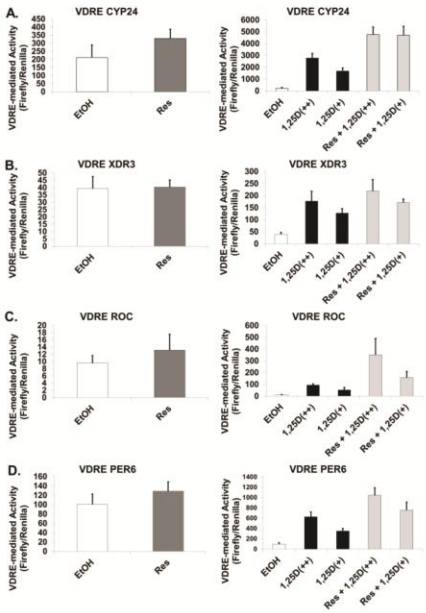

Figure 2

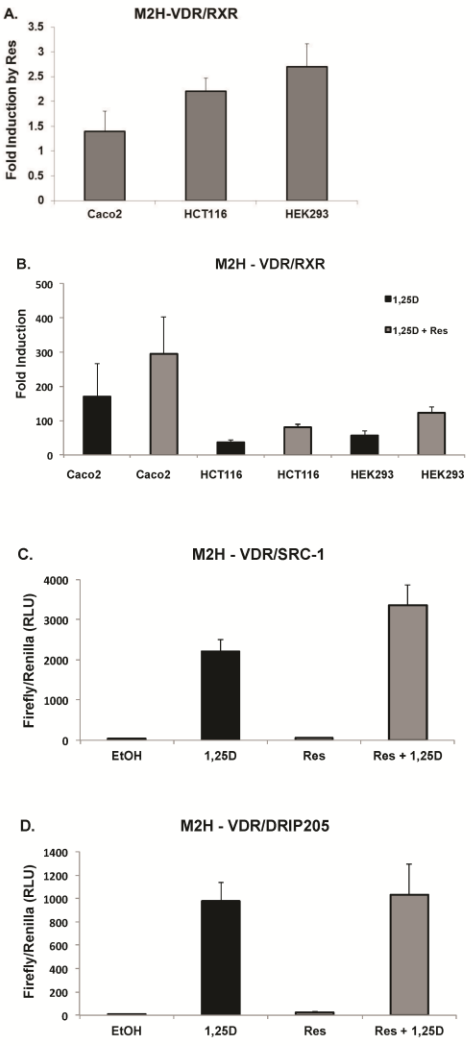

Figure 3

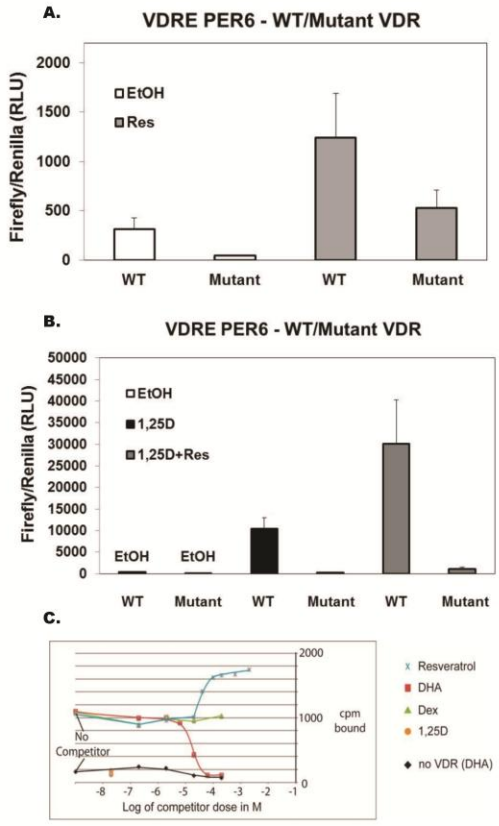

Figure 4

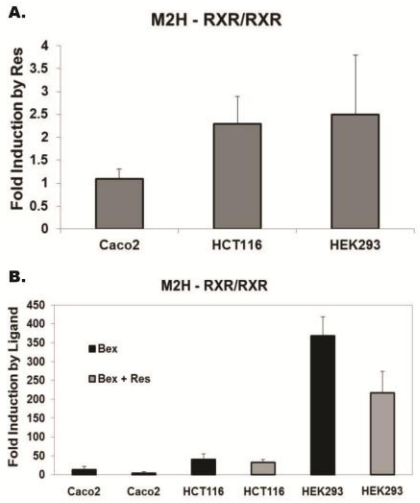

Figure 5

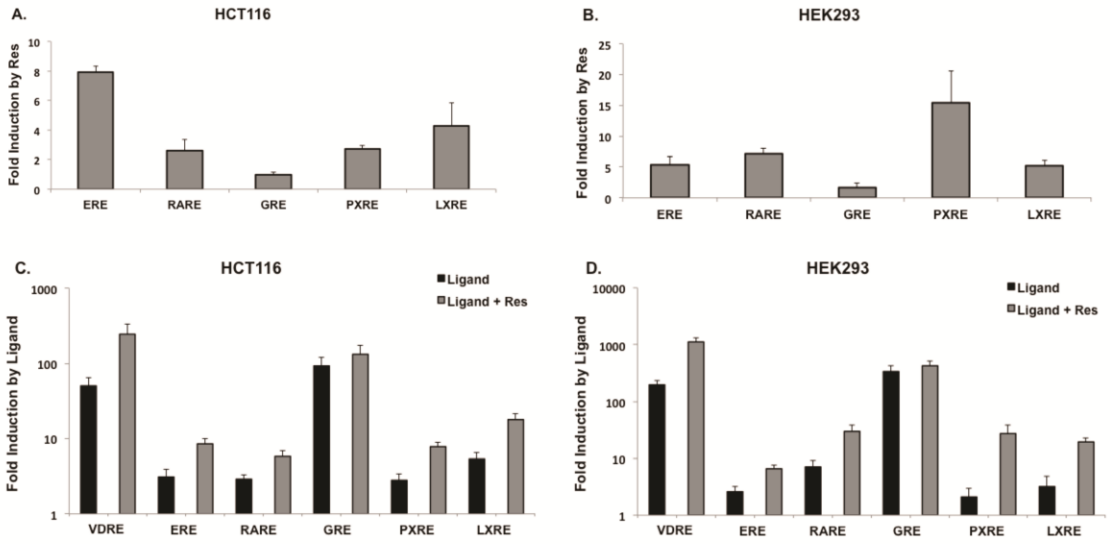

Figure 6