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Brief Communication

Synthetically Accessible Non-Secosteroidal Hybrid Molecules Combining Vitamin D Receptor Agonism and Histone Deacetylase Inhibition

Joshua Fischer,[1,5] Tian-Tian Wang,[2,5] Dainis Kaldre,[1] Natacha Rochel,[4] Dino Moras,[4] John H. White,[2,3,*] and James L. Gleason[1,*]
[1]Department of Chemistry
[2]Department of Physiology
[3]Department of Medicine
McGill University, Montreal, Quebec H3A 0G4, Canada
[4]Department of Structural Biology and Genomics, Institut de Gènètique et de Biologie Molèculaire et Cellulaire, 67404 Illkirch, France
[5]These authors contributed equally to this work
*Correspondence: john.white@mcgill.ca (J.H.W.), jim.gleason@mcgill.ca (J.L.G.)
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SUMMARY

1,25-Dihydroxyvitamin D_3 (1,25D), the hormonal form of vitamin D, and several analogs have failed as monotherapies for cancer because of poor efficacy or acquired resistance. However, 1,25D analogs are amenable to bifunctionalization. Preclinical studies have revealed combinatorial effects of 1,25D analogs and histone deacetylase inhibitors (HDACi). Secosteroidal hybrid molecules combining vitamin D receptor (VDR) agonism with HDACi displayed enhanced efficacy but are laborious to synthesize. Here, we have developed easily assembled, fully integrated, non-secosteroidal VDR agonist/HDACi hybrids. The most promising are full VDR agonists with ~10-fold lower potency than 1,25D. Structure/function studies revealed that antiproliferative activity against 1,25D-resistant squamous carcinoma cells required VDR agonism and HDACi. Remarkably, modeling and X-ray crystallography reveal non-secosteroidal hybrids bind in the VDR ligand binding domain in the opposite orientation of their secosteroidal counterparts.

INTRODUCTION

Vitamin D was identified as the cure for nutritional rickets and is a critical regulator of calcium homeostasis. However, it is now considered to have pleiotropic actions, including rapidly growing evidence for function as a cancer chemopreventive agent and as a key modulator of immune system function (Deeb et al., 2007; Lin and White, 2004, 2008). These properties are of considerable clinical significance in light of several studies suggesting that vitamin D insufficiency and deficiency are widespread globally (Arabi et al., 2010; Holick, 2007). Vitamin D is converted by hepatic 25-hydroxylation, followed by 1α-hydroxylation in several peripheral tissues into the active metabolite, 1,25-dihydroxyvitamin D_3 (1,25D; Figure 1). Hormonal 1,25D activates the vitamin D receptor (VDR), which functions as a ligand-regulated transcription factor (Afonja et al., 2004). In a classic negative feedback loop, 1,25D stimulates its own metabolism by inducing transcription of the gene encoding the CYP24 enzyme essential for 1,25D catabolism.

The therapeutic potential of 1,25D is limited by its induction of elevated high circulating calcium concentrations (hypercalcemia). A number of 1,25D analogs have been developed that are non-calcemic while still retaining the potential therapeutic effects of 1,25D (Eelen et al., 2007). However, these have generally failed in clinical trials because of poor efficacy or acquired resistance (Deeb et al., 2007). We have been studying the capacity of 1,25D, and its analogs, to inhibit proliferation of head and neck squamous carcinoma cells (HNSCC) (Akutsu et al., 2001; Lin et al., 2002; Prudencio et al., 2001). Whereas proliferation of well-differentiated human SCC25 cells is arrested in G0/G1 (Akutsu et al., 2001), poorly differentiated SCC4 cells are 1,25D-resistant. However, this resistance can be overcome by coadministration of the histone deacetylase inhibitor (HDACi) trichostatin A (TSA). Indeed, SCC4 cells are particularly sensitive to the combination of 1,25D and TSA, consistent with combinatory effects on prostate and breast cancer cell proliferation and survival (Banwell et al., 2004, 2006; Khanim et al., 2004; Rashid et al., 2001).

HDACs are a broad family of nuclear or cytoplasmic proteins that, in combination with histone acetyltransferases (HATs), control the state of acetylation of several histone and nonhistone proteins (Venugopal and Evans, 2011; Walkinshaw and Yang, 2008). Nuclear HDACs can modulate gene transcription by controlling DNA-histone interactions in the nucleosome and through regulation of components of the transcription machinery. HDACis, such as TSA and suberoylanilide hydroxamic acid (SAHA), have been investigated as anticancer agents and, similar to VDR agonists, HDACs induce cell cycle arrest, cellular differentiation and/or apoptosis (Kim et al., 2006; McLaughlin and La Thangue, 2004; Villar-Garea and Esteller, 2004).

Based on the synergistic effects of TSA and 1,25D, we developed a class of therapeutic agents which are hybrids of 1,25D and HDACs (Lamblin et al., 2010a; Tavera-Mendoza et al., 2008). The first molecule in this class, triciferol, merged the secosteroidal backbone of 1,25D with a side-chain derived from TSA. In this design, the terminal hydroxamic acid zinc-binding

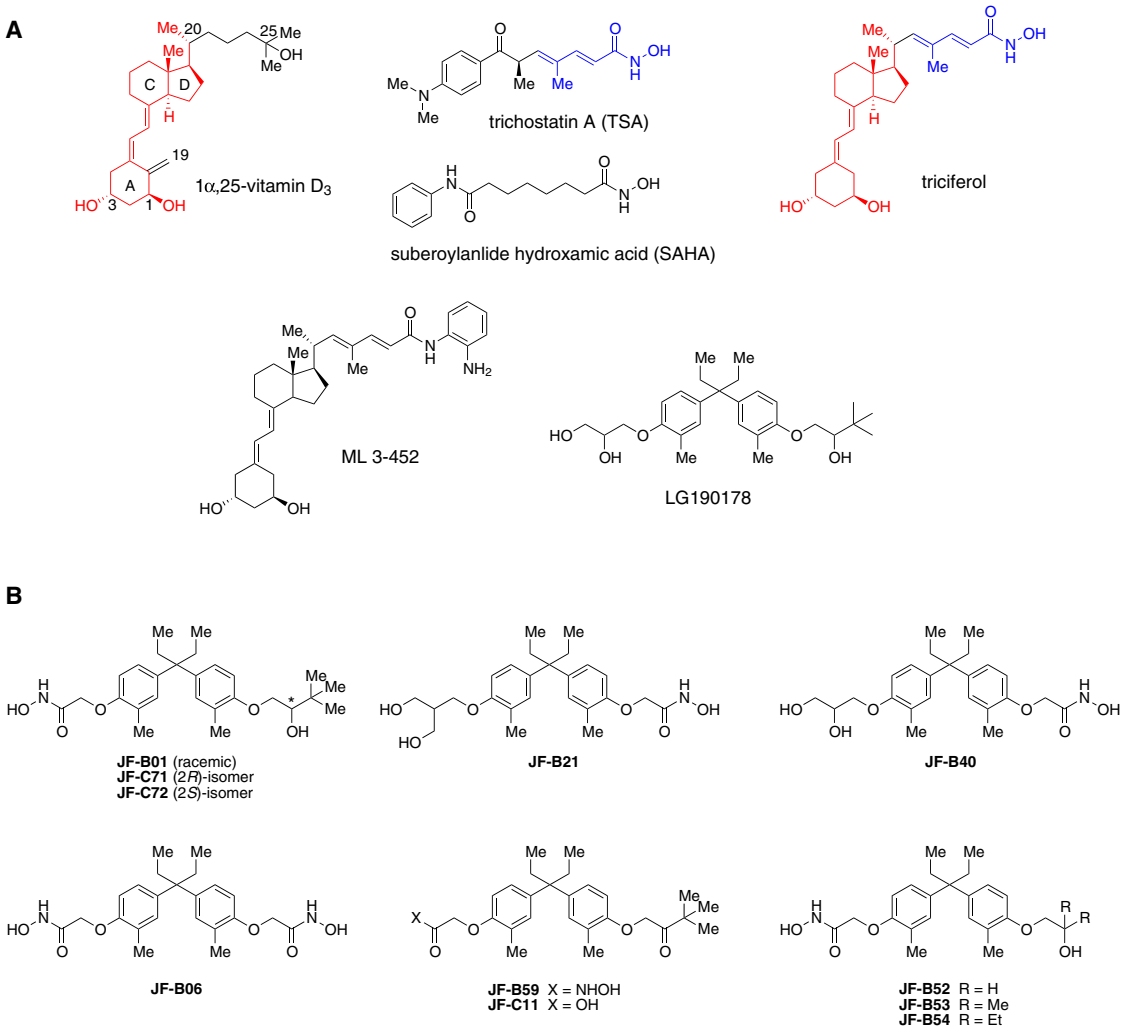

Figure 1. Structures of VDR Agonists, HDACi, and VDR Agonist/HDACi Hybrids

(A) Structures of 1,25D, HDACis TSA and SAHA, secosteroidal 1,25D/HDACi hybrid triciferol, VDR antaognist ML-3-452, and non-secosteroidal VDR agonist LG190178.

(B) Structures of novel non-secosteroidal VDR agonist/HDACi hybrids.

group (ZBG) critical for HDAC inhibition replaced the 25-hydroxy end of 1,25D (Tavera-Mendoza et al., 2008). Triciferol is both a VDR agonist and an HDACi in cells and in culture, and was more efficacious in vitro than 1,25D in inhibition of SCC4 cell proliferation. Further studies developed a series of bifunctional secosteroidal hybrids incorporating variations in side-chain length and substitution, and a variety of ZBGs targeting HDACs (Lamblin et al., 2010a). This revealed that the choice of ZBG could influence HDACi activity and HDAC specificity substantially, with some hybrids inducing histone hyperacetylation (either H3 or H4, or both) and others inducing tubulin hyperacetylation. This work also led to the identification of ML 3-452, which is a strong antagonist due to the combination of its bulky ortho-aminoanilide side group and more rigid side-chain (Lamblin et al., 2010b). A major drawback, however, was that all compounds were based on the structure of 1,25D, and as such, assembly of these hybrids all required lengthy synthetic sequences in excess of 20 steps.

1,25D analog development has largely moved from synthetically challenging secosteroidal backbones to more easily assembled structures identified in high-throughput screens. The 3,3-diarylpentanes, as typified by LG190178 (Boehm et al., 1999; Ma et al., 2006; Swann et al., 2002), are a class of easily synthesized VDR agonists that display potent prodifferentiation and antiproliferative effects, while generally lacking calcemic activity in mice. We sought to synthesize compounds that combined these synthetically accessible backbones with ZBGs necessary for HDAC inhibition.

We report here on the design, synthesis, and functional analysis of several compounds developed in this effort. We have found that non-secosteroidal VDR agonists are readily amenable to bifunctionalization, generating compounds that are efficacious inhibitors of vitamin D-resistant SCC4 cell proliferation and survival. Remarkably, and in stark contrast to secosteroidal hybrids, we find that the hybrid molecules bind in the VDR with the ZBG occupying the A-ring binding pocket. These studies

reveal an unexpected flexibility in our capacity to convert synthetically accessible vitamin D analogs into fully integrated bifunctional compounds with enhanced cytostatic/cytotoxic efficacy. Additionally, this suggests that such a hybrid approach may be amenable to other combinations of pharmocophores.

RESULTS

Design, Synthesis, and VDR Agonism of Non-Secosteroidal Hybrids

Our hybrids were designed using the LG190178 skeleton as a guide. X-ray crystallographic analysis has shown that the 2-hydroxy-3,3-dimethylbutyl group in LG190178 acts as a mimic for the side-chain of 1,25D while the 2,3-dihydroxypropyl group is a mimic for the A-ring (Hakamata et al., 2008). In our design of triciferol, we replaced the side-chain of 1,25D with a dienylhydroxamic acid and we hypothesized that replacement of the 2-hydroxy-3,3-dimethylbutyl group with an acetic hydroxamic acid would yield a bifunctional molecule. Thus, initially, we prepared several hybrids of this form and possessing a variety of A-ring mimics including the one found in LG190178 (JF-B40, -21, and -52). For comparison, we also prepared a symmetric bis-hydroxamic acid (JF-B06) and an analog (JF-B01) where the A-ring mimic was replaced with an acetic-hydroxamic acid. The synthesis of these analogs was easily achieved in 5-8 steps by alkylation of a symmetrical diol prepared by condensation of *o*-cresol with 3-pentanone under acidic conditions (see Supplemental Information available online).

Test compounds (10 μM) were first screened for VDR agonism by analysis of induction of *CYP24* gene expression in 1,25D-sensitive human SCC25 cells. *CYP24* was chosen because its expression is highly induced and exquisitely sensitive to the presence of VDR agonists. Quite unexpectedly, only JF-B01 showed any VDR agonist activity (Figure 2A). In contrast, compounds JF-B21, JF-B40, and JF-B52, all designed using triciferol as a guide, as well as symmetric JF-B06, were completely inactive as VDR agonists at 10 μM. JF-B01 is an efficacious agonist, with a potency equal to or greater than that of LG190178, and approximately 10-fold lower than that of 1,25D (Figure 2B). The carboxylic acid analog of JF-B01 (JF-C11) also showed some agonist activity.

With the observation of VDR agonism of hybrids where the A-ring mimic is replaced with a hydroxamic acid, we prepared several similar hybrids. These showed that the secondary alcohol of JF-B01 is clearly important for VDR agonism, as its ketone derivative (JF-B59) functioned with a >10-fold lower potency (Figure 2B). Similarly, compounds JF-B53 and JF-B54 also showed at least partial VDR agonist activity, but with reduced potency (Figure 2B). They are analogous in structure to JF-B01, but possess tertiary alcohols more similar to those in the side-chains of 1,25D and its analog EB1089 (Colston et al., 1992), respectively. Consistent with binding of hybrids to the VDR, induction of *CYP24* by JF-B01, JF-B53 or JF-B54 was blocked (Figure 2C) by recently developed VDR antagonist ML 3-452 (Lamblin et al., 2010b). JF-B01 also functioned as a full agonist of 1,25D target genes *IL1RL1*, and *CAMP* (Lin et al., 2002; Wang et al., 2004), whereas it consistently superinduced expression of *GADD45* (Figure S1), in agreement with combined effects of VDR agonists and TSA on *GADD45* expression seen in other studies (Banwell et al., 2004; Khanim et al., 2004).

Lead compound JF-B01 was initially prepared in racemic form. However, its VDR agonism, along with the knowledge that the 2′*R* isomer of LG190178 is the more active form (Hakamata et al., 2008), prompted preparation of enantioenriched forms. This was easily accomplished by reduction of the corresponding ketone using the Corey oxazaborolidine catalysts (Corey et al., 1987a, 1987b), to afford the (*R*)- and (*S*)-isomers, JF-C71 and JF-C72 respectively, each in >95% ee (see Supplemental Information). Both enantiomers proved to be VDR agonists with similar potencies (Figure 2D), suggesting that the side-chain can adopt multiple binding conformations in the VDR ligand binding pocket. Moreover, both enantiomers induced binding of the VDR to the vitamin D response element located in the proximal promoter of the *CYP24* gene, as assessed by chromatin immunoprecipitation (ChIP) assay (Figure 2E), consistent with their function as VDR agonists. Direct binding to the VDR of the most potent hybrids JF-C71 and -C72 was confirmed by a fluorescence polarization competition assay (Tavera-Mendoza et al., 2008). JF-C71 and -C72 competed for binding with an IC_{50} of approximately 100 and 35 nM (Figure 2F), respectively, or less than one order of magnitude lower than the 13 nM IC_{50} of 1,25D in this assay (Tavera-Mendoza et al., 2008), and in excellent agreement with studies of VDR agonism above. Taken together, VDR agonist activities and structures of JF-B01, JF-B53, and JF-B54, along with those of inactive compounds, suggest, surprisingly, that the hydroxamic acid moiety is a viable surrogate for the A-ring of 1,25D.

The above structure/function studies were strongly supported by the results of molecular modeling. We used FITTED, a program which proved useful for the design of triciferol, for our studies. Using the protein structure of rat VDR bound to LG190178 (Hakamata et al., 2008), we docked the (*R*)-enantiomer JF-C71 and the (2*S*,2′*R*)-enantiomer of JF-B40 and compared them to the (2*S*,2′*R*)-enantiomer of LG190178 (Figure 3A). Notably, both hybrids docked with the hydroxamic acid in the A-ring binding pocket (Figure 3A, i and ii) despite the large difference in the second substituents. The hydroxamic acid of JF-C71 made significant hydrogen bond contacts to Arg270, Tyr143, and Ser274, while JF-B40 made close contact with Arg270 and Ser233. Of the two, the hydroxamic acid JF-B40 more closely resembled the conformation of the diol in LG190178 (Figure 3A, iii and iv). Importantly, the overall orientation puts the polar diol of JF-B40 in the side-chain binding pocket. This forcing of the diol into the side-chain pocket is presumably responsible for the poorer binding score observed in FITTED and its lack of activity described above. Importantly, while the secondary alcohol makes a similar hydrogen bond contact with His301 as LG190178, the primary alcohol docks in the same location as the hydrophobic *tert*-butyl group (Figure 3A, iv). This mismatch is presumably the most significant factor in the loss of activity of JF-B40 and related hybrids.

Crystal Structure of JF-C71 and JF-C72 Bound to the Zebrafish-VDR Ligand Binding Domain

The modeling data above were substantially corroborated by crystal structures of JF-C71 and JF-C72 bound to the

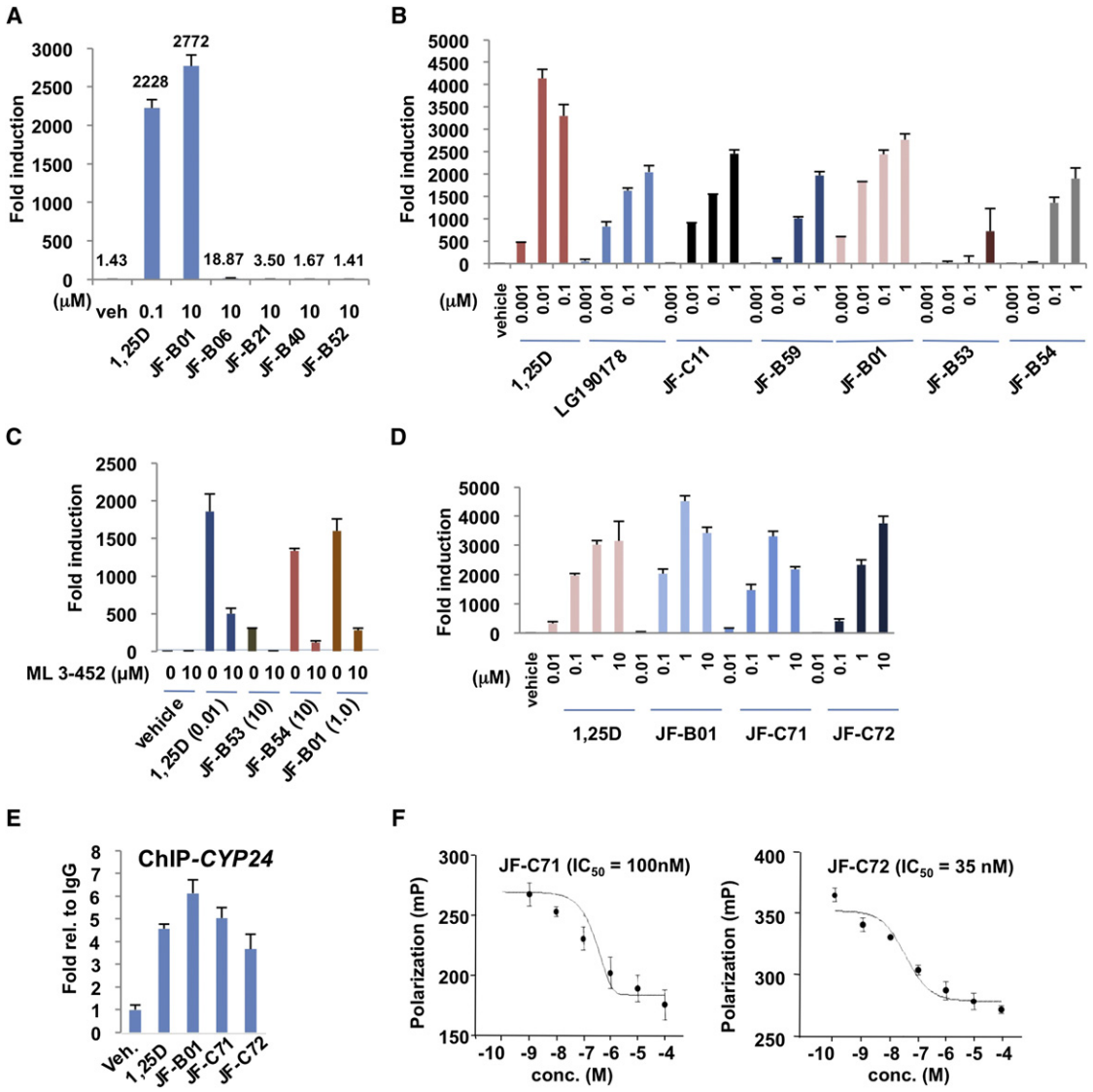

Figure 2. Identification and Characterization of Non-Secosteroidal VDR Agonists

(A) Initial screening by RT/qPCR for induction of *CYP24* expression in SCC25 cells by JF-B01, -B06, -B21, -B40, and -B52 (10 μM).

(B) Dose-dependent *CYP24* induction by JF-B01, -B04, -B11, -B53, -B54, and -B59, along with 1,25D as a positive control.

(C) *CYP24* expression induced by JF-B01 (1 μM), or -B53 and -B54 (10 μM) is inhibited by VDR antagonist ML-3-452 (3; 10 μM). See also Figure S1A.

(D) Enantiomers of JF-B01, JF-C71 and -C72, are agonists of the VDR as measured by CYP24 induction.

(E) JF-B01, -C71, and -C72 (1 μM) induce DNA binding of the VDR to the proximal promoter region of the *CYP24* promoter as measured by chromatin immunoprecipitation (ChIP) assay.

(F) JF-C71 and -C72 compete for binding of a fluorescent tracer to the VDR ligand binding domain with apparent IC_{50} of 100 and 35 nM, respectively, as measured by fluorescence polarization assay.

Error bars represent SEM.

zebrafish-VDR (zVDR) LBD, solved at a resolution of 2.4 and 2.33 Å, respectively (see Figure 3B, i and ii; Supplemental Information; and Table S1 for details). Note that the human-, rat-, and zebrafish-VDR ligand binding pockets are lined with identical residues and have homologous structures (Ciesielski et al., 2007). Both complexes display the canonical agonist conformation of all previously reported structures of VDR bound to agonist and superagonist ligands with helix H12 folded in the agonistic position (Antony et al., 2010; Ciesielski et al., 2007; Rochel and Moras, 2006). When compared to the zVDR LBD-1,25D structure, the Cα atoms of the zVDR LBD-JF-C71 and zVDR LBD-JF-C72 complexes have a root mean square deviation (rmsd) of 0.382 and 0.386 Å, respectively over 248 residues. The ligands are buried in the predominantly hydrophobic pocket and adopt the same orientation as YR301, the (*2S,2′R*)-stereoisomer of LG190178 (Figure 3B, iii; Kakuda et al., 2008). The diethyl groups occupy the space filled by the CD-rings of the natural 1,25D ligand. JF-C71 and JF-C72 form similar

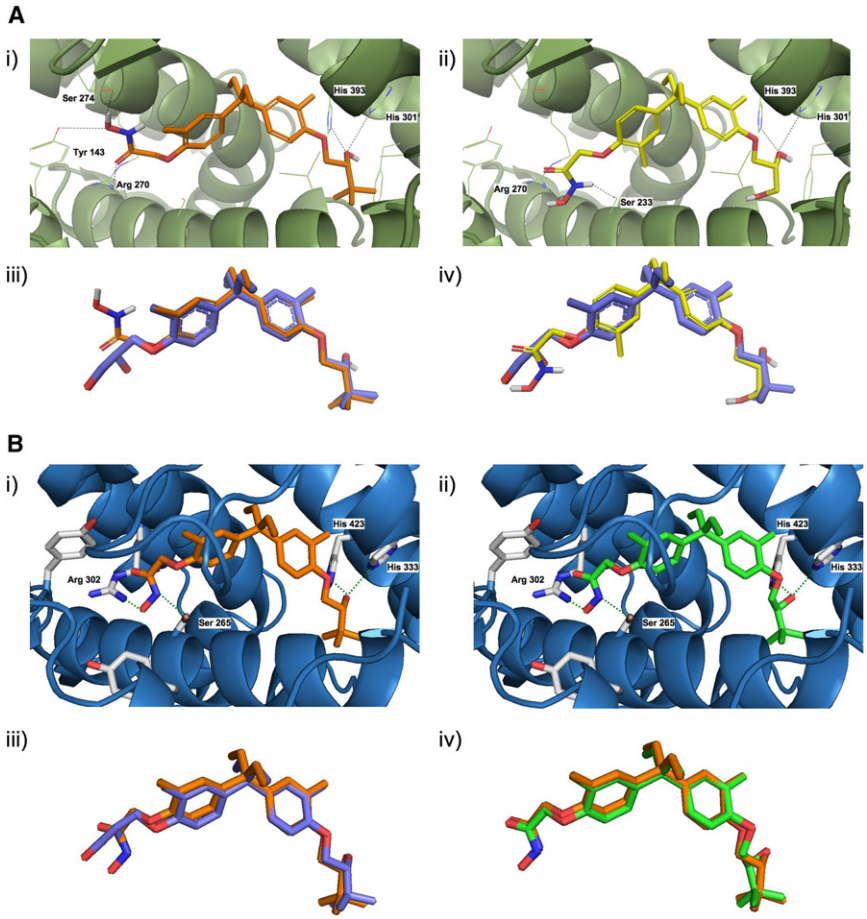

Figure 3. Modeling and X-Ray Structural Data

(A) Structures obtained from docking of ligands to VDR (PDB file 2ZFX) with helix 6 deleted for clarity. Docking solutions of (i) JF-B01 and (ii) JF-B40 in VDR indicating the binding of the hydroxamic acid in the A-ring binding pocket. Note that protein chain between amino acids 284–291 has been removed for clarity. Overlays of (iii) JF-B01 (orange) and (iv) JF-B40 (yellow) with LG190178 (blue) showing the correlation of the hydroxamic acid in JF-B01 with the diol of LG190178 and the placement of the 2-OH group of JF-B40 in the same space as a methyl group in LG190178.

(B) X-ray crystallographic structures of (i) JF-C71 and (ii) JF-C72 bound to zVDR LBD. (iii) Overlay of JF-C71 (orange) with YR-301 (blue), the (*2S,2′R*)-stereoisomer of LG190178, showing excellent correlation of binding conformation. (iv) Overlay of JF-C71 (orange) with JF-C72 (green) showing minor conformational changes at the C2-OH group.

interactions with the zVDR ligand binding pocket with the hydroxamate groups pointing toward helix H1 of the zVDR LBD and occupying the 1,25D A-ring binding pocket. The hydroxamic acid side-chain adopts a conformation slightly different than that predicted by the modeling. The hydroxy group interacts through an H-bond with Arg302 (2.6 Å for JF-C71, 3.0 Å for JF-C72). This interaction directly mimics that of the C1-OH of 1,25D in the LBP (Ciesielski et al., 2007). In addition, the NH forms a hydrogen bond with Ser265 (2.5–2.6 Å). This hydroxamic acid conformation was predicted in the modeling above for JF-B40 but not for JF-C71 where the hydroxamate OH adopted a similar position to C3-OH in 1,25D. Given the polarity of the binding pockets for both C1-OH and C3-OH in 1,25D, this minor discrepancy between modeling and X-ray data was not surprising. Van der Waals interactions of the OH and carbonyl group are observed with Tyr264, Arg302, and Tyr175. Those residues are also interacting with water molecules forming the water channel that was previously described near the C2 position of 1,25D. Due to the inverse configuration of the alcohol at C2′, the stereoisomers adopt slightly different side-chain conformations (Figure 3B, iv) that result in a similar position of the hydroxyl group interacting through hydrogen bonds with His333 and His423 (distances of interactions of 2.6 Å and 3.0 Å for JF-C71, and 2.7 Å and 3.2 Å for JF-C72). Taken together, the above structural studies demonstrate that the hydroxamic acid moiety is a viable surrogate for the A-ring of 1,25D, and confirm the remarkable structural flexibility in conversion of 1,25D analogs into fully integrated bifunctional molecules.

HDACi Activities of Test Compounds

Several of the compounds developed above were initially tested for their capacity to inhibit purified class I HDAC3 and class II enzyme HDAC6 in vitro (Wegener et al., 2003a, 2003b) using SAHA as a positive control. Compounds tested inhibited HDAC3 in vitro with IC_{50} values between 13 and 36 μM, which compares very favorably with the IC_{50} of triciferol for this enzyme (13.3 μM; Lamblin et al., 2010a), but about 500- to 1000-fold lower than that of SAHA (Table 1). With the exception of JF-B06, IC_{50} values for inhibition of HDAC6 in vitro were in a very similar range at around 30 μM, or 50-fold lower than SAHA. JF-B06, unlike other compounds, is flanked by dual hydroxamic ZBGs and displayed an approximate 10-fold improved potency for HDAC6 (4.7 μM). Finally, we assessed JF-C71 for its activity against HDAC1 and HDAC2, where it showed 14.3 μM and 6.0 μM potency, respectively. Thus these hybrids appear to be effective against a range of HDACs.

Several VDR agonists as well as JF-B40, which was inactive as an agonist, were also tested along with 1,25D for HDACi activity using a fluorogenic substrate in vivo (Ciossek et al., 2008) in SCC25 and SCC4 cells (Figures 4A and 4B). This assay measures the inhibition of total intracellular HDAC activity and determines the capacity of compounds to inhibit HDACs in the form of their naturally occurring complexes. While an increasing concentration of 1,25D had no effect on substrate deacetylation in either cell line, all compounds tested displayed dose-dependent HDAC inhibition, with JF-B54 being the most potent. Notably, unlike 1,25D, JF-B01, -C71, and -C72 also induced histone H3 acetylation on the *CYP24* promoter as assessed by ChIP assay, which is consistent with their HDACi activity (Figure S2).

Table 1. HDACi Activity of Selected Compounds Determined Using a Standard Fluorescence Assay against Purified HDACs

Compound	HDAC3	HDAC6
SAHA	33.8 nM	580 nM
JF-B06	13.4 μM	4.7 μM
JF-B21	36.1 μM	37.9 μM
JF-B53	17.0 μM	33.3 μM
JF-B54	29.9 μM	31.5 μM
JF-C71	22.0 μM	26.1 μM
	HDAC1	HDAC2
SAHA	287 nM	516 nM
JF-C71	14.3 μM	6.0 μM

Purified HDACs were obtained from Cayman Chemical. See Wegener et al., 2003a and 2003b.

Cytostatic/Cytotoxic Activities of Bifunctional Compounds

JF-B01, JF-B53, and JF-B54 were further tested for their capacity to inhibit the proliferation of 1,25D-sensitive (SCC25 and AT84) or 1,25D-resistant (SCC4) HNSCC lines. As expected (Akutsu et al., 2001; Lin et al., 2002; Prudencio et al., 2001), proliferation of SCC25 or AT84 cells was strongly inhibited by 1,25D (Figures 4C and 4D), as measured by incorporation of the nucleoside analog EdU. All test compounds blocked EdU incorporation with relative potencies that were roughly parallel to their capacity to induce *CYP24* expression. JF-B01 was 10-fold less potent than 1,25D in these assays. JF-B06 and JF-B21, which possess HDACi activity but are devoid of VDR agonism, failed to block AT84 proliferation (Figure 4D). Remarkably, whereas SCC4 cell proliferation was resistant to 1,25D, it was found that JF-B01, JF-B53, or JF-B54 partially or totally blocked EdU incorporation (Figure 4E; Figure S3). Significantly, LG190178 and JF-C11, the carboxylic acid analog of JF-B01 which displays VDR agonism, and HDACi JF-B21 all failed to block SCC4 proliferation (Figure S4), consistent with the efficacy of JF-B01 being dependent on both VDR agonist and HDACi activity. It is also noteworthy that, unlike 1,25D, hybrid compounds completely abolished EdU incorporation in SCC25 cells and in SCC4 cells at higher concentrations. Corresponding plates treated with compounds were free of cells after 48h of treatment, consistent with dose-dependent reduction in live cells as measured by a fluorescence-based cytotoxicity assay (Figure 4F) or by liberation of lactate dehydrogenase activity from dying cells (Figure S5). Death appeared to occur by apoptosis, as, like positive control staurosporin, hybrid compounds induced substantial levels of apoptosis-associated caspase activity (Figure S6; data not shown). Finally, JF-B01 was assessed by the National Cancer Institute in their 60 cell panel of human carcinoma cell lines. It displayed antiproliferative activity in all cell lines, with the most promising results in melanoma cell lines with full growth inhibition at 7 μM or less in eight out of nine cell lines (see Supplemental Information; Figure S7).

DISCUSSION

In our original work, we developed triciferol, designed to combine nuclear receptor agonism with HDACi activity in a single stable (i.e., non-prodrug) entity (Tavera-Mendoza et al., 2008). While this molecule provided excellent proof-of-principle for the concept and possessed enhanced cytostatic and cytotoxic activity, its lengthy synthesis beginning from ozonolytic degradation of vitamin D_2 made analog development laborious and expensive and limited significant variation to mainly the side-chain. The non-secosteroidal hybrids described herein have several advantages. They are easily prepared in 5–10 steps from inexpensive starting materials. In addition, because they differ structurally from 1,25D, they are very likely to be resistant to catabolism by CYP24 activity, which is strongly induced by VDR agonists. The most promising of these novel hybrids, JF-B01 and its enantioenriched forms JF-C71 and JF-C72 are at least as efficacious as 1,25D in inducing VDR DNA binding as assessed by ChIP assay, in stimulating *CYP24* gene transcription, and they induce *CYP24* expression with a potency of ~10-fold lower than that of 1,25D. This profile is highly comparable to the VDR agonist activity observed for triciferol (Tavera-Mendoza et al., 2008). While the potencies of non-secosteroidal compounds as HDAC6 inhibitors are reduced by about one order of magnitude relative to triciferol and other secosteroidal hybrids (Lamblin et al., 2010a; Tavera-Mendoza et al., 2008), their HDAC3i activity in vitro is essentially the same.

Comparative studies in 1,25D-sensitive and 1,25D-resistant HNSCC lines provided several insights into the biochemical activities required for antiproliferative activity. JF-B01 inhibited proliferation of 1,25D-sensititve SCC25 and AT84 cells about 10-fold less potently than 1,25D. However, JF-B06 and JF-B21, which possess HDACi activity, but are not VDR agonists, were devoid of activity in this assay. JF-B21 was also inactive against 1,25D-resistant SCC4 cells, suggesting that HDAC inhibition on its own does not afford cytostatic activity in this assay. Similarly, JF-C11, the precursor of JF-B01 which is a VDR agonist but lacks a ZBG, and the parent compound LG190178 were inactive against SCC4 cells. These data strongly argue that the cytostatic efficacy of hybrids JF-B01, -B53 and -B54 in SCC4 cells stems from their specific combination of both VDR agonism and HDACi activity. In addition, the gain of function in these hybrids apparently does not require potency for HDAC inhibition to be in the nanomolar range: all three hybrids are 50–1000 times less potent than SAHA in in vitro assays yet function effectively in cells.

A surprising revelation of these studies was that hybrids were active as VDR agonists only when the A-ring mimic diol of LG190178 was replaced with a hydroxamic acid. This observation was strongly supported by docking studies in the human VDR, which placed the hydroxamic acid in the A-ring binding pocket and confirmed by X-ray crystallography of JF-C71 and JF-C72 bound to the zebrafish-VDR. JF-C71 and JF-C72 form similar interactions with the zVDR LBD with the hydroxamate groups pointing toward the end of helix H1. The stereoisomers adopt different side-chain conformations that result in a similar position of the hydroxyl group interacting through hydrogen bonds with His333 and His423. These findings stand in sharp contrast to both triciferol and its analogs where the ZBG takes the place of the C25-OH in 1,25D, and highlights the remarkably high synthetic latitude in creating hybrid VDR agonist/HDACi hybrids.

Part of this flexibility arises from the structural latitude available in the generation of HDACis. HDACis are composed of

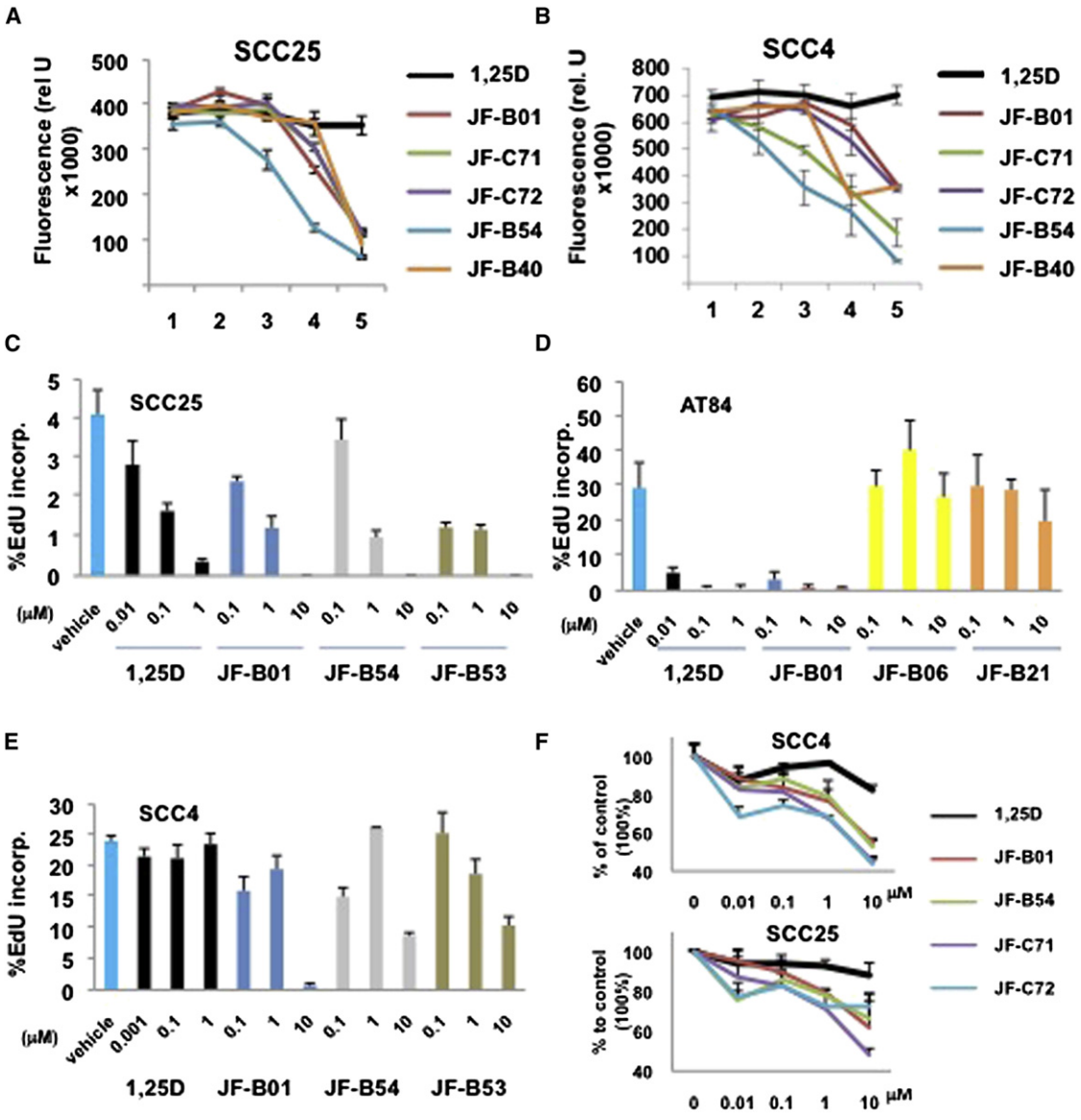

Figure 4. HDACi and Cytostatic Activities of Hybrid Compounds

(A and B) HDACi activity of compounds was measured in live SCC25 or SCC4 cells, as indicated, as described in Supplemental Experimental Procedures. HDACi activity of non-secosteroidal compounds was measured in 10-fold increments between 1nM (1) and 10 μM (5), whereas 1,25D was tested between 0.1 nM (1) and 1 μM (5). See also Figure S2.

(C–E) The dose-dependent antiproliferative activities of 1,25D or non-secosteroidal compounds were assessed by measuring incorporation of fluorescent nucleoside analog EdU in 1,25D-sensitive SCC25 cells (C), or AT84 cells (D), or 1,25D-resistant SCC4 cells (E). See also Figures S3 and S4.

(F) Measurement of SCC4 or SCC25 viability in the presence of increasing concentrations of 1,25D or hybrid molecules indicated (Supplemental Experimental Procedures). See also Figures S5 and S6.

Error bars represent SEM.

ZBGs fused to linkers coupled to so-called cap groups, which can be highly heterogeneous in structure, ranging from derivatized aromatic rings (e.g., SAHA) to cyclic peptides (Paris et al., 2008; Villar-Garea and Esteller, 2004). In the molecules developed here, the derivatized 3,3-diarylpentane would serve as both side-chain and cap group. However, the finding that, depending on the hybrid, the ZBG can either occupy A-ring binding site of the VDR ligand binding domain as in the non-secosteroidal hybrids described here, or replace the 25-OH group of 1,25D as found for triciferol and its analogs, was unexpected. This suggests therefore that there remains substantial latitude for structure-guided optimization of the potencies of both the VDR agonist and HDACi activities of hybrids. It also suggests that it is reasonable to expect that other classes of drugs can be bifunctionalized. Although bifunctionalization would likely lead to a loss of potency for the primary target, addition of activity against a second, sympathetic biochemical target would be expected to increase clinical efficacy.

SIGNIFICANCE

The active form of vitamin D, 1,25-dihydroxyvitamin D (1,25D) has attracted broad interest because of its anticancer properties and its potential as a chemopreventive agent. However, 1,25D and several analogs have failed as cancer therapies because of poor efficacy or acquired resistance. Previous work has shown that 1,25D/HDACi hybrids in which zinc-binding groups necessary for HDACi replaced the 25-hydroxy terminus of the 1,25D secosteroid are more effacious than 1,25D against numerous cancer cell lines, including 1,25D-resistant cell lines. These hybrids are remarkable, as HDACi activity was fully integrated into the secosteroidal backbone of a VDR agonist. However, the secosteroidal nature of the hybrids severely limited their synthetic accessibility. Here, we have shown that it is possible to create easily assembled non-secosteroid-based hybrids that function efficaciously as both VDR agonists and HDACis. Moreover, our studies show that, contrary to secosteroidal hybrids, the optimal location for incorporating the highly hydrophilic hydroxamic acid corresponds to the portion of the molecules that serve as secosteroidal A-ring mimetics. The best hybrid, JF-B01, is a full VDR agonist as assessed by several criteria, and an efficacious antiproliferative agent against both 1,25D-sensitive (SCC25, AT84) and 1,25D-resistant (SCC4) squamous carcinoma cell lines. Importantly, activity in 1,25D-resistant SCC4 cells required both the VDR agonism and HDACi activity of JF-B01. Furthermore, profiling by the NCI in a 60-cell line screen revealed it to be active against a wide-range of cancer cell lines, including melanoma where it displayed full growth inhibition at low micromolar concentrations in eight out of nine cell lines. Finally, in conjunction with previous studies on secosteroidal hybrids, the work presented here reveals the remarkable flexibility in conversion of 1,25D analogs into fully integrated bifunctional molecules, suggesting that it may be possible to extend fully integrated bifunctionalization to other pharmacophores.

EXPERIMENTAL PROCEDURES

All details about the Experimental Procedures used are given in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2012.05.024.

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