Bioorganic & Medicinal Chemistry 21 (2013) 2241–2249

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

VBP15: Preclinical characterization of a novel anti-inflammatory delta 9,11 steroid

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ARTICLE INFO

Article history:
Received 24 December 2012
Revised 3 February 2013
Accepted 11 February 2013
Available online 18 February 2013

Keywords:
Glucocorticoids
VBP15
Steroids
Anti-inflammatories
Neuromuscular disease

ABSTRACT

Δ9,11 modifications of glucocorticoids (21-aminosteroids) have been developed as drugs for protection against cell damage (lipid peroxidation; lazaroids) and inhibition of neovascularization (anecortave). Part of the rationale for developing these compounds has been the loss of glucocorticoid receptor binding due to the Δ9,11 modification, thus avoiding many immunosuppressive activities and deleterious side effect profiles associated with binding to glucocorticoid and mineralocorticoid receptors. We recently demonstrated that anecortave acetate and its 21-hydroxy analog (VBP1) do, in fact, show glucocorticoid and mineralocorticoid receptor binding activities, with potent translocation of the glucocorticoid receptor to the cell nucleus. We concluded that Δ9,11 steroids showed novel anti-inflammatory properties, retaining NF-κB inhibition, but losing deleterious glucocorticoid side effect profiles. Evidence for this was developed in pre-clinical trials of chronic muscle inflammation. Here, we describe a drug development program aimed at optimizing the Δ9,11 chemistry. Twenty Δ9,11 derivatives were tested in in vitro screens for NF-κB inhibition and GR translocation to the nucleus, and low cell toxicity. VBP15 was selected as the lead compound due to potent NF-κB inhibition and GR translocation similar to prednisone and dexamethasone, lack of transactivation properties, and good bioavailability. Phamacokinetics were similar to traditional glucocorticoid drugs with terminal half-life of 0.35 h (mice), 0.58 h (rats), 5.42 h (dogs), and bioavailability of 74.5% (mice), and 53.2% (dogs). Metabolic stability showed ⩾80% remaining at 1 h of VBP6 and VBP15 in human, dog, and monkey liver microsomes. Solubility, permeability and plasma protein binding were within acceptable limits. VBP15 moderately induced CYP3A4 across the three human hepatocyte donors (24–42%), similar to other steroids. VBP15 is currently under development for treatment of Duchenne muscular dystrophy.

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1. Introduction

Δ9,11 Steroids (21-aminosteroids) have been designed and developed to stably incorporate into cell membranes and inhibit lipid peroxidation without glucocorticoid or mineralocorticoid activities, thus avoiding side effects associated with traditional corticosteroids.[1] A few Δ9,11 steroids brought to clinical trials were Lazaroids and Anecortave. Lazaroids have extensive hydrophilic head groups designed to optimize stability in membranes and lipid peroxidation inhibitory activities (Fig. 1). Randomized controlled trials of tirilazad mesylate in acute spinal cord injury,[2,3] and in stroke[4–6] showed good safety profiles, but failed to show clear evidence of efficacy. Pre-clinical work in additional indications continues, including multiple wound states and lung disease.[7–9]

Anecortave was developed for inhibition of neo-vascularization in age-related macular degeneration as a molecule that lacked immunomodulatory glucocorticoid activity.[10,11] The mechanism of action is thought to be through inhibition of proliferation of vascular endothelial cells, inhibition of uPA and matrix metalloproteinase 3, and stimulation of plasminogen activator inhibitor-1.[11] Controlled clinical trials of posterior juxtascleral depot (delivered at 6 month intervals) showed no safety issues, but marginal clinical benefit.[12,13] More recently, blinded controlled trials of anecortave in steroid-induced elevated intraocular pressure (IOP) have been carried out, with similar results of good safety profile.[14] The best dose of anecortave acetate showed a 31% reduction in IOP and this benefit lasted a mean duration of 56 days from a single administration.[15,16]

Previous drug development programs pursuing lazaroids and anecortave as prototype Δ9,11 glucocorticoids (21-aminosteroids) have assumed that the Δ9,11 modification inhibited drug binding to the glucocorticoid receptor (GR) and mineralocorticoid receptor

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http://dx.doi.org/10.1016/j.bmc.2013.02.009

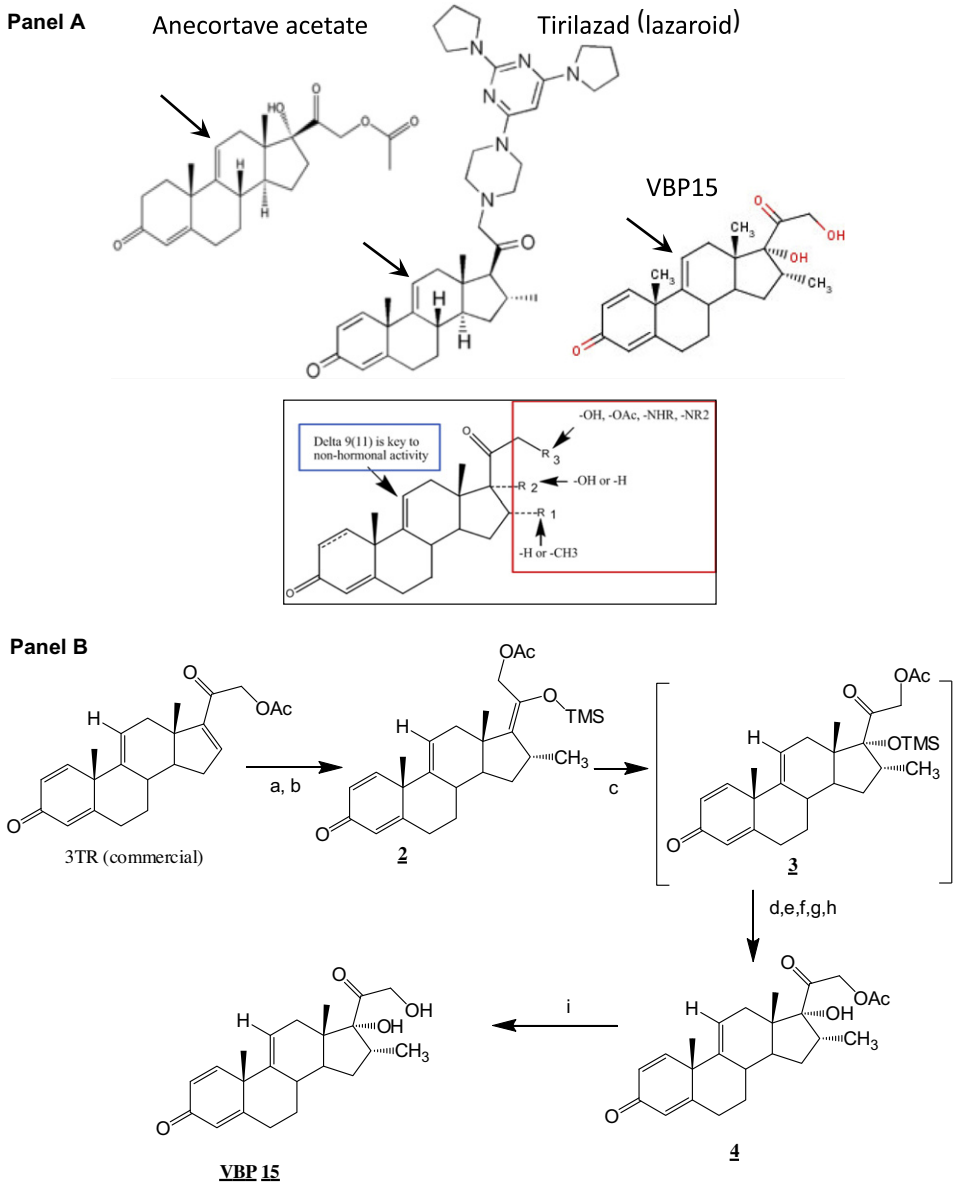

Figure 1. Structures and synthesis of Δ9,11 compounds. Panel A: Shown are two previously developed Δ9,11 steroids (Anecortave, Tirilizad), and the lead compound identified in the current study (VBP15). The Δ9,11 bond distinguishing these drugs from other steroidal compounds is shown by the black arrows. The lower panel shows the chemical modifications to the chemical backbone. The R1, R2, and R3 residues shown are those that were chemically modified with alternative chemical structures (see red box). Panel B: The synthesis of VBP15 that is pictured is typical for the synthesis of analogs in this series. (a) TMS imidazole, MeMgCl, THF; (b) $CuAc_2$, H_2O, DMPU, MeMgCl, THF; (c) peracetic acid, toluene, −10 °C; (d) $NaHSO_3$, TFA; (e) EtOAc, heptanes; (f) acetonitrile trituaration; (g) HBr, HC_2Cl_2, 40 °C; (h) MeOH crystallization; (i) K_2CO_3, MeOH, followed by HCL and crystallization from $MeOH/H_2O$.

(MR). This assumption was based largely on the lack of efficacy of the drugs in inhibiting LPS-induced inflammation in pre-clinical models—traditional glucocorticoids are impressively effective at blocking lipopolysaccharide (LPS)-induced acute inflammation.[10] However, we recently showed that both anecortave acetate and its 21-hydroxy analog (VBP1) showed relatively high affinity binding to both the GR and MR.[17] The Δ9,11 drugs induced GR translocation from the cell cytoplasm to cell nucleus, shown by both reporter and immunostaining assays, again suggesting effective ligand/receptor interactions. Nuclear translocation of steroid hormone receptors is a hallmark of classic glucocorticoid transcriptional activity, and is necessary for downstream activation of gene promoters via binding of ligand/receptor complexes to glucocorticoid response elements (GREs). However, neither Δ9,11 drug showed significant GRE-mediated transcriptional activities, either by mRNA microarray, targeted RT-PCR, or luciferase reporter constructs.[17] On the other hand, both drugs retained anti-inflammatory activity via inhibition of NF-κB pathways, and showed efficacy in inhibiting inflammation in vivo in two mouse models of chronic inflammation in muscle (dystrophin-deficient *mdx* and dysferlin-deficient *SJL* mice).[17] The discrepancy between previous findings of lack of anti-inflammatory activity, and our findings of retention of anti-inflammatory activity may be explained by the assays utilized. For example, McNatt et al. studied an acute model of LPS-induced IL-1 induction, where anecortave failed to induce IL-1, whereas glucocorticoids were effective in induction.[10] Our studies focused on in vitro assays of NF-κB inhibition and models of chronic immunity not utilizing LPS.[17]

Here, we queried the chemical space around the Δ9,11 steroid backbone, optimizing for anti-inflammatory properties (NF-κB

inhibition and GR nuclear translocation). The lead compound, VBP15, shows excellent drug properties, and is in development for Duchenne muscular dystrophy as the initial indication.

2. Materials and methods

2.1. Chemistry

All compounds were synthesized by Bridge Organics Co. (Kalamazoo, Michigan). Systematic (IUPAC) names: Anecortave ([2-[(8*R*,10*S*,13*S*,14*R*,17*R*)-17-hydroxy-10,13-dimethyl-3-oxo-2,6,7,8,12,14,15,16-octahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl]-2-oxo-ethyl] acetate). Tirilazad ((16α)-21-[4-(2,6-dipyrrolidin-1-yl-pyrimidin-4-yl)piperazin-1-yl]-16-methylpregna-1,4,9(11)-triene-3,20-dione). Additional chemical structures studied are described in Table 1.

Traditional glucocorticoids have an 11-beta-hydroxy or an 11-keto group whereas we used a double bond between carbons 9 and 11, leading to a Δ9,11 C ring as the backbone, then developed a series of steroids based on this scaffold (Fig. 1, Panel A; Table 1). These scaffolds are not oxidatively metabolized to 11-beta hydroxy compounds, and should have less off-target effects related to active metabolites. We varied the D ring of the steroid, probing the effect of different lipophilic groups at C16 and either hydroxy or hydrogen at C17. Twenty compounds were synthesized in the VBP series of Δ9,11 compounds varying in structure at the three positions (R1, R2, R3) (Fig. 1; Table 1).

The synthesis of the Δ9,11 steroid begins with commercially available steroid 3TR (Fig. 1; Panel B). Copper catalyzed Michael addition of MeMgCl with TMSCl through a 1,4 addition introduced a 16-alpha methyl group to give silyl enol ether **2** with 93% purity by hplc/ms and in nearly quantitative yield. The product was isolated as a toluene slurry which was oxidized with 32% by weight peracetic acid in acetic acid to yield intermediate **3** which was not isolated but was converted to compound **4** as a powder that precipitated from EtOAc/heptane trituration in 60% yield from 3TR and with 86% purity. In order to remove a small amount of the delta 9,11 epoxide, compound **4** was treated with HBr in methylene chloride. Yield from the HBr treatment after MeOH/water crystallization was 90% with 97% purity and an overall yield from 3TR of 54%. Finally, the acetate of compound **4** is removed by K_2CO_3 hydrolysis. Methanol/water crystallization of VBP15 gave a 79% yield with 98.3% purity. Overall yield from 3TR is 43%. Purity is assigned by hplc/ms. Structure was confirmed by proton and carbon NMR.

2.2. NF-κB screen

C_2C_{12} skeletal muscle cells stably transfected with a luciferase reporter construct regulated under NF-κB response element (Panomics, Fremont, CA) were used for screening NF-κB inhibitors, as previously described.[18] Myoblasts and myotubes grown in growth and differentiating medium respectively were pretreated with various concentrations (vehicle [DMSO]; 0.01–100 μg/ml) of the drugs for a 24 h duration before stimulating with tumor necrosis factor-α (TNF-α) (10 ng/ml) for another 24 h. After the completion of incubation, cells were washed once with PBS and lysed with cell lysis buffer to measure luciferase activity (Promega Corp, Madison, WI) using Centro LB 960 luminometer (Berthold technologies, GmbH & Co, Bad Wildbad, Germany) and IC_{50} values are calculated for each compound.

2.3. Cell viability

Cell viability was determine by MTT assay (3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (Sigma, St., Louis, Missouri) as per manufacturer's protocols. Percent cell viability was calculated relative to untreated cells, whereas relative luminescence units with TNF-α stimulation in the absence of drugs were considered as 100% percent. Data are represented as % inhibition relative to TNF-α induced NF-κB activation. Significance was calculated using a one-way repeated measure ANOVA to determine if there is an effect of drug concentration on cell viability.

2.4. Nuclear translocation assays

Translocation assays were performed by DiscoveRx (Fremont, CA) using GR Nuclear Translocation PathHunter cells (DiscoveRx; Fremont, CA). This assay is based on the detection of protein–protein interactions between the GR and a nuclear fusion protein containing steroid receptor co-activator peptide. The receptor is tagged with the ProLink component of enzyme fragment complementation assay system and the steroid receptor co-activator peptide (SRCP) is fused to the enzyme acceptor component. When the receptor is bound by ligand it translocates to the nucleus where it

Table 1
Chemical structures of compounds tested

VBP#	A	16	17	21	NF-κB inhibition EC_{50} (M)	Rank NF-κB	GR nuclear translocation (%)	Rank GR translocation	Sum rank
Pred/Dex					1.67E-08	1	100.00	1	2
6	Delta 1,4	CH_3	H	OH	5.64E-08	3	54.20	4	7
15	Delta 1,4	CH_3	OH	OH	6.59E-08	6	80.40	2	8
7	Delta 1,4	CH_3	H	OAc	5.41E-08	2	48.80	7	9
1	Delta 4	H	OH	OH	6.29E-08	5	49.20	6	11
16	Delta 1,4	Beta CH_3	OH	OH	6.23E-08	4	44.50	8	12
2	Delta 1,4	H	OH	OH	1.29E-07	9	57.60	3	12
3	Delta 4	H	OH	Oac	2.12E-07	10	51.50	5	15
41	Delta 1,4	Ethyl	H	OH	8.95E-08	7	27.00	11	18
17	Delta 4	CH_3	H	Dipropylamino	7.23E-07	13	37.70	9	22
11	Delta 4	H	OH	Morpholino	1.74E-06	15	28.70	10	25
5	Delta 1,4	ene	ene	OAc	9.36E-08	8	15.30	18	26
13	Delta 4	H	OH	Dipropylamino	4.35E-07	11	21.80	15	26
10	Delta 4	H	OH	Pyrrolidine	1.85E-06	16	24.70	12	28
4	Delta 1,4	ene	ene	OH	4.95E-07	12	16.70	17	29
18	Delta 4	CH_3	H	Pyrrolidine	9.88E-07	14	19.90	16	30
14	Delta 4	CH_3	H	Morpholino	5.24E-06	17	22.70	14	31
9	Delta 4	H	OH	N-Me Piperazine	5.78E-06	18	23.50	13	31
12	Delta 4	Me	H	N-Me Piperazine	>1e-5	21	14.70	19	40
59	Delta 4	*n*Butyl	H	OH	>1e-5	21	8.50	20	41
58	Delta 1,4	*n*Butyl	H	OH	>1e-5	21	6.90	21	42

recruits the SRCP and complementation occurs producing a chemiluminescent signal. A ten point agonist dose curve, ranging from 1×10^{-5} to 1.69×10^{-10} M was performed on all VBP compounds. Dexamethasone was performed in parallel as the standard reference control. Percentage activity is calculated using the following formula: % Activity = 100% × (Mean Relative Light Units [RLU] of test sample—mean RLU of vehicle control)/(mean MAX RLU control ligand—mean RLU of vehicle control)). EC_{50} is calculated for all the compounds.

2.5. Transactivation assays

HEK-293 cells stably transfected with a luciferase reporter construct regulated under glucocorticoid response element (GRE) (Panomics, Fremont, CA) were grown according to manufacturer's instructions. Cells were treated with various concentrations (0.01 to 100 μg/ml) of the drug for 6 h. Cells were washed once with PBS and lysed with cell lysis buffer to measure luciferase activity (Promega Corp, Madison, WI) using Centro LB 960 luminometer (Berthold technologies, GmbH & Co, Bad Wildbad, Germany).

2.6. Pharmacokinetics

Pharmacokinetic studies were carried out at Pharmaron (US headquarters 6 Venture, Suite 250, Irvine, CA 92618; work performed in Pharmaron-Beijing, 6 Taihe Road, BDA Beijing, 100176). Mice were purchased from Huafukang Bioscience, Beijing, China. Dogs were purchased from Marshall Inc., Beijing, China. Monkeys were purchased from Hainan, Inc., Jinggang, China and Guangxi Inc., Guidong China.

2.6.1. Analysis

All analyses were conducted on a Shimadzu liquid chromatograph separation system equipped with degasser DGU-20A3, solvent delivery unit LC-20AD, system controller CBM-20A, column oven CTO-10ASVP and CTC Analytics HTC PAL System. . Samples were loaded onto a Phenomenex Luna 5μ C18 (2) (2.0 × 50 mm) coupled with a preguard column with a mobile phase of 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water. Mass spectrometric analysis was performed using an API 4000 instrument from AB Inc (Canada) with an ESI interfaceThe data acquisition and control system were created using Analyst 1.5 software from ABI Inc.

2.6.2. Mouse

CD1 mice (n = 3/drug) were injected via tail vein with a solution of 10 mg/kg VBP6 (10% ethanol and 40% PEG400; pH 7.0) or 10 mg/kg VBP15 (10% ethanol, 10% DMSO, and 30% PEG400; pH 7.0). Oral administration (PO) was conducted by feeding CD1 mice (n = 3/drug) an aqueous emulsion of either VBP6 or VBP15 in 30% Labrafil. Blood samples were taken at 0-, 5-, 15-, 30 min and 1-, 2-, 4-, 8-, and 24 h.

2.6.3. Dog and rat

Sprague–Dawley rats (n = 3) were intravenously injected with a solution of 10 mg/kg VBP15 (10% ethanol, 10% DMSO and 30% PEG400; pH 7.0). Beagle dogs (n = 3) were intravenously injected with a solution of 10 mg/kg VBP15 (8% ethanol, 8% DMSO, 50% PEG400 and 34% HP-β-CD (20%W/V in water). Oral administration for SD rats (n = 3) and beagles (n = 3) was conducted by feeding an emulsion of VBP15 in 30% Labrafil. Blood samples were taken at 0-5-, 15-, 30 min and 1-, 2-, 4-, 8-, and 24 h.

2.7. Metabolic stability

Stability studies were conducted by Pharmaron using liver microsomes from human, monkey, dog, rat and mice. Either VBP compound or positive control (Verapamil) was added to microsomal solutions at a final concentration of 2 μM VBP compound, 0.5 mg/mL microsomes, 5 mM $MgCl_2$ and 5 mM PBS. The reaction was started with the addition NADPH solution at a final concentration of 1 mM and carried out at 37 °C. H_2O was used instead of NADPH solutions in the negative control. Aliquots were taken from the reaction solution at 0 and 60 min. The reaction was stopped by the addition of 3 volume of cold methanol. Aliquots of the supernatant were used for LC/MS/MS analysis for metabolite analysis and identification was performed as described above.

2.8. CYP induction

CYP1A2 and CYP3A4 induction studies were conducted by Pharmaron. Cryopreserved human liver microsomes from 3 donors (separate incubations) were obtained commercially from CellzDirect (Invitrogen). Hepatocytes were cultured on a collagen substratum for three days prior to study initiation according to manufacturer's instructions. Hepatocyte cultures were treated daily with fresh media containing 1-, 10-, and 100 μM VBP15, vehicle (negative control) or appropriate positive control (rifampin for CYP3A4 and omeprazole for CYP1A2). 24-h after the final treatment, CYP3A4 and CYP1A2 activity was determined using the FDA recommended probe substrates testosterone and phenacetin respectively. Assay analysis was conducted via LC/MS/MS. The percentage inductions relative to positive control were calculated. A greater than 40% of positive control in any one of the three donors for a CYP was considered a potential inducer of that CYP.

3. Results

3.1. In vitro screening of VBP compounds for NF-κB transrepression and GR nuclear translocation

We screened the 20 compounds for their ability to inhibit NF-κB using a luciferase reporter construct stably transfected into C_2C_{12} myogenic cells.[18] Both undifferentiated myoblasts, and differentiated, multi-nucleated myotubes were studied. VBP compounds were able to inhibit TNF-alpha induced NF-κB to varying degrees resulting in reduced percent NF-κB activity. Figure 2 displays the top 5 NF-κB inhibitors in myoblasts and myotubes. These results indicate that several of the VBP compounds are able to inhibit NF-κB at potency similar to prednisolone in vitro in muscle cells.

Cell viability was assayed on C_2C_{12} cells grown and treated concurrently to those measuring NF-κB activity. We discovered that a C16-methyl group showed strong inhibition of NF-κB and the lowest cell toxicity, while larger alkyl groups (ethyl and butyl) either decreased activity or enhanced toxicity. We now favor a Δ1,4 in the A ring because of improved metabolic stability, a Δ9,11 in the C ring, a methyl at C16, and either hydroxyl or hydrogen at C17.

Compounds were tested for potency in causing translocation of the GR from the cytoplasm to nucleus using a reporter assay in both myoblasts and myotubes.[18] EC_{50} for GR Translocation [Log M] was then plotted against the IC_{50} for NF-κB inhibition (Figure 3). VBP6, VBP7 and VBP15 clustered with prednisone and dexamethasone, showing high activities for both assays. VBP6 and VBP15 are both 21-hydroxyls with VBP7 being the acetate (prodrug) of VBP6.

3.2. VBP compounds lack transactivation activity

To determine if VBP compounds dissociate transrepression from transactivation, we screened the top NF-κB inhibiting compounds for their ability to induce GRE-dependent gene transcription using a GRE-luciferase reporter construct as we have previously

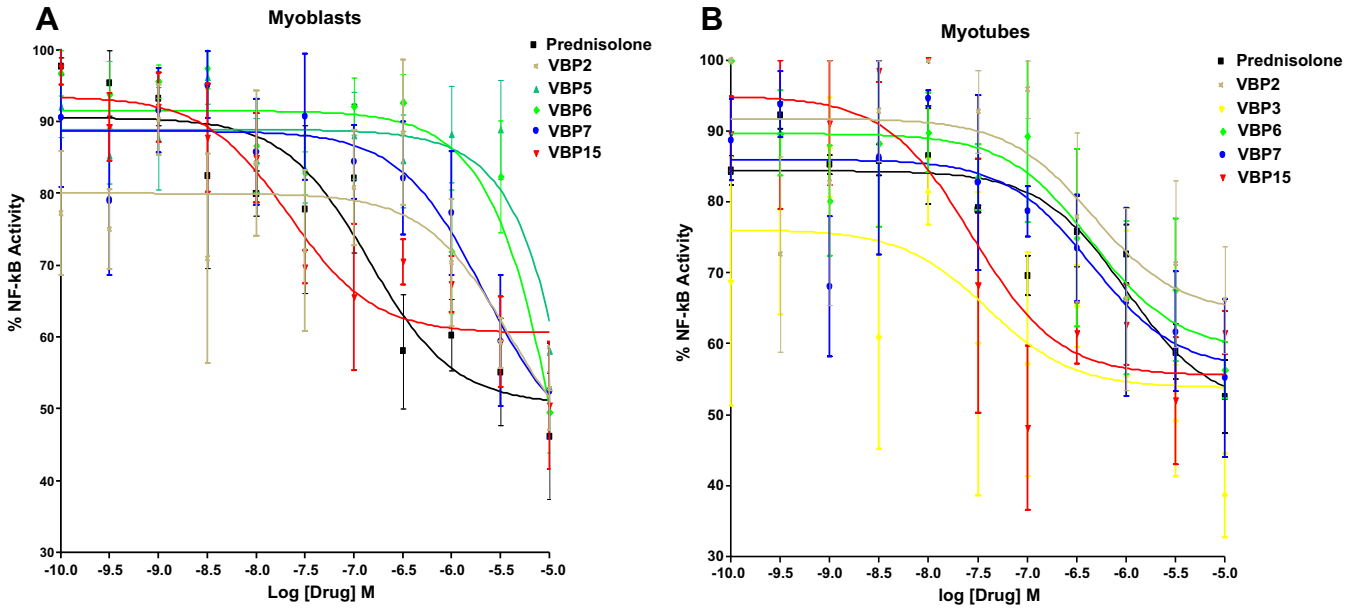

Figure 2. NF-κB inhibition in myogenic cells. (A) Dose response of the top NF-κB inhibiting VBP compounds in C_2C_{12} myoblasts. (B) Dose response of the top NF-κB inhibiting VBP compounds in C_2C_{12} differentiated myotubes. Standard deviations are shown.

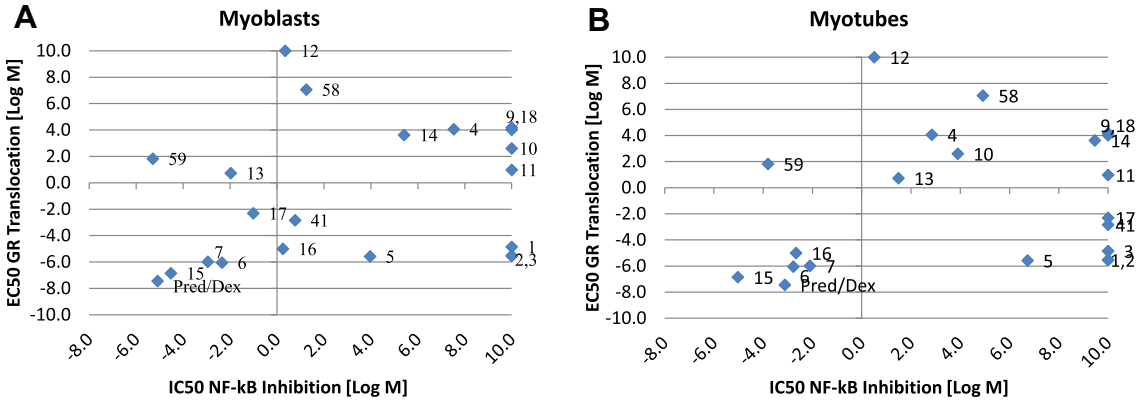

Figure 3. Correlation of NF-κB and GR nuclear translocation potency in (A) myoblasts and (B) myotubes. IC_{50} values for NF-kB inhibition activity (*X* axis) and GR nuclear translocation activity (*Y* axis) were plotted for all VBP series compounds, relative to prednisone and dexamethasone.

described.[18] Results show that there was a significant dose-dependent response in prednisolone treated cells (p <0.0001) but there was no increase in luciferase transcription at even the highest doses tested for any of the VBP compounds (Fig. 4). These data suggest we are achieving our desired SAR goals of dissociating the gene transcriptional (classic glucocorticoid) properties from the anti-inflammatory (NF-κB) properties.

3.3. Pharmacokinetics in CD1 mice following intravenous and oral administration

The two top candidates from the in vitro assays (VBP6 and VBP15) were compared for bioavailability through PK studies. The relatively poor solubility of VBP15 required formulation in 8% DMSO + 8% Ethanol + 50% PEG400 + 34% HP-β-CD (20% W/V).

PK results for VBP6 indicate that it had a relatively high clearance of 48.2 ml/min/kg, which was about 50% of the mouse blood hepatic flow. The terminal half-life was 0.67 h. Volume of distribution (V_{ss}) was 0.87 L/kg, indicating that it did not have high tissue accumulation. In the PO arm, C_{max} = 10203 ng/ml and percentage bioavailability (*F*%) was 128% (Table 2, Fig. 5). The *F*% was calculated by comparing the dose-corrected area under curve (AUC) non-intravenous divided by AUC intravenous. IV AUC was considered 100%. We had different formulations for the oral versus IV which improved solubility, and hence a larger AUC, for the oral formulation compared to the IV formulation leading to *F*% >100%.

PK results for VBP15 indicate that it had low-medium clearance 18.8 ml/min/kg. The terminal half-life was 0.35 h. Volume of distribution was 0.75 L/kg indicating that it also did not have high tissue accumulation. In the PO arm, C_{max} = 6787 ng/ml at 2 h, and bioavailability (*F*%) was 74.5% (Table 2; Fig. 5).

3.4. Metabolic stability across multiple species

The in vitro metabolic stability of VBP6 and VBP15 was studied in different species of liver microsomes. Results show ⩾80% remaining at 1 h of VBP6 and VBP15 in human, dog, and monkey microsomes (Fig. 6). VBP6 had poor stability in mouse liver microsomes (34%) compared to VBP15 (88%). Both compounds showed poor stability in rat, presumably due to the high first pass clearance traditionally seen with glucocorticoids in rats.[19]

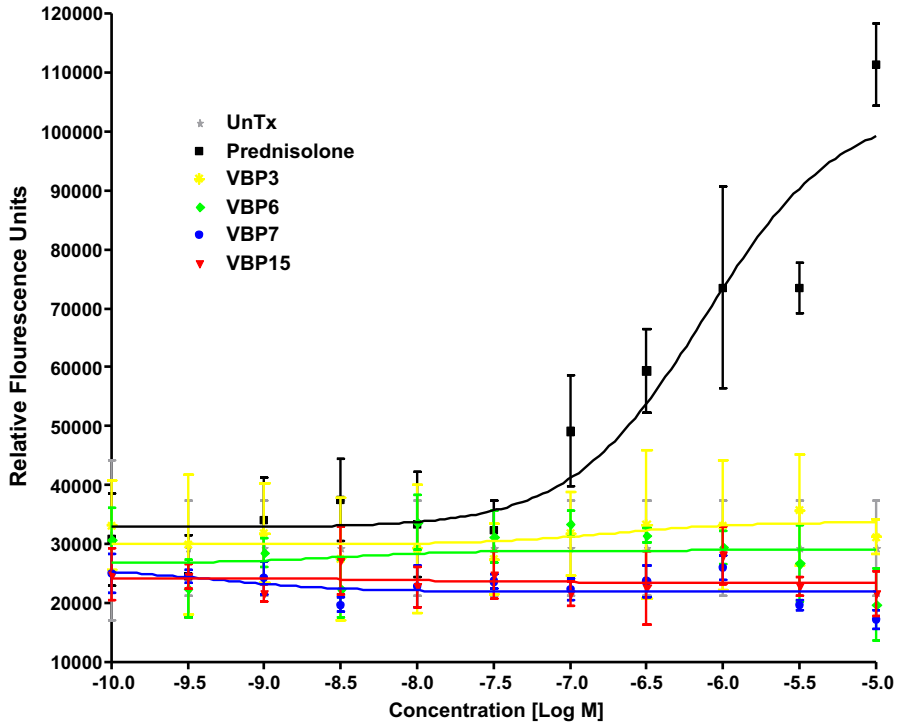

Figure 4. VBP compounds do not induce GRE-dependent gene transcription. A reporter construct with luciferase driven by glucocorticoid response elements (GREs) was studied, as previously described.[18] Prednisone induced luciferase expression, while none of the VBP series did. Standard deviations are shown.

Table 2
PK analysis of VBP6 and VBP15 following IV and PO administration in CD1 mice

Drug	Route	Dose (mg/kg)	Formulation	Cl_{obs} (mL/min/kg)	$T_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/mL)	AUC_{last} (h ng/mL)	AUC_{Inf} (h ng/mL)	MRT (h)	AUC/D (h ng/mL)	Vss_{obs} (L/kg)	*F* (%)
VBP6	IV	10	10% EtOH + 40% PEG400	48.2	0.667	0.0833	8.637	3.447	3.457	0.289	345	0.874	NA
	PO	50	30% Labrafil	NA	0.659	1.00	10.203	22.023	22.031	NA	440	NA	128
VBP15	IV	10	10% EtOH 10% DMSO 40% PEG400	18.8	0.354	0.0833	11.167	8.838	8.842	0.667	884	0.757	NA
	PO	50	30% Labrafil	NA	0.678	2.00	6.787	32.912	32.932	NA	658	NA	74.5

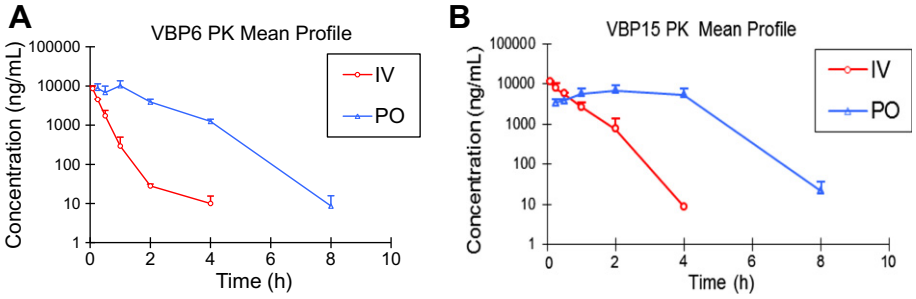

Figure 5. PK mean profiles for (A) VBP6 and (B) VBP15 in mice. Error bars indicate standard deviation.

3.5. Lead compound selection

Potential lead compounds were narrowed to VBP6 and VBP15 based on superior activities (high NF-κB inhibition, low cytotoxicity, high GR binding and GR translocation). PK data showed that VBP15 had a longer T_{max} and lower C_{max} across species, leading to a greater and preferred AUC compared to VBP6. Thus, VBP15 was selected as the lead compound for all further studies.

3.6. Absorption, distribution, metabolism and excretion (ADME) and pharmacokinetics of VBP15

We performed a series of ADME and pharmacokinetic studies to characterize our lead compound. Table 3 summarizes the results of these ADME experiments. PK results for VBP15 in rats indicate that it had a clearance of 20.2 mL/min/kg. The terminal half-life was 0.58 h. Volume of distribution (V_{ss}) was 0.77 L/kg, indicating that it did not

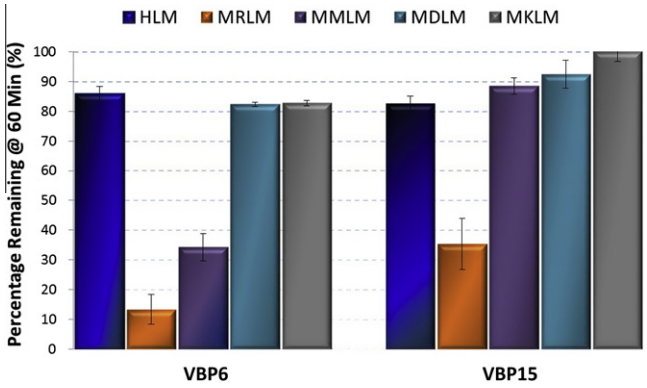

Figure 6. Metabolic stability of VBP6 and VBP15 in liver microsomes across multiple species. VBP15 shows better stability in rat and mouse microsomes compared to VBP6. HLM = human liver microsomes, MRLM = male rat liver microsomes, MMLM = male mouse liver microsomes, MDLM = male dog liver microsomes, MKLM = male monkey liver microsomes.

have high tissue accumulation. In the PO arm, C_{max} = 2543 ng/mL and percentage bioavailability (*F*%) was 47.8% (Table 4).

VBP15 in beagle dogs had medium-high clearance of 24.7 mL/min/kg. The terminal half-life was 5.42 h. Volume of distribution was 1.93 L/kg indicating that it had certain tissue distribution. In the PO arm, C_{max} = 814 ng/mL, and bioavailability (*F*%) was 53.2% (Table 4).

3.7. Metabolite identification

Human, monkey, dog, rat and mouse hepatocytes were incubated at a final concentration of 10 μM VBP15 for 2 and 4 h. Four metabolites were identified in the human and monkey hepatocytes, 3 were identified in the dog (peak 4 is the major metabolite), 5 metabolites were identified in rat (peak 3 is the major metabolite) and 2 in the mouse (Table 5).

3.8. CYP induction

There was no induction of CYP1A2 by VBP15 seen across the three donors therefore VBP15 is not considered an inducer of CYP1A2. VBP15 moderately induced CYP3A4 across the three donors (24–42%). This indicates that VBP15 is a potential inducer of CYP3A4, similar to other steroidal compounds.

4. Discussion

We have recently shown that an existing Δ9,11 steroidal compound (anecortave acetate) was able to bind and translocate the glucocorticoid receptor (GR) and inhibit anti-inflammatory pathways (NF-κB).[17] Based on these data we synthesized 20 new compounds not previously known to the literature, with the goal of carrying out a lead optimization program using the Δ9,11 backbone, in vitro screening assays included potent inhibition of NF-κB, potent translocation of the GR to the nucleus, loss of GRE-mediated transcriptional activity, and low cytotoxicity. We found that a C16-methyl group was important for NF-κB inhibitory activity, while larger alkyl groups (ethyl and butyl) either decreased activity or enhanced cytotoxicity of myogenic cells. Other optimizations of chemistry included a Δ1,4 in the A ring (improved metabolic stability), a Δ9,11 in the C ring, a methyl at C16, and either hydroxyl or hydrogen at C17. VBP15 was selected as the lead compound based on these findings. VBP15 also showed desirable ADME and PK properties. Like many steroids, VBP15 shows oral absorption in a target range acceptable for oral delivery. The drug can be formulated for PK and other studies in vehicles that are typical for related steroids. PK and metabolite work suggest that the primate and mouse will probably be the preferred species for PK.

Synthetic glucocorticoids are among the most commonly prescribed drugs due to their potent anti-inflammatory properties, and remain standard of care in many conditions such as arthritis, dermatitis, asthma, muscular dystrophy, and auto-immune disorders.[20–22] However, glucocorticoids have many off-target effects that together contribute to a relatively broad side effect profile. These include but are not limited to disruption of glucose metabolism, immune suppression, adrenal suppression, thymocyte apoptosis, osteoporosis, erythroblast proliferation, elevation of intraocular pressure, cataract, and mood changes. Many of these side effect profiles are associated with GRE-mediated transcriptional activities of steroids (classic glucocorticoid activity). We have shown that VBP15 lacks GRE-mediated transcriptional

Table 3
ADME properties of VBP15

In vitro properties	Units	Value & class	Target range
Solubility (pH, media)	(μM)	187.18 μM	>60
Stability—microsomes	$t_{1/2}$ (min)	Human = 82% Rat = 35.4% Mouse = 88.8% Dog = 92.5% Monkey = 100%	>30
Stability—plasma (species)	% Remaining at 1 h	88.06%	>80%
CYP450 Inhibition (2C9,2D6,3A4)	% Inhibition at 10 mM IC_{50} (mM)	1.9%, 7.9%, 15.3% >50 μM for all	<40% >10
Permeability—Caco-2	P_{app} (a–b, 10^{-6} cm/s) Efflux ratio	11.2 0.61	>10 <2
hERG— (method)	IC_{50} (mM)	20 μM	>10
Free C_{max}—plasma	Total C_{max} (mM) $^{*}F_{u,\ plasma}$	0.12	
Ames test	Positive/negative	Negative	Negative
Micronucleus test	Positive/negative	Negative	Negative
Blood Brain Barrier (rats)[a]	Ratio (brain/plasma) (h)	0.25 = 0.648 0.5 = 0.620 1.0 = 0.614 2.0 = 0.593 4.0 = 0.583 6.0 = 0.581 8.0 = 0.433	

[a] The brain concentration was not corrected for vascular content.

Table 4
PK properties of VBP15 across species

PK properties	Units	Mouse 10 mpk IV 50 mpk PO	Rat 10 mpk IV 50 mpk PO	Dog 10 mpk IV 30 mpk PO	Target range
$t_{1/2}$	h	0.354 (IV) 0.678 (PO)	0.58 (IV) 2.29 (PO)	5.42 (IV) 2.25 (PO)	>3
$AUC_{0-\infty,\ total,\ unbound}$	h ng/mL	8842 (IV) 32932 (PO)	8339 (IV) 19937 (PO)	6791 (IV) 10844 (PO)	>500 (PO)
CL	mL/min/kg	18.8 (IV)	20.2 (IV)	24.7 (IV)	<25% HBF
$C_{max,\ total,\ unbound}$	ng/mL (nM)	11167 (IV) 6787 (PO)	2543 (PO)	814 (PO)	
T_{max}	h	0.083 (IV) 2.0 (PO)	4.00 (PO)	6.00 (PO)	
V_{ss}	L/kg	0.757 (IV)	0.770 (IV)	1.93 (IV)	
F	%	74.5	47.8	53.2	>20%

Table 5
VBP15 metabolites identified across species

Peak	Rt	Expected	Mass	Biotransformation	Metabolites in hepatocytes				
no.	(min)	m/z	shift		Human	Monkey	Dog	Rat	Mouse
1	5.52	371.2	16	Oxidation	+	+	+	+	+
2	6.37	373.2	18	Oxidation + hydrogenation	+	+	–	+	–
3	7.78	371.2	16	Oxidation	–	–	–	+++	–
4	9.69	369.2	14	Methylation	+	+	+++	+	+
5	10.42	355.2	0	Parent drug	+++	+++	+	+	+++
6	12.86	357.2	2	Hydrogenation	+	+	+	+	–

activity, although it inhibits NF-κB and causes translocation of the GR to the nucleus. We should note that classic glucocorticoids result in nuclear translocation as ligand/GR dimmers able to bind GRE elements (transactivation properties), as well as ligand/GR complexes with NF-κB transcriptional complexes that target NF-κB promoter elements (transrepression properties). VBP15 appears to disassociate these two subactivities, with GR translocation likely due to NF-κB transcriptional complexes. Thus, we expect that preclinical studies will show superior side effect profiles of VBP15 compared to other steroid-based anti-inflammatories, as we have previously shown for anecortave acetate.[17]

The extensive effect profiles of pharmacological glucocorticoids often limit prescription despite proven efficacy, particularly in children. For example, in Duchenne muscular dystrophy, daily prednisone is considered standard of care in some countries, but the efficacy if offset by increased bone fragility, mood changes, weight gain, and muscle catabolic pathways.[23–26] Thus, there is non-adoption of this standard in some countries, and relatively poor adherence even in those countries adopting daily glucocorticoids.[27] The VBP15 described here is being developed for Duchenne muscular dystrophy as the initial indication, in collaboration with the Muscular Dystrophy Association Venture Philanthropy, and National Institutes of Health Therapeutics for Rare and Neglected Disease program. It is our hope that VBP15 not only provides a superior side effect profile relative to prednisone, but will also improve efficacy by opening the therapeutic window (allowing increased dosing relative to prednisone).

Acknowledgments

Supported in part by Grants from the Congressionally Directed Medical Research Program of the US Department of Defense (W81XWH-09-1-0218; W81XWH-11-1-0754), Muscular Dystrophy Association Venture Philanthropy (Phase 1 and Phase 2), Foundation to Eradicate Duchenne, NIH Wellstone Muscular Dystrophy Research Center (1U54HD053177-01A1), and NIH Therapeutics for Rare and Neglected Disease (TRND) program.

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