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1α,25-dihydroxyvitamin D_3 Stimulates Steroid Sulphatase Activity in HL60 and NB4 Acute Myeloid Leukaemia Cell Lines by Different Receptor-Mediated Mechanisms

Philip J. Hughes,[1]* Andreas Steinmeyer,[2] Roshantha A.S. Chandraratna,[3] and Geoffrey Brown[1]

[1]Division of Immunity and Infection, The Medical School, University of Birmingham, Edgbaston, Birmingham B15 2TT, United Kingdom
[2]Medicinal Chemistry III, Research Centre Europe, Schering AG, Berlin D-13342, Germany
[3]Retinoid Research, Allergan, Inc. Irvine, California 92612

Abstract Steroid sulphatase is a key enzyme in the biosynthesis of bioactive estrogens and androgens from highly abundant inactive circulating sulphated steroid precursors. Little is known about how the expression/activity of this enzyme is regulated. In this article, we show that of 1α,25$(OH)_2D_3$ stimulates an increase steroid sulphatase activity in the HL60 myeloid leukaemic cell line that is inhibited by a specific nuclear VDR (VDR_{nuc}) antagonist and unaffected by plasma membrane-associated vitamin D receptor (VDR_{mem}) agonists and antagonists. 1α,25$(OH)_2D_3$-mediated up-regulation of steroid sulphatase activity in HL60 cells was augmented by RXR agonists, blocked by RXR-specific antagonists, and RAR specific agonists and antagonists had no effect. In contrast, the 1α,25$(OH)_2D_3$-mediated up-regulation of steroid sulphatase activity in the NB4 myeloid leukaemic cell line was unaffected by the specific VDR_{nuc} and RXR antagonists, but was blocked by a VDR_{mem}-specific antagonist and was increased by VDR_{mem}-specific agonists. The findings reveal that VDR_{nuc}-RXR-heterodimers play a key role in the 1α,25$(OH)_2D_3$-mediated up-regulation of steroid sulphatase activity in HL60 cells. However, in NB4 cells, VDR_{nuc}-derived signals do not play an obligatory role, and non-genomic VDR_{mem}-derived signals are important. J. Cell. Biochem. 94: 1175–1189, 2005.

Key words: 1α,25-dihydroxyvitamin D_3; nuclear vitamin D receptor; membrane-associated vitamin D receptor; steroid sulphatase; myeloid cells

The 1α,25-dihydroxyvitamin D_3-nuclear vitamin D receptor (1α,25$(OH)_2D_3$-VDR_{nuc}) signalling system plays a role in regulating estrogen biosynthesis. Knockout of VDR_{nuc} causes uterine hypoplasia, impaired folliculogenesis and incomplete spermatogenesis, which can be reversed by exogenous estrogens. A defect in aromatase expression is also associated with loss of VDR_{nuc} [Yoshizawa et al., 1997; Kinuta et al., 2000]. More direct experiments show that 1α,25$(OH)_2D_3$ regulates the expression and activity of key enzymes that generate bioactive estrogens (including aromatase and 17β-hydroxysteroid dehydrogenase) [Jacob et al., 1995; Yoshizawa et al., 1997; Mountford et al., 1999; Kinuta et al., 2000; Enjuanes et al., 2003; Yanase et al., 2003]. We have shown that 1α,25$(OH)D_3$ regulates the activity of steroid sulphatase in HL60 promyeloid cells [Hughes et al., 2001]. Steroid sulphatase catalyses the

Abbreviations used: 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; AML, acute myeloid leukaemia; APL, acute promyelocytic leukaemia; E_1S, estrone sulphate; EC_{50}, concentration required to produce a half maximal response; FBS, fetal bovine serum; RAR, retinoic acid receptor; RXR, retinoid-X receptor; STS, steroid sulphatase; VDR_{nuc}, classical nuclear vitamin D receptor; VDR_{mem}, plasma membrane-associated vitamin D receptor.

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*Correspondence to: Philip J. Hughes, Rm EG16, Division of Immunity and Infection, The Medical School, University of Birmingham, Edgbaston B15 2TT, UK.
E-mail: P.J.Hughes@bham.ac.uk

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first step of the local conversion of "inert" circulating steroid sulphates into biologically active steroids within peripheral tissues and enzyme activity or expression is often elevated in hormone-dependent tumours [Nakata et al., 2003]. At present the mechanism by which $1\alpha,25(OH)D_3$ increases steroid sulphatase activity is poorly understood.

The VDR_{nuc} is a member of the nuclear receptor superfamily and is a ligand-activated transcription factor. The ligated VDR_{nuc} associates with other nuclear hormone receptors, most notably the retinoid-X receptor (RXR). Binding of VDR/RXR heterodimers to specific vitamin D response elements (VDREs) recruits transactivation co-activators and chromatin remodelling complexes that facilitate engagement of the basal transcription machinery [Belandia and Parker, 2003; Bettoun et al., 2003; Carlberg, 2003a,b]. VDR/RXR heterodimer-mediated signalling is relatively slow, taking hours to days before the consequences of activation of this pathway becomes apparent. Also, a large number of the genes that are activated by $1\alpha,25(OH)_2D_3$ do not have a classical canonical VDRE in their promoter [Carlberg, 2003a], and other control mechanisms may regulate the expression of these genes.

$1\alpha,25(OH)_2D_3$ can provoke biological effects in cells that become apparent within seconds or minutes of exposure to the seco-steroid. These include increases in calcium and phosphate uptake in intestinal cells (transcaltachia) [Nemere et al., 1984], the opening of voltage-gated calcium and chloride channels in osteoblasts [Zanello and Norman, 1997, 2003, 2004a,b] and skeletal and cardiac muscle [De Boland and Boland, 1994], the opening of Ca^{2+}-dependent K^+ channels in kidney proximal tubules [Edelman et al., 1986], increased superoxide generation in osteoblasts [Berger et al., 1999] and potentiation of insulin secretion from pancreatic β-cells [Kajikawa et al., 1999]. Rapid $1\alpha,25(OH)_2D_3$-stimulated non-genomic signals are also thought to be involved in regulating growth arrest and differentiation of chondrocyte growth plates [Schwartz et al., 2002a,b], vascular smooth cells [Rebsamen et al., 2002], keratinocytes [Gniadecki, 1996; Johansen et al., 2003; De Haes et al., 2004] and breast cancer cell lines [Capiati et al., 2004]. Similarly, the phosphatidylinositol 3-kinase/Akt/PDK-1 [Hmama et al., 1999; Marcinkowska and Kutner, 2002; Rebsamen et al., 2002; De Haes et al., 2004; Lee et al., 2004], the phospholipase $C\gamma$-Ca^{2+}-protein kinase C [Berry et al., 1996; Xie and Bikle, 2001; Schwartz et al., 2002a,b], cyclic AMP-Protein kinase A [Massheimer et al., 1994; Vazquez et al., 1997; Lopez-Lluch et al., 1998; Takahashi et al., 1998] and the Ras/Raf/MEK/ERK 1/2 [Gniadecki, 1996; Song et al., 1998; Buitrago et al., 2002; Chae et al., 2002; Marcinkowska and Kutner, 2002; Johansen et al., 2003; De Haes et al., 2004] intracellular signalling pathways are activated within a few seconds or minutes of exposure of cells to $1\alpha,25(OH)_2D_3$.

We show that $1\alpha,25(OH)_2D_3$ stimulates an increase steroid sulphatase activity in the HL60 myeloid leukaemic cell line and that this is inhibited by a specific VDR_{nuc} antagonist and unaffected by specific agonists and antagonists of a plasma-membrane-associated vitamin D receptor (VDR_{mem}). In HL60 cells the $1\alpha,25(OH)_2D_3$-mediated up-regulation of steroid sulphatase activity was augmented by RXR agonists, blocked by RXR-specific antagonists, and RAR specific agonists and antagonists had no effect. In contrast, the $1\alpha,25(OH)_2D_3$-mediated up-regulation of steroid sulphatase activity in the NB4 myeloid leukaemic cell line was unaffected by the specific VDR_{nuc} and RXR antagonists, but was blocked by a VDR_{mem}-specific antagonist and was increased by VDR_{mem}-specific agonists.

EXPERIMENTAL PROCEDURES

Chemicals

$1\alpha,25$-dihydroxyvitamin D_3 was a gift from Dr. Lise Binderup (Leo Pharmaceutical Products, Ballerup, Denmark). ZK 159,222 was synthesised by the Department of Medicinal Chemistry of Schering AG (Berlin, Germany). $1\alpha,25$-dihydroxylumisterol$_3$ and $1\beta,25$-dihydroxyvitamin D_3 were kindly provided by Prof. A. Norman (Department of Biochemistry, University of California, Riverside, CA). The RXR antagonist AGN195393, the pan-RAR agonist TTNPB and the pan-RAR antagonist AGN194310 [Hammond et al., 2001; Xiao et al., 2003] were synthesised by the Retinoid Research Division (Allergan, Inc., Irvine, CA). Radiolabelled steroids were from Perkin-Elmer Life Science Products-UK Ltd., (Cambridge, UK). All cell culture medium and supplements were purchased from Invitrogen Ltd., (Paisley, Scotland). All other chemicals were purchased from Sigma (Poole, Dorset, UK).

Cell Culture

The HL60 cell line was provided by Dr. R.C. Gallo (NIH, Bethesda, MD). The variant HL60-Pager D cell line was obtained from Prof. Farzin Farzaneh (Division of Cancer Studies, Kings College, London, UK). HL60-Pager D cells express a truncated RARα that exerts a dominant negative effect that blocks retinoid-driven RARα-mediated granulocytic differentiation. However, HL60-Pager D cells retain their responsiveness to 1α,25-dihydroxyvitamin D_3, dimethylsulfoxide, sodium butyrate or phorbol ester [Brown and Farzaneh, unpublished observations]. The dominant negative effect is achieved by quenching accessory transcription factors. The NB4 cell line was obtained from Dr. K. Mills (Dept of Haematology, University of Wales College of Medicine, Cardiff, UK). HL60 and NB4 cell lines were grown in RPMI 1640 medium containing glutamine, 10% heat-inactivated fetal bovine serum (FBS) and antibiotics in 95% air/5% CO_2 at 37°C. Cells were seeded at 2.5×10^5 cells per ml as 10 ml cultures in 25 cm^2 flasks and treated with 0.1–500 nM 1α,25$(OH)_2D_3$ for 72 h, unless otherwise stated.

Depletion of Plasma-Membrane Cholesterol

HL60 cells that had been grown for several years serum free in RPMI 1640 supplemented with ITS^{+1} (Sigma) were used in experiments in which the cholesterol sequestering agents methyl-β-cyclodextrin (MCD) and filipin were used to disrupt cholesterol-rich lipid raft formation. NB4 cells were adapted to growth in RPMI 1640 containing 2% FBS by reducing the percentage of FBS over a period of 28 days, and were maintained in 2% FBS-containing media for 14 days prior to experiments. The use of these cells minimises repletion of plasma membrane cholesterol from the high levels of cholesterol in FBS. To confirm that a change in 1α,25$(OH)_2D_3$-stimulation of steroid sulphatase activity was related to cholesterol depletion, MCD- or filipin-treated cells were incubated at 37°C for 2 h in RPMI 1640 containing 50 μg/ml of water-soluble cholesterol prior to stimulation with 1α,25$(OH)_2D_3$. The general membrane lipid binding agent xylazine was used as a negative control [Huo et al., 2003]. Cells were stimulated in serum-free RPMI 1640 with 100 nM 1α,25$(OH)_2D_3$ for 24 h and processed as below. This short incubation time with 1α,25$(OH)_2D_3$ was employed because of toxicity when acute myeloid leukaemic cell lines were exposed to the cholesterol-modulating agents for >36 h [Li et al., 2003].

Measurement of Steroid Sulphatase Activity in Cell Fractions

Cells were collected by centrifugation (5 min at 400 × g, 4°C) and re-suspended in 1 ml of hypotonic homogenisation buffer (50 mM HEPES, 0.1 mM EDTA, pH 7.4 supplemented with Sigma Protease inhibitor cocktail 1 and Sigma Phosphatase inhibitor cocktail). After 30 min on ice, the cells were disrupted by repeated sonication and centrifuged at 100,000 × g at 4°C for 60 min. The resultant pellet was re-suspended in 0.5 ml of the above buffer.

Steroid sulphatase activity was assayed at 37°C in 0.02 M Tris-HCl and 0.1 mM EDTA (pH 7.5) containing 20 μM E_1S (spiked with ~100,000 dpm of [^{3}H]-E_1S) in a final volume of 100 μl. Reactions were started with 30–80 μg protein and quenched after 60 min with 900 μl of ice-cold 0.1 M sodium bicarbonate containing 5000 dpm of [^{14}C]-E_1 (to determine the recovery of the extraction process). 950 μl of the resulting mixture were extracted with 3 mls of toluene and the organic and aqueous layers were separated. Eight millilitre of Emulsifier Safe scintillant (Canberra Packard, Pangborne, Berks, UK) were added to each fraction and radioactivity was determined in a Packard 2000CA TriCarb liquid scintillation counter. Blank incubations in the absence of protein were used to correct for carry-over of radiolabelled substrate into the organic layer. The protein concentration of each sample was measured in triplicate using Bio-Rad protein assay kit using bovine γ-globulin as the standard (Bio-Rad, Hemel Hempstead, Herts, UK).

Data Manipulation and Statistical Analysis

Multiple experiments were performed with quadruplicate replicates. Results are expressed as means ± standard errors of means (SEM). The statistical significance between groups of data was analysed by the Student's *t*-test or where appropriate by the Mann–Whitney rank sum or Kruskal–Wallis one way analysis of variance tests using either the Minitab™ or the SigmaStat™ statistical software packages. Irrespective of the test used a P-value < 0.05 was considered to be a significant difference.

Dose response curves were fitted to Sigmoidal dose-response curves using the 'pharmacology'

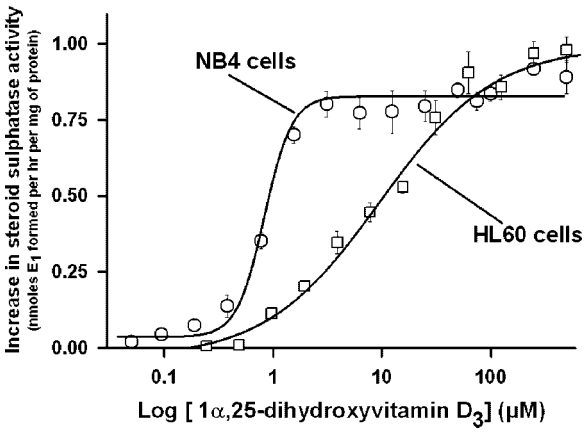

Fig. 1. The 1α,25-dihydroxyvitamin D_3 stimulates steroid sulphatase activity in HL60 and NB4 acute myeloid leukaemic cells. Concentration-response curves were obtained for 1α,25$(OH)_2D_3$-stimulated increases in steroid sulphatase activity in HL60 cells (n = 12) and NB4 cells (n = 7). All data points were acquired after 72 h exposure to 1α,25$(OH)_2D_3$ and are shown as means ± SEM from quadruplicate determinations. Response curves were fitted to a sigmoidal curve, and EC_{50}'s and Hill coefficients were estimated using the 'pharmacology' module of the Sigmaplot™ (version 8.0) graphical software package.

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RESULTS

The 1α,25-dihydroxyvitamin D_3 Stimulates Steroid Sulphatase Activity in HL60 and NB4 Acute Myeloid Leukaemic Cells

The effect of 1α,25$(OH)_2D_3$ on steroid sulphatase activity in HL60 and NB4 acute myeloid leukaemic cells is shown in Figure 1. 1α,25$(OH)_2D_3$ stimulated steroid sulphatase activity in both cell lines and after 72 h treatment with 100 nM 1α,25$(OH)_2D_3$ steroid sulphatase activity had increased ~6- to 8-fold over basal. In HL60 cells, the 1α,25$(OH)_2D_3$ concentration-response curve was best fitted by a sigmoidal curve with a Hill coefficient not significantly different from unity ($N_H = 0.90 \pm 0.18$, n = 12, $r^2 = 0.93$), and the concentration 1α,25$(OH)_2D_3$ required to produce a half-maximal increase (EC_{50}) was 10.2 ± 1.2 nM. 1α,25$(OH)_2D_3$ also produced a similar time and concentration related increase in steroid sulphatase activity in the NB4 acute promyelocytic leukaemia cell line. In this cell line, the EC_{50} was significantly lower being 1.02 ± 0.4 nM ($P > 0.03$, n = 7). Similarly, the Hill slope for the 1α,25$(OH)_2D_3$ dose response curve in NB4 cells was significantly steeper at 2.87 ± 0.2 (n = 7, $r^2 = 0.88$, $P > 0.01$).

The differing shapes of the 1α,25$(OH)_2D_3$ dose-response curves in HL60 and NB4 cells may indicate that the 1α,25$(OH)_2D_3$-stimulated increases in steroid sulphatase activity are mediated by different 1α,25$(OH)_2D_3$ receptors in the two cell lines.

Pharmacological Characterisation of the Vitamin D Receptors Involved in Up-regulating Steroid Sulphatase Activity in the NB4 Myeloid Leukaemia Cell Line

1α,25$(OH)_2D_3$ analogues can be used to pharmacologically differentiate between cellular responses initiated by VDR_{nuc} and VDR_{mem}. For example, the planar 6-s-*cis* locked vitamin D_3 analogue 1α,25-dihydroxylumisterol$_3$ specifically activates non-genomic signalling pathways but fails to stimulate transactivation of 1α,25$(OH)_2D_3$-sensitive genes via the VDR_{nuc} [Norman et al., 2001]. Figure 2A shows the effects on steroid sulphatase activity of treating NB4 cells for 72 h with increasing concentrations of 1α,25$(OH)_2D_3$ and the 6-s-*cis* locked 'non-genomic' vitamin D_3 agonist 1α,25-dihydroxylumisterol$_3$. As already shown in Figure 1, treatment of NB4 cells with 1α,25$(OH)_2D_3$ produced a co-operative dose-related increase in steroid sulphatase activity ($EC_{50} = 0.94 \pm 0.17$ nM, $N_H = 2.75 \pm 0.34$,

Fig. 2. Pharmacological characterisation of the vitamin D receptors involved in up-regulating steroid sulphatase activity in the NB4 myeloid leukaemia cell line. **Panel** (**A**) shows the concentration-response curves for the 1α,25$(OH)_2D_3$- or 1α,25-dihydroxylumisterol$_3$-stimulated increase in steroid sulphatase activity in NB4 cells (n = 4). **Panels** (**B**) and (**C**) show the effects of the VDR_{mem} non-genomic signalling antagonist 1β,25$(OH)_2D_3$ on the 1α,25$(OH)_2D_3$- (n = 7) and 1α,25-dihydroxylumisterol$_3$-stimulated (n = 3) increase in steroid sulphatase activity in NB4 cells. **Panel** (**D**) shows the effects of the VDR_{nuc} genomic antagonist ZK159,222 on the 1α,25$(OH)_2D_3$- stimulated increase in steroid sulphatase activity in NB4 cells (n = 8). Panel E shows the effects of ZK159,222 on the 1α,25-dihydroxylumisterol$_3$-stimulated increase in steroid sulphatase activity in NB4 cells (n = 3). All data points were acquired after 72 h exposure to 1α,25$(OH)_2D_3$ or 1α,25-dihydroxylumisterol$_3$. The VDR_{nuc} and VDR_{mem} antagonists were added 2 h prior to the addition of the agonists. All results are shown as means ± SEM from quadruplicate determinations. The concentration-response curves were fitted to a sigmoidal curve, and EC_{50}'s and Hill coefficients and their respective errors were estimated using the 'pharmacology' module of the Sigmaplot™ (version 8.0) graphical software package.

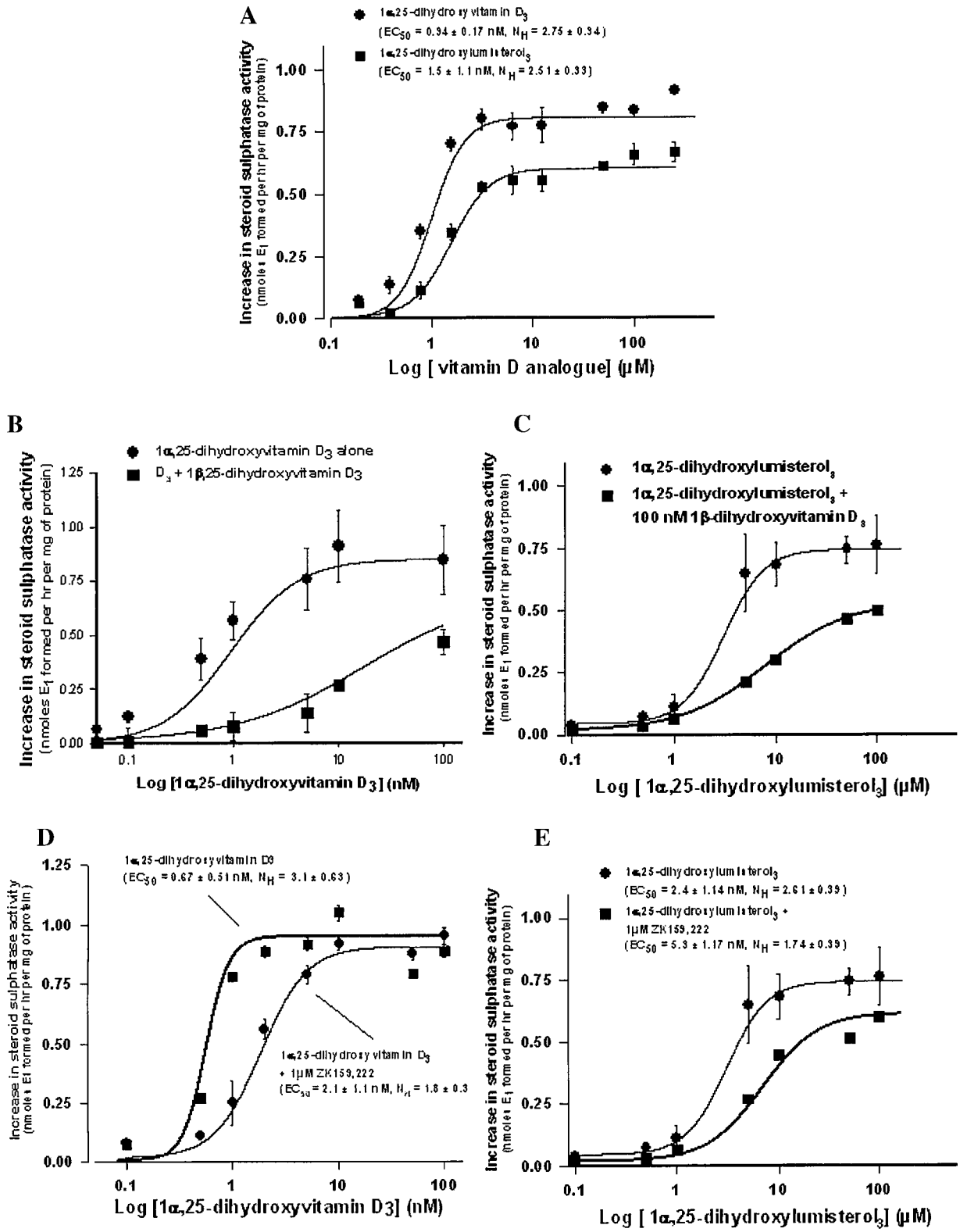
A
1α,25-dihydroxyvitamin D3
(EC50 = 0.94 ± 0.17 nM, NH = 2.75 ± 0.34)
1α,25-dihydroxylumisterol3
(EC50 = 1.5 ± 1.1 nM, NH = 2.51 ± 0.33)
Increase in steroid sulphatase activity
(nmoles E1 formed per hr per mg of protein)
Log [vitamin D analogue] (μM)
B
1α,25-dihydroxyvitamin D3 alone
D3 + 1β,25-dihydroxyvitamin D3
Log [1α,25-dihydroxyvitamin D3] (nM)
C
1α,25-dihydroxylumisterol3
1α,25-dihydroxylumisterol3 +
100 nM 1β-dihydroxyvitamin D3
Log [1α,25-dihydroxylumisterol3] (μM)
D
1α,25-dihydroxyvitamin D3
(EC50 = 0.67 ± 0.51 nM, NH = 3.1 ± 0.63)
1α,25-dihydroxyvitamin D3
+ 1μM ZK159,222
Log [1α,25-dihydroxyvitamin D3] (nM)
E
1α,25-dihydroxylumisterol3
(EC50 = 2.4 ± 1.14 nM, NH = 2.61 ± 0.39)
1α,25-dihydroxylumisterol3 +
1μM ZK159,222
(EC50 = 5.3 ± 1.17 nM, NH = 1.74 ± 0.39)
Log [1α,25-dihydroxylumisterol3] (μM)

Fig. 2.

n = 4). The 'non-genomic' agonist 1α,25-dihydroxylumisterol$_3$ also stimulated a dose-related increase in steroid sulphatase activity and in four experiments was slightly less effective than 1α,25(OH)$_2$D$_3$. The maximum response produced by 100 nM 1α,25-dihydroxylumisterol$_3$ was ~80% of that observed with 1α,25(OH)$_2$D$_3$ and the EC_{50} for 1α,25-dihydroxylumisterol$_3$ stimulated steroid sulphatase activity was 1.5 ± 1.1 nM. The Hill slope of the 1α,25-dihydroxylumisterol$_3$ dose response displayed significant co-operativity being 2.51 ± 0.33 (n = 4, $r^2 = 0.76$).

1β,25(OH)$_2$D$_3$ is a potent antagonist of 1α,25(OH)$_2$D$_3$-stimulated rapid non-genomic responses and has no effect on 1α,25(OH)$_2$D$_3$-stimulated genomic signalling via the VDR$_{nuc}$ [Norman et al., 2001]. 1β,25(OH)$_2$D$_3$ has previously been shown to inhibit 1α,25(OH)$_2$D$_3$-mediated differentiation of NB4 cells [Miura et al., 1999]. Pre-treatment of NB4 cells with 100 nM 1β,25(OH)$_2$D$_3$ significantly inhibited the increases in steroid sulphatase activity produced by both 1α,25(OH)$_2$D$_3$ (n = 7) and 1α,25-dihydroxylumisterol$_3$ (n = 3) (Fig. 2B,C). A value for the concentration of 1β,25(OH)$_2$D$_3$ that is required to inhibit the increase in steroid sulphatase activity produced by both 1α,25(OH)$_2$D$_3$ and 1α,25-dihydroxylumisterol$_3$ can be estimated by visual inspection of the curves and is approximately 20–50 nM.

Two classes of VDR$_{nuc}$ antagonists have been described: the 25-carboxylic esters (e.g., ZK159,222) and the 26,23-lactones (e.g., TEI-9647) [Toell et al., 2001; Carlberg, 2003b]. TEI-9647 fails to block 1α,25(OH)$_2$D$_3$-stimulated differentiation of NB4 cells [Miura et al., 1999]. Treatment of NB4 cells with 1 μM ZK159,222 had only a minimal effect on the maximal increase in steroid sulphatase activity produced by either 100 nM 1α,25(OH)$_2$D$_3$ and 1α,25-dihydroxylumisterol$_3$ (Fig. 2D,E). However, in both cases the mid section of the dose response curves were slightly right-shifted by ZK159,222, such that the slopes of the respective dose response curves were slightly flattened. The concentration of 1α,25(OH)$_2$D$_3$ required for half maximal stimulation of steroid sulphatase activity in NB4 cells was 0.67 ± 0.51 nM (n = 8) and the Hill slope was 3.1 ± 0.6. In the presence of 1 μM ZK159,222, the EC_{50} was slightly increased to 2.1 ± 1.1 nM (n = 8, $P = 0.11$) and the Hill slope was slightly decreased to 1.8 ± 0.35 (n = 8, $P = 0.08$). Similarly, the concentration of 1α,25-dihydroxylumisterol$_3$ required for half maximal stimulation of steroid sulphatase activity in NB4 cells was 2.4 ± 1.14 nM (n = 3) and the Hill slope was 2.61 ± 0.4 In the presence of 1 μM ZK159,222, the EC_{50} was slightly increased to 5.3 ± 1.17 nM (n = 3, $P = 0.05$), and the Hill slope was slightly decreased to 1.74 ± 0.4 (n = 3, $P = 0.03$).

Pharmacological Characterisation of the Vitamin D Receptors Involved in Up-regulating Steroid Sulphatase Activity in the HL60 Myeloid Leukaemia Cell Line

Figure 3A shows that treatment of HL60 cells for 72 h with 1α,25(OH)$_2$D$_3$ or the 'non-genomic' agonist 1α,25-dihydroxylumisterol$_3$ produced dose responsive increases in steroid sulphatase activity. However, the increase in steroid sulphatase activity produced by 1α,25-dihydroxylumisterol$_3$ was significantly lower than that produced by an identical concentration of 1α,25(OH)$_2$D$_3$. For example, the maximum increase in steroid sulphatase activity induced by treatment with 1α,25-dihydroxylumisterol$_3$ was only 25% of the maximum response produced by 1α,25(OH)$_2$D$_3$ (n = 6).

Pre-treatment of HL60 cells with increasing concentrations of the VDR$_{mem}$-specific inhibitor 1β,25(OH)$_2$D$_3$ had only a minimal effect on the

Fig. 3. Pharmacological characterisation of the vitamin D receptors involved in up-regulating steroid sulphatase activity in the HL60 myeloid leukaemia cell line. **Panel** (**A**) shows a comparison of the concentration-response curves for the 1α,25(OH)$_2$D$_3$- or 1α,25-dihydroxylumisterol$_3$-stimulated increase in steroid sulphatase activity in HL60 cells (n = 6). **Panel** (**B**) shows the effects of increasing concentrations of 1β,25(OH)$_2$D$_3$ on the increase in steroid sulphatase activity produced by incubating HL60 cells for 72 h incubation with 100 nM 1α,25(OH)$_2$D$_3$ (n = 4). The effect of pre-treatment with 100 nM 1β,25(OH)$_2$D$_3$ on the 1α,25(OH)$_2$D$_3$ concentration-response curve in HL60 cells is shown in **panel** (**C**) (n = 5). **Panel** (**D**) shows the effects of increasing concentrations of ZK159,222 on the increase in steroid sulphatase activity produced by incubating HL60 cells for 72 h incubation with 100 nM 1α,25(OH)$_2$D$_3$ (n = 4). The effect of pre-treatment with 1 μM ZK159,22 on the 1α,25(OH)$_2$D$_3$ concentration-response curve in HL60 cells is shown in **panel** (**E**) (n = 4). All data points were acquired after 72 h exposure to 1α,25(OH)$_2$D$_3$ or 1α,25-dihydroxylumisterol$_3$. The VDR$_{nuc}$ and VDR$_{mem}$ antagonists were added 2 h prior to the addition of the agonists. All results are shown as means ± SEM from quadruplicate determinations. The concentration-response curves were fitted to a sigmoidal curve, and EC_{50}'s and Hill coefficients and their respective errors were estimated using the 'pharmacology' module of the Sigmaplot™ (version 8.0) graphical software package.

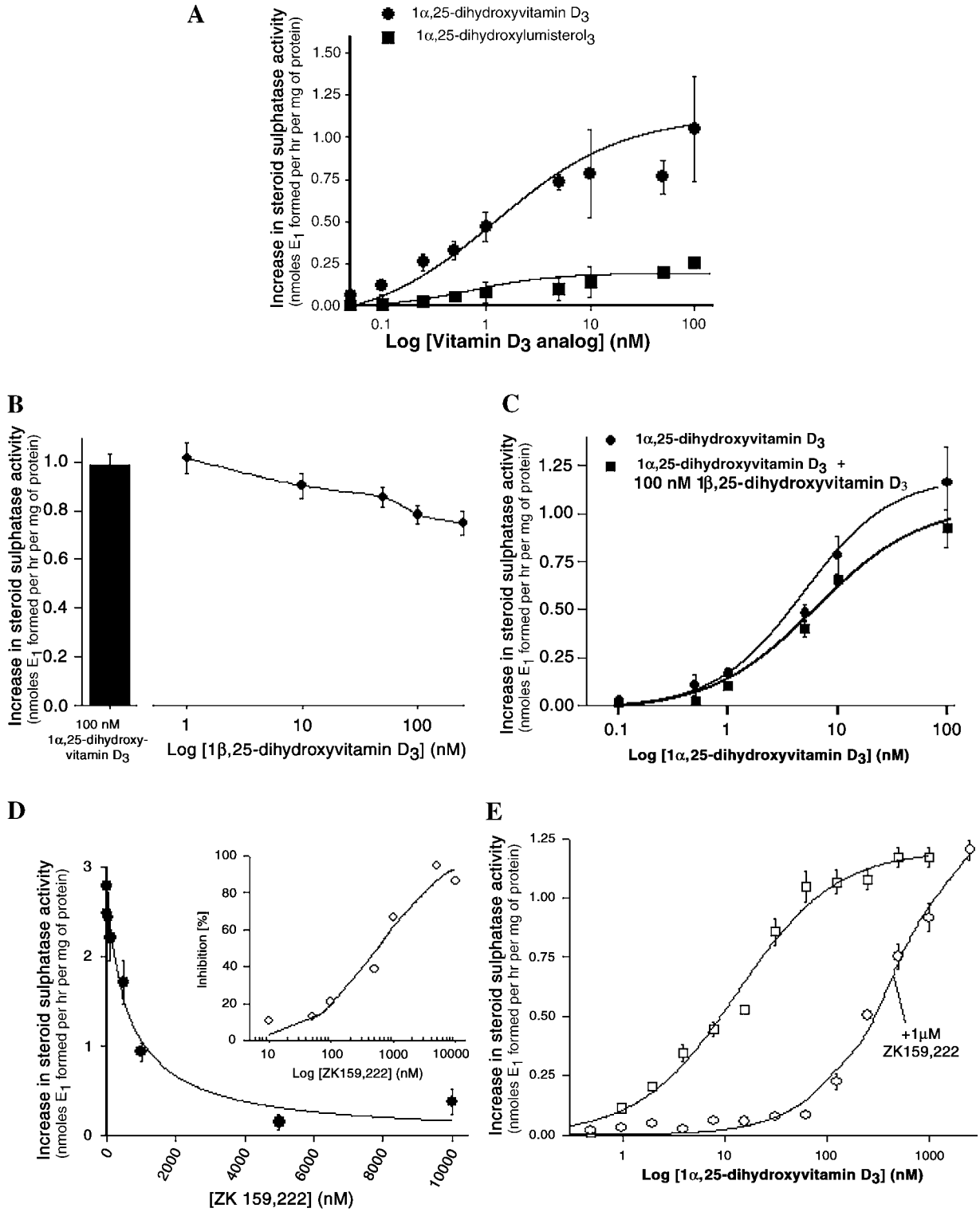

Fig. 3.

increase in steroid sulphatase activity induced by $1\alpha,25(OH)_2D_3$ (Fig. 3B, n = 4 and Fig. 3C, n = 5). In contrast, the VDR_{nuc}-stimulated genomic signalling inhibitor ZK159,222 prevented the increase in steroid sulphatase activity in HL60 cells produced by 100 nM $1\alpha,25(OH)_2D_3$ in a dose responsive fashion. In these experiments, the concentration of

ZK159,222 required to inhibit the increase in steroid sulphatase activity produced by 100 nM $1\alpha,25(OH)_2D_3$ by 50% was 705 ± 89 nM (Fig. 3D, $n = 4$). Figure 3E shows that pre-incubation of HL60 cells with 1 μM ZK159,222 produced a parallel ~30 fold right shift in the $1\alpha,25(OH)_2D_3$ concentration-response curve in HL60 cells. The concentration of $1\alpha,25(OH)_2D_3$ required for half maximal stimulation of steroid sulphatase activity was 13.2 ± 1.1 μM and was significantly increased to 421.9 ± 55.9 μM ($n = 4$, $P < 0.01$) by the presence of ZK159,222. ZK159,222 has been shown to inhibit the $1\alpha,25(OH)_2D_3$-stimulated monocytic differentiation of HL60 cells [Ji and Studzinski, 2004].

RXR Antagonists Block the $1\alpha,25(OH)_2D_3$-Stimulated Increase in Steroid Sulphatase Activity in HL60 Cells but not in NB4 Cells

The $1\alpha,25(OH)_2D_3$-stimulated increase in steroid sulphatase activity in HL60 cells was significantly inhibited by a specific RXR antagonist (AGN195393, 100 nM, Fig. 4A) and was unaffected by a specific RAR antagonist (AGN194310, 100 nM). HL60-Pager D cells express a dominant-negative truncated RARα and do not differentiate in response to RAR agonists but retain their sensitivity to $1\alpha,25(OH)_2D_3$ (F. Farzaneh, unpublished communication). The RAR specific agonist TTNPB did not produce a significant increase in steroid sulphatase activity in HL60-Pager D cells even at concentrations as high as 250–500 nM. 100 nM $1\alpha,25(OH)_2D_3$-stimulated similar increases in steroid sulphatase activity in HL60-Pager D cells and HL60 cells (Fig. 4C). RXR and RAR receptor antagonists had no effect on the $1\alpha,25(OH)_2D_3$-stimulated increase in steroid sulphatase activity in NB4 cells (Fig. 4B).

Depletion of Plasma Membrane Cholesterol Blocks $1\alpha,25$-Dihydroxyvitamin D_3 Stimulation of Steroid Sulphatase Activity in Both HL60 and NB4 Cells

Many of the putative candidates (annexin II, ERp57, PKC) for the VDR_{mem} may be located in specialised caveolin-containing cholesterol-rich micro-domains in the plasma membrane. Similarly, VDR_{nuc} has been shown to be located in caveolin-containing cholesterol-rich plasma membrane micro-domains (caveolae) in NB4 cells and several other cell types [Norman et al., 2002, 2004; Zanello and Norman, 2003, 2004a,b; Huhtakangas et al., 2004; Mizwicki et al., 2004]. Therefore, to test whether caveolae-located VDR_{mem} or VDR_{nuc} play any role in the $1\alpha,25(OH)_2D_3$-stimulated increase in steroid sulphatase activity in NB4 and HL60 cells methyl-β-cyclodextrin (MβCD) and filipin were used to prevent the formation or disrupt the structure of caveolae [Huo et al., 2003].

Prolonged incubation of the myeloid leukaemia cell lines to MβCD or filipin led to unacceptable levels of toxicity (data not shown). Therefore, cells were exposed to the cholesterol depleting agents for 2 h prior to 24 h stimulation with 100 nM $1\alpha,25(OH)_2D_3$ or $1\alpha,25$-dihydroxylumisterol$_3$. Using this protocol cell viability remained at >90% during the time course of the experiment. Figure 5A,B shows that MβCD (10 mM) and filipin (1.2 μg/ml) inhibited the $1\alpha,25(OH)_2D_3$-stimulated increase in steroid sulphatase in a dose-related fashion in both HL60 and NB4 cells. Short-term treatment of HL60 cells with the 'non-genomic' agonist $1\alpha,25$-dihydroxylumisterol$_3$ had a marginal and variable effect on steroid sulphatase activity in HL60 cells (data not shown) so that estimating the effects of cholesterol depletion on the $1\alpha,25$-dihydroxylumisterol$_3$ response was not feasible. However, $1\alpha,25$-dihydroxylumisterol$_3$ stimulated a robust response in NB4 cells and this was significantly reduced by both MβCD and filipin treatment. Re-addition of cholesterol partially reversed the MβCD- (Fig. 5A) and filipin- (Fig. 5B) mediated inhibitions of the $1\alpha,25(OH)_2D_3$-stimulated increase in steroid sulphatase activity in both cell lines and the $1\alpha,25$-dihydroxylumisterol$_3$-stimulated increase in steroid sulphatase activity in NB4 cells. Xylazine, a membrane inserting lipid-binding agent, which does not disrupt lipid raft formation or function [Huo et al., 2003], did not affect the $1\alpha,25(OH)_2D_3$-stimulated increase in steroid sulphatase in either HL60 or NB4 cells (data not shown).

DISCUSSION

In this paper we have shown that $1\alpha,25(OH)_2D_3$ stimulates a concentration dependent increase in steroid sulphatase activity in both HL60 and NB4 acute myeloid leukaemic cell lines. The pharmacological characteristics of $1\alpha,25(OH)_2D_3$ dose-response curves were markedly different in the two cell lines. This suggests that different

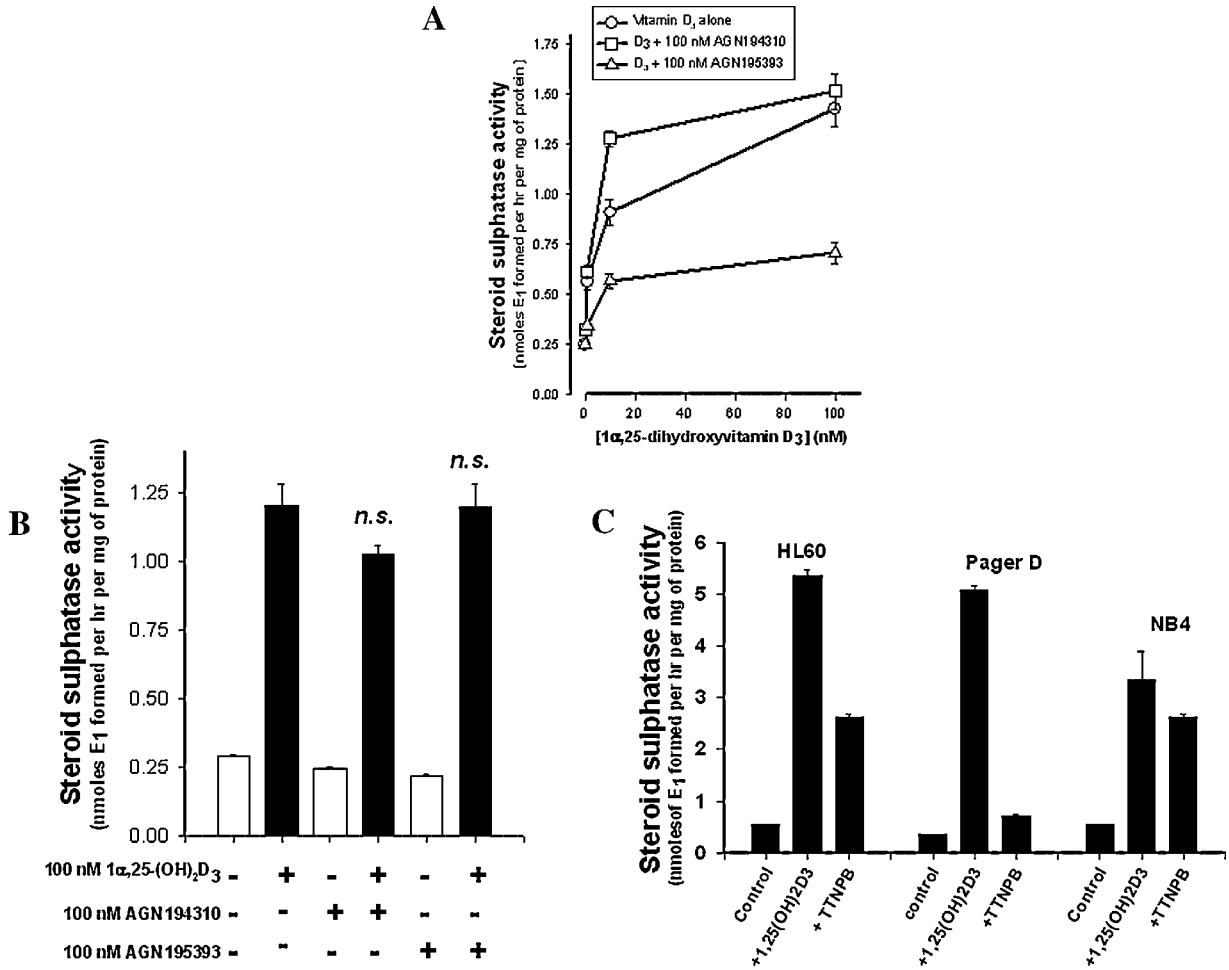

Fig. 4. Comparison of the effects of RXR or RAR specific agonists and antagonists on 1α,25-dihydroxyvitamin D_3-driven steroid sulphatase activity in HL60 and NB4 cells. **Panel** (**A**) shows the effect of 2 h pre-incubation with either 100 nM AGN195393 (RXR specific antagonist □) or 100 nM AGN194310 (RAR specific antagonist, △) on the 1α,25$(OH)_2D_3$-driven increase in steroid sulphatase activity in HL60 cells. **Panel** (**B**) shows the effects of the same compounds on the 1α,25$(OH)_2D_3$-driven increase in steroid sulphatase activity in NB4 cells. Cells were harvested after 72 h treatment with 1α,25$(OH)_2D_3$ and microsomal extracts were prepared as described in the "Experimental Procedures." Data are expressed as the mean ± SEM increase in steroid sulphatase activity obtained in nine experiments (panel A) or six experiments (panel B), that were each performed in quadruplicate. The data in **panel** (**C**) shows that RAR is not required for 1α,25-dihydroxyvitamin D_3 stimulation of steroid sulphatase activity in myeloid cells. HL60, HL60-Pager D and NB4 were treated with either 100 nM 1α,25$(OH)_2D_3$ or 100 nM TTNPB (RAR specific agonist). HL60-Pager D express a dominant negative truncated RAR which blocks RAR-dependent signalling. Cells were harvested after 72 h incubation with the agonist and microsomal extracts were prepared as described in the "Experimental Procedures." Data are expressed as the mean ± SEM increase in steroid sulphatase activity, and were obtained in four experiments, that were each performed in quadruplicate.

receptor-driven mechanisms underpin the 1α,25$(OH)_2D_3$-stimulated elevation of steroid sulphatase activity in NB4 and HL60 cells. Furthermore, the 1α,25$(OH)_2D_3$-stimulated increase in steroid sulphatase activity in both cell lines was abrogated by depletion of plasma membrane cholesterol. This indicates that plasma membrane-derived signals play regulatory roles in the 1α,25$(OH)_2D_3$-mediated increase in steroid sulphatase activity in both cell types.

In NB4 cells, both 1α,25$(OH)_2D_3$ and the 'non-genomic agonist' 1α,25-dihydroxylumisterol$_3$ were equally effective in increasing steroid sulphatase activity (Fig. 2A,B). Similarly, the non-genomic antagonist 1β,25-dihydroxyvitamin D_3 abrogated the increase in steroid sulphatase activity that was stimulated by both 1α,25$(OH)_2D_3$ and 1α,25-dihydroxylumisterol$_3$ (Fig. 2B,C). The VDR_{nuc} genomic antagonist ZK159,222 had a complex effect on the 1α,25$(OH)_2D_3$ and 1α,25-dihydroxylumisterol$_3$-

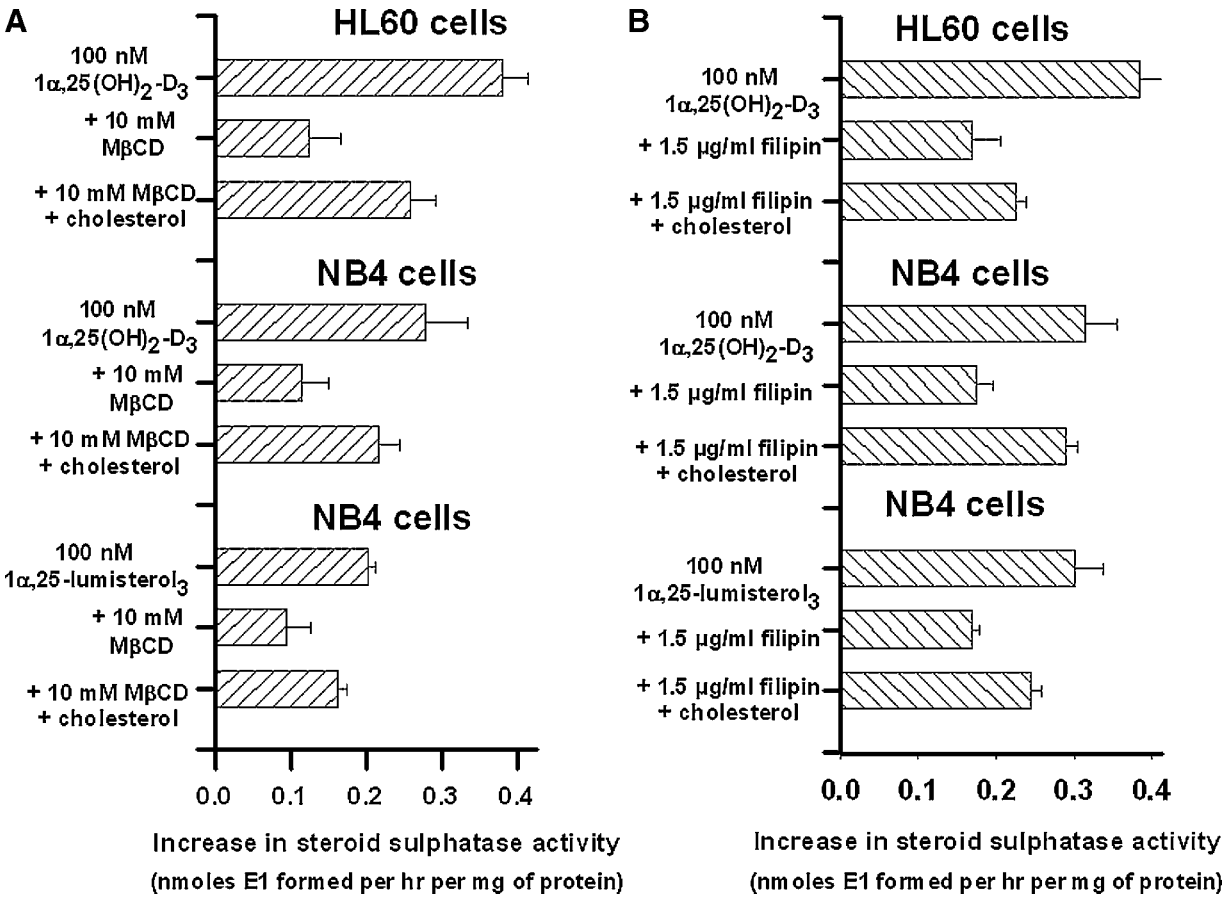

Fig. 5. Disruption of cholesterol-rich lipid rafts blocks 1α,25-dihydroxyvitamin D_3 stimulation of steroid sulphatase activity in both HL60 and NB4 cells. The experiments shown below were performed in serum-free grown HL60 or NB4 cells adapted to growth in 2% FBS. The cell lines were treated with 10 mM methyl-β-cyclodextrin (**panel A**) or 1.5 μg/ml filipin (**panel B**) for 2 h prior to stimulation with 100 nM 1α,25-dihydroxyvitamin D_3 or 100 nM 1α,25-dihydroxylumisterol$_3$. To reverse the depletion of cholesterol 100 μg/ml water-soluble cholesterol was added 30 min before methyl-β-cyclodextrin or filipin. Cells were harvested after 24 h incubation with 1α,25-dihydroxyvitamin D_3 or 1α,25-dihydroxylumisterol$_3$ and microsomal extracts were prepared and steroid sulphatase activity measured as described in the "Experimental Procedures." Data are expressed as the mean ± SEM increase in steroid sulphatase activity over basal and were obtained in six separate experiments for 1α,25-dihydroxyvitamin D_3 and in two experiments for 1α,25-dihydroxylumisterol$_3$. Each performed was performed in quadruplicate.

mediated increases in steroid sulphatase activity in NB4 cells. The maximum increase in steroid sulphatase activity produced by both agonists was not affected by a 100-fold excess of ZK159,222. However, following pre-treatment with ZK159,222 the slopes of the dose-response curve for each agonist were significantly decreased. These results suggest that VDR_{nuc} derived signals do not play an obligatory role in the 1α,25(OH)$_2$D$_3$- or 1α,25-dihydroxylumisterol$_3$-mediated up-regulation of steroid sulphatase activity in NB4 cells and that any loss of genomic or non-genomic signalling via the VDR_{nuc} can be compensated for by non-genomic signals generated from alternate 1α,25(OH)$_2$D$_3$-binding proteins. Other workers have argued that VDR_{nuc}-derived genomic signals are not mandatory for 1α,25(OH)$_2$D$_3$-mediated differentiation of NB4 cells [Bhatia et al., 1995; Berry and Meckling-Gill, 1999; Miura et al., 1999; Berry et al., 2002]. In fact, VDR_{nuc} forms a complex with the PML-RARα fusion protein in NB4 cells, and the latter is unable to interact with the genomic signalling machinery in the nucleus [Puccetti et al., 2002].

In HL60 cells, the 'non-genomic agonist' 1α,25-dihydroxylumisterol$_3$ was significantly less effective than 1α,25(OH)$_2$D$_3$ at increasing steroid sulphatase activity (Fig. 3A). Similarly, the non-genomic antagonist 1β,25-dihydroxyvitamin D_3 failed to have any significant effect on the 1α,25(OH)$_2$D$_3$-mediated increase in steroid

sulphatase activity (Fig. 3B,C). Conversely, ZK159,222 blocked the $1\alpha,25(OH)_2D_3$-mediated increase in steroid sulphatase activity (Fig. 3D,E) suggesting that VDR_{nuc}, rather than an alternative membrane associated vitamin D_3 binding protein, plays a major role in the up-regulation of steroid sulphatase activity in HL60 cells. This is consistent with previous observations that specific VDR_{nuc} antagonists [Miura et al., 1999; Ji and Studzinski, 2004] and DR3-VDRE oligonucleotide decoys [Ji et al., 2002] block $1\alpha,25(OH)_2D_3$-mediated monocytic differentiation of HL60 cells. Observations that RXR antagonists but not RAR antagonists abrogate the $1\alpha,25(OH)_2D_3$-mediated increase in steroid sulphatase activity suggests that formation of and signalling via VDR_{nuc}-RXR heterodimers in the $1\alpha,25(OH)_2D_3$-mediated stimulation of steroid sulphatase activity in HL60 cells. $1\alpha,25(OH)_2D_3$-stimulated VDR_{nuc}-mediated rapid non-genomic signals play only a minor modulatory role in the $1\alpha,25(OH)_2D_3$-stimulated increase in HL60 cells.

A novel plasma membrane protein(s) (VDR_{mem}) has been postulated to be involved in initiating $1\alpha,25(OH)_2D_3$-stimulated rapid non-genomic effects. This hypothesis is supported by observations that several non-VDR_{nuc} binding $1\alpha,25(OH)_2D_3$ analogues act as specific agonists and antagonists of non-genomic signalling pathway [Norman et al., 2001]. The exact molecular nature of the VDR_{mem} is still uncertain. It has been suggested that $1\alpha,25(OH)_2D_3$ binds to proteins such as annexin II [Baran et al., 2000], protein kinase C [Slater et al., 1995] or ERp57 [Nemere et al., 2004]. These proteins are all located in cholesterol-rich plasma membrane micro-domains, and when ligated they couple to and activate a variety of intracellular signalling pathways.

VDR_{nuc}-mediated pathways may play a key role in $1\alpha,25(OH)_2D_3$-activated that are rapidly activated in some cell types. This hypothesis is based on observations that $1\alpha,25(OH)_2D_3$ cannot stimulate ion channels in osteoblasts obtained from VDR_{nuc} knockout mouse [Zanello and Norman, 2004a,b]. Similarly, rapid intracellular signalling pathways are abrogated in human fibroblasts that harbour VDR_{nuc} with inactivating mutations in the DNA or ligand binding domains [Nguyen et al., 2004]. $1\alpha,25(OH)_2D_3$-ligated VDR_{nuc} can associate with specific acceptor sites at the plasma membrane [Kim et al., 1996; Boland et al., 2002]. In fact, in some cell types the plasma membrane specific binding of $1\alpha,25(OH)_2D_3$ is decreased by up to 80% in VDR_{nuc}-knockout mice [Zanello and Norman, 2004a,b]. In avian smooth muscle cells [Capiati et al., 2002] and human breast cancer cells [Capiati et al., 2004], the $1\alpha,25(OH)_2D_3$-ligated VDR_{nuc} is able to shuttle from the nucleus into the plasma membrane. The VDR_{nuc}-chaperones Hsp70 and Hsp90 shuttle between the nucleus and acceptor sites located in cholesterol-rich plasma membrane micro-domains called caveolae [Broquet et al., 2003] and, VDR_{nuc} appears to accumulate in caveolae in avian intestinal cells [Norman et al., 2002] and in NB4 cells [Huhtakangas et al., 2004]. Hence, VDR_{nuc} may be delivered to the plasma membrane as part of a multi-protein complex that contains components of intracellular signalling pathways. It is, perhaps not surprising that microtubule disrupting agents (colchicine) and Hsp90 inhibitors (geldanomycin or 17-allylamino-17-demethoxygeldanomycin) block the $1\alpha,25(OH)_2D_3$-mediated elevation of steroid sulphatase activity in both NB4 and HL60 cells (Hughes and Brown, unpublished observations).

Localisation of VDR_{nuc} in or around caveolae would place it in close proximity to molecules that are involved in regulating many intracellular signalling pathways. The serine/threonine phosphatases PP1C and PP2A are located in caveolae in a complex with unligated VDR_{nuc} that also contains inactive p70S6-kinase. When VDR_{nuc} binds $1\alpha,25(OH)_2D_3$, PP1C and PP2A are released from the complex and p70S6-kinase is activated; this is essential to $1\alpha,25(OH)_2D_3$-monocytic differentiation of HL60 cell [Bettoun et al., 2004]. In THP-1 cells, $1\alpha,25(OH)_2D_3$-ligated VDR_{nuc} has been shown to associate with the cholesterol-rich micro-domain located p85 regulatory subunit of phosphatidylinositol 3-kinase and activate the p110 catalytic subunit [Hmama et al., 1999]. Inhibition of phosphatidylinositol 3-kinase activity blocks $1\alpha,25(OH)_2D_3$-driven differentiation of these cells [Hmama et al., 1999; Lee et al., 2004; Hughes and Brown, unpublished observations].

A new model (the conformation ensemble model) has been developed that explains how rapid non-genomic signalling pathways are activated by plasma membrane located VDR_{nuc} [Norman et al., 2004; Mizwicki et al., 2004a]. The conformation ensemble model proposes that the ability of $1\alpha,25(OH)_2D_3$ to drive both non-genomic and genomic signalling pathways

is determined by the flexibility of 1α,25(OH)$_2$D$_3$ about the 6–7 carbon bond and conformational malleability of VDR$_{nuc}$. Studies with 1α,25(OH)$_2$D$_3$ and conformationally restricted analogues show that 1α,25(OH)$_2$D$_3$ must adopt a 'basket-like' 6-s-*trans* conformation to activate VDR$_{nuc}$-driven genomic responses. Conversely, when 1α,25(OH)$_2$D$_3$ assumes a 'planar' 6-s-*cis* configuration the activation of rapid non-genomic signalling pathways is favoured [Norman et al., 2001]. A second ligand binding pocket has been identified in the VDR$_{nuc}$, which preferentially recognises 1α,25(OH)$_2$D$_3$ in its 6-s-*cis* conformation [Mizwicki et al., 2004a]. Once this pocket is ligated by 1α,25(OH)$_2$D$_3$ in its 6-s-*cis* conformation, the VDR$_{nuc}$ adopts a conformation that permits it to interact with components of intracellular signalling pathways. Therefore, by adopting multiple interchangeable ligand- and environment-determined conformations VDR$_{nuc}$ can activate both 'slow' genomic and 'fast' non-genomic signalling pathways. It must be noted that this hypothesis does not preclude the possibility that other membrane/cytosolic functional 1α,25(OH)$_2$D$_3$ binding protein(s) exist, and does not imply that the VDR$_{nuc}$ is the only 1α,25(OH)$_2$D$_3$ receptor that activates non-genomic signalling events.

In summary, 1α,25(OH)$_2$D$_3$ stimulates the activity of steroid sulphatase in NB4 cells in a complex manner that involves signals that emanate from cholesterol-rich plasma membrane micro-domains rather than a nuclear-located VDR$_{nuc}$-driven genomic signal. These signals may be generated by one or more 1α,25(OH)$_2$D$_3$-binding protein that preferentially recognise 1α,25(OH)$_2$D$_3$ in it 6-s-*cis* non-genomic signalling configuration. One of these binding proteins is likely to be a caveolae-localised VDR$_{nuc}$ but signals emanating from a plasma-membrane localised VDR$_{nuc}$ does not appear to be an absolute requirement for 1α,25(OH)$_2$D$_3$-mediated stimulation of steroid sulphatase activity in NB4 cells. In HL60 cells, a mixture of VDR$_{nuc}$-stimulated genomic and non-genomic signals is important for maximal 1α,25(OH)$_2$D$_3$-stimulated increase in steroid sulphatase activity. In this case the VDR$_{nuc}$-stimulated genomic signals seem to be the dominant component. Several studies have shown that VDR$_{nuc}$/RXR-mediated genomic signals are an absolute requirement for 1α,25(OH)$_2$D$_3$-driven monocytic differentiation of HL60 cells [Miura et al., 1999; Ji et al., 2002; Ji and Studzinski, 2004]. However, 1α,25(OH)$_2$D$_3$-stimulated VDR$_{nuc}$-mediated activation of the ERK and JNK MAP kinase [Ji and Studzinski, 2004], phosphatidylinositol 3-kinase [Lee et al., 2004] and p70 S6 kinase [Bettoun et al., 2004] signalling pathways are also required for 1α,25(OH)$_2$D$_3$-driven monocytic differentiation of myeloid leukaemic cell lines. In fact, for efficient VDR$_{nuc}$/RXR-mediated transactivation of some genes, e.g. the rat and human *CYP24* genes [Dwivedi et al., 2002; Nguyen et al., 2004], 1α,25(OH)$_2$D$_3$ ligated-VDR$_{nuc}$ must activate both the slow genomic and rapid non-genomic signalling pathways. We are currently investigating whether 1α,25(OH)$_2$D$_3$-mediated stimulation of VDR$_{nuc}$ in HL60 cells and VDR$_{mem}$ in NB4 cells activate the same or different signalling pathways and the involvement of these pathways in the 1α,25(OH)$_2$D$_3$-driven increase in steroid sulphatase activity.

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