Inhibition of Histone Deacetylase Activity by Valproic Acid Blocks Adipogenesis

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Summary

Adipogenesis is dependent on the sequential activation of transcription factors including the CCAAT/enhancer binding proteins (C/EBP), peroxisome proliferator-activated receptor gamma (PPAR\(\gamma\)), and steroid regulatory element binding protein (SREBP). We show that the mood stabilizing drug valproic acid (VPA; 0.5-2 mM) inhibits mouse 3T3 L1 and human preadipocyte differentiation, likely through its histone deacetylase (HDAC) inhibitory properties. The HDAC inhibitor trichostatin A (TSA) also inhibited adipogenesis, whereas the VPA analog valpromide (VPM), which does not possess HDAC inhibitory effects, did not prevent adipogenesis. Acute or chronic VPA treatment inhibited differentiation yet did not affect mitotic clonal expansion. VPA (1 mM) inhibited PPAR\(\gamma\)-induced differentiation, but does not activate a PPAR\(\gamma\)reporter gene, suggesting it is not a PPAR\(\gamma\)ligand. VPA or TSA treatment reduced mRNA and protein levels of PPAR\(\gamma\) SREBP1a. TSA reduced C/EBP\(\alpha\) mRNA and protein levels, whereas VPA only produced a decrease in C/EBP\(\alpha\) protein expression. Overall our results highlight a role for HDAC activity in adipogenesis that can be blocked by treatment with VPA.

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1 CCAAT/enhancer binding proteins (C/EBP), peroxisome proliferator-activated receptor gamma (PPAR\(\gamma\)), steroid regulatory element binding protein (SREBP), valproic acid (VPA), histone deacetylase (HDAC), trichostatin A (TSA), 4-phenylbutyrate (4PB), valpromide (VPM), triacylglycerol (TAG), differentiation inducing medium (MDI), rosiglitazone (ROS), troglitazone (TGZ), DNA binding domain (DBD), ligand binding domain (LBD), tumor necrosis factor alpha (TNF\(\alpha\)), untreated (UT).
Introduction

Valproic acid (VPA) has been used as an anticonvulsant agent for the treatment of epilepsy, as well as a mood stabilizer for the treatment of bipolar disorder, for several decades. Overall VPA is well tolerated, with common side effects including tremor, sedation, alopecia, and weight gain (1). The mechanism of action for these efficacious and deleterious effects remains to be elucidated and is most likely multifactorial. The specific effects of VPA on neurotransmitter systems, most notably GABA signaling, as well as other cell signaling pathways explains some of the mechanisms by which VPA exerts its effects in humans, rodents, and cell culture systems (2). For example, VPA has histone deacetylase (HDAC) inhibitory effects that are the likely cause of VPA teratogenicity (3,4). In addition, inositol depletion contributes to VPA inhibition of sensory neuronal growth cone formation in vitro (5).

Up to 70% of adult patients receiving VPA treatment gain weight (5-14 kgs.) (6). This is cause for concern due to the increasing number of off-label uses for VPA, such as migraine headache and neuropathic pain, and the increased health risk and decreased compliance rate associated with weight gain (6-8). Clinically evaluating patient age, sex, familial predisposition to weight gain, changes in dietary habits, and VPA dosage have failed to reveal predictive factors for VPA-induced weight gain. In attempting to generate animal models of VPA-induced weight gain, we and others have demonstrated that VPA does not cause weight gain in mice (9) or rats (10,11), however VPA can induce a significant increase in body weight in female rhesus monkeys (12), suggesting that this side-effect may be a characteristic of primate physiology. There is currently no clear mechanism to explain how VPA causes weight gain.

VPA can inhibit both class I and II HDACs, with a high potency for class I HDACs (4). The role of HDAC activity in adipocyte differentiation is not well defined, however recent work has
highlighted a requirement for reduced HDAC activity for transcriptional activation of adipogenic genes in vitro (13). We demonstrate that VPA treatment inhibits mouse 3T3-L1 and human adipocyte differentiation. Adipocytes differentiate from preadipocytes via a well-defined series of transcriptional events involving several key transcription factors, including members of the CCAAT/enhancer binding protein (C/EBP) family, peroxisome proliferator-activated receptor gamma (PPARγ) and steroid regulatory element binding protein (SREBP) (14). Inhibition of differentiation by VPA is characterized by diminished PPARγ and SREBP1a mRNA and protein. The mRNA levels for C/EBPa were not affected by VPA treatment, however there was a significant reduction in C/EBPa protein levels. Experiments using either the structurally unrelated HDAC inhibitor trichostatin A (TSA) or valpromide (VPM), an amide analog of VPA that does not inhibit HDACs, support the hypothesis that HDAC activity is required for initiation of adipogenesis.

Experimental Procedures

Cell culture and differentiation

Mouse 3T3-L1 were obtained from the American Type Culture Collection and subcultured in 5% CO₂ at 37°C. Cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen), with 10% heat inactivated calf serum (Invitrogen) and penicillin G/streptomycin sulfate (Invitrogen). Media was changed every two days and preadipocytes were maintained at <50% confluence.

For 3T3-L1 differentiation experiments, 2 days after preadipocytes reached confluence they were treated with medium to induce differentiation [MDI: DMEM, 10% fetal bovine serum (FBS; CanSera), 250 nmol/L dexamethasone (DEX; Sigma), 0.5 nmol/L 3-isobutyl-1-
methylxanthine (IBMX, Sigma), and 100 nmol/L (0.58 µg/ml) human insulin (Roche Molecular Biochemicals)]. After 2 days in MDI preadipocytes were cultured in DMEM containing 10% FBS, and 5 µg/ml insulin. Subsequent medium changes occurred every second day. Valproic acid (VPA) and 4 phenylbutyrate (4PB) were dissolved in phosphate buffered saline (PBS), whereas valpromide (VPM) and trichostatin A (TSA) were dissolved in DMSO, and applied as indicated in figure legends. DMSO levels were kept under 0.1%. For PPAR\[agonist induced differentiation, preadipocytes (2 days post confluent) were treated with DMEM, 10% FBS, 100 nmol/L insulin, and a PPAR\[agonist [troglitazone (TGZ; 5 nM or 10 nM) or rosiglitazone (ROS; BRL49653, 250 nM or 1 µM)]. After 2 days medium was changed to DMEM, 10% FBS, 5 ug/ml insulin, and the PPAR\[agonist. Subsequent media changes occurred every 2 days with medium containing the PPAR\[agonist.

Human adipocyte experiments were conducted by Zen-Bio Inc. (Contract #DAL040403). Primary human preadipocytes were obtained from patients undergoing liposuction surgery. Two lots of cells were used including those from an individual [L091901, male, 48 years old, body mass index (BMI) 25.07] and a mixed lot SL0023 (6 female individuals, average age 48, average BMI: 26.07). Details for preadipocyte differentiation can be found at www.zen-bio.com. For each experiment, a control consisting of a triplicate set of cells treated with 5 ng/ml TNF\[ was included. Addition of ROS, Zen-Bio PPAR\[agonist, or TNF\[ was included with each medium change.

**Oil Red O staining**

Cells were stained with Oil Red O and quantified as previously described by Kasturi and Joshi (15). Stain bound to lipid droplets was extracted with isopropanol and the absorbance of the dye-triglyceride complex was measured at 520 nm.
**[³H] thymidine incorporation**

Cells were pulsed labeled with [³H] thymidine (2 µCi/ml Amersham) at 37°C for 1 hour prior to harvesting (1h, 10h, 20h, or 30h after the induction of differentiation), similar to the method of Tang and Lane (16). Briefly, cells were placed on ice and rinsed twice with cold PBS. To precipitate the DNA, cells were incubated with 10% trichloroacetic acid (TCA) for 1 h at 4°C. Cells were then washed with absolute alcohol at room temperature (RT) and allowed to air dry at RT for 2 h. DNA was extracted using 0.1M NaOH for at least 1 h at RT and radioactivity was counted in acidified scintillation fluid.

**Analysis of lipid synthesis**

Cells were pulse labeled with [¹⁴C] acetic acid (250 nCi/ml, Amersham) for 2 h at 37°C. [¹⁴C] acetic acid will be metabolized and incorporated into acetyl CoA, which is subsequently incorporated into newly synthesized triacylglycerol. Cells were rinsed twice with cold PBS and scraped into 1ml methanol:water (5:4, v/v) and sonicated. The organic and aqueous phases were extracted using chloroform:methanol (1:2, v/v) with 0.58% NaCl. The organic phase was washed three times with ideal upper phase buffer (0.57% methanol:NaCl:choloroform, 45:47:3, v/v/v), evaporated and then resuspended in chloroform. Radiolabeled lipids were resolved by thin layer chromatography (TLC) in petroleum ether:diethyl ether:acetic acid (60:40:1, v/v/v). Standards were identified using iodine staining and TLC plates were exposed to film. Radiolabeled lipids identified by co-migration with authentic standards were scraped into vials and quantified by scintillation counting.

**Northern analysis**

RNA was isolated using Trizol reagent (Invitrogen). Twenty micrograms of total RNA was separated on a 1.5% agarose/0.67% formaldehyde gel and transferred to BrightStar Plus
membrane (Ambion Inc.). The mouse PPAR[], C/EBP[], and SREBP1α cDNAs were labeled with $[\alpha-^{32}P]dATP$ (2 µCi/ml) using the Strip-EZ DNA Probe Synthesis and Removal Kit (Ambion). Blots were incubated with probe (1x10⁶ cpm/ml) in ULTRAhyb buffer (Ambion) overnight, blots washed and signals visualized by autoradiography and quantified using a Molecular Dynamics Storm Phosphoimager.

**Western analysis**

Cells were washed 2 times in ice cold PBS, extracts isolated using high-salt lysis buffer [10 mM Tris-Cl pH 7.4, 500 mM NaCl, 2 mM EDTA, 1 mM DTT, 1% Triton X 100, 1x complete protease inhibitors (Roche), 1 mM PMSF], sonicated, clarified by centrifugation (10 minutes at 15 000 x g), and quantified by Bradford analysis (BioRad Laboratories). For SREBP1α analysis, cells were treated for 4 hours before harvesting and again during lysis with 25 µg/ml of the proteasome inhibitor, N-acetyl-leucine-leucine-norleucinal (ALLN, Sigma). Twenty to forty micrograms of protein per lane were used for Western analysis. Proteins were detected with antibodies from Santa Cruz Biotechnology Inc. including anti-PPAR[](E-8), anti-SREBP1α (2A4), anti-C/EBP[] (14AA) or rabbit polyclonal anti-actin (Sigma). After washing in Tris-buffered saline, blots were incubated with sheep anti-rabbit or sheep anti-mouse IgG-HRP conjugated secondary antibody (Chemicon International). Protein expression was visualized with enhanced chemiluminescence (Perkin Elmer Corp) and signal quantified using NIH Image software. Western blots were stripped in stripping solution (62.5mM Tris pH 6.8, 2% SDS, 100mM ß- mercaptoethanol) for 15 min at 50°C, washed, blocked and re-probed.

**Transient transfection and PPAR-LBD assay**

Reporter gene assays were completed in HepG2 cells maintained in DMEM with 10% FBS. Cells were plated in 24-well plates at 7.5x10⁴ cells/well the day prior to transfection. Cells were
transfected in triplicate with the mammalian transfection mixture of 200 ng reporter plasmid FR-luc, 100 ng internal reference plasmid pCMV β-galactosidase, and 12.5 ng of either the GAL4 DNA binding domain (DBD) expression plasmid (BD-Gal4) or the expression plasmid for GAL4 DBD fused with the PPARβ ligand binding domain (PPAR-Gal4) using FuGENE6 (Roche Molecular Biochemicals). The subsequent day, cells were treated overnight with compounds. Twenty hours after addition of drugs cells were harvested and luciferase activity determined using the Enhanced Luciferase Assay kit (BD Pharmingen). Results were normalized using β-galactosidase activity and represent the mean data from three independent experiments.

## Results

**VPA inhibits adipocyte differentiation**

To determine whether VPA could induce adipocyte differentiation, mouse 3T3-L1 cells were cultured for two days in DMEM medium containing 10% FBS, insulin and VPA (1 or 5 mM). After 13 days in culture there were very few cells staining red with Oil Red O, which binds to triacylglycerol (TAG) in fat droplets, a hallmark of adipocyte formation (Figure 1A, left panel; higher magnification 1B). As a positive control, cells induced to differentiate in medium consisting of DMEM containing 10% FBS, IBMX, dexamethasone (0.25 µM) and insulin (MDI) but no VPA, contained numerous lipid droplets (Figure 1A). These data show that chronic VPA treatment was unable to induce differentiation of 3T3-L1 cells into adipocytes.

We next examined the effect of VPA treatment on MDI-induced preadipocyte differentiation. Daily treatment with VPA significantly inhibited MDI-induced adipocyte differentiation in a dose-dependent manner; shown by the decrease in lipid content measured 13 days after the initiation of differentiation (Figure 1A & 1C). Under higher magnification, almost 100% of
MDI-treated cells displayed lipid droplet formation, whereas in the presence of MDI and VPA there was a reduction in the number of cells that had lipid droplet formation (Figure 1B, right panels). To quantify the decrease in TAG levels following daily treatment with VPA, cells were metabolically labeled with $^{14}$C-acetic acid at day 13 post differentiation in the presence or absence of VPA. Chronic VPA treatment resulted in an average significant decrease in TAG synthesis by 51% (untreated 21,723±4,782 dpm/mg protein vs. 1 mM VPA 10,698±2,855 dpm/mg protein, p<0.001).

In addition to working with 3T3-L1 cells, Zen-Bio Inc. were contracted to conduct a double-blind study to examine the effects of VPA on differentiation of primary human subcutaneous preadipocyte cultures in vitro. Similar to the studies in 3T3-L1 cells, the effect of VPA on adipocyte differentiation was measured both in the absence and presence of differentiation inducing media (Zen-DIM). These studies were conducted on two cell lots, one from a single individual (L091901) and the other from a pooled sample containing adipocytes from 6 individuals (SL0023). In both lots VPA by itself did not induce differentiation of human preadipocytes, rather it inhibited differentiation in the presence of Zen-DIM (Figure 2A). Lot L091901 exhibited some differentiation in the absence of Zen-DIM, which was also inhibited by VPA treatment (Figure 2A). Quantification of TAG levels revealed that VPA inhibited differentiation in a dose dependent manner more effectively than tumor necrosis factor α, a known inhibitor of preadipocyte differentiation (Figure 2B). Collectively these data show that in both mouse and human cells chronic VPA treatment inhibits adipocyte differentiation.

We then examined whether the inhibitory effects of VPA on 3T3-L1 differentiation occurred following a single (acute) treatment with VPA. When 1 mM VPA was added once concurrent with MDI at the onset of differentiation, VPA reduced adipocyte formation (Figure 3A). This
finding suggested that VPA treatment might affect critical steps that occur at the onset of differentiation. After reaching confluence preadipocytes become quiescent, however in response to differentiation media they undergo mitotic clonal expansion. Since inhibition of mitotic clonal expansion can inhibit differentiation we measured whether VPA affected reentry into the cell cycle. Measuring $[^{3}H]$-thymidine incorporation into cellular DNA, differentiating preadipocytes exhibited a well-characterized entry into, and exit from, S phase of the cell cycle at ~12-16 hours following addition of the MDI (Figure 3B). In the presence of VPA, this curve remains unchanged, indicating that VPA does not affect mitotic clonal expansion. Daily VPA treatment beginning after the removal of the MDI (days 3-13) caused inhibition of differentiation to the same extent as VPA treated concurrently with the MDI (day 0-2) (Figure 3C). Daily VPA treatment beginning 7 days after the initiation of differentiation (day 7-13) also reduced Oil Red O levels, however this reduction was not significant compared to untreated cells. These results suggest that VPA is affecting critical events that occur both at the onset of differentiation, as well as events required during the initial maturation of the adipocytes.

**VPA inhibits PPAR$\gamma$-induced differentiation**

PPAR$\gamma$ agonists induce differentiation of adipocytes, whereas PPAR$\gamma$ antagonists inhibit differentiation (17). The PPAR$\gamma$ ligands troglitazone (TGZ) and the more potent rosiglitazone (ROS) were used to induce differentiation of 3T3-L1 and human preadipocytes. When co-treated with VPA and either TGZ or ROS, differentiation of 3T3-L1 cells was inhibited by 60% and 43% respectively (Figure 4A), suggesting that VPA can inhibit differentiation mediated by PPAR$\gamma$. In support of these findings, VPA was also able to inhibit ROS-induced differentiation of human preadipocytes; assessed by TAG levels (Figure 4B). Furthermore, VPA inhibited differentiation of human preadipocytes when differentiation was induced with 10 $\mu$M Zen-Bio
proprietary non-thiazolidinedione PPAR$g$ agonist. These data show that VPA inhibits PPAR$g$-induced differentiation in mouse and human preadipocytes.

**VPA is not a PPAR$g$ ligand**

Due to VPA’s ability to block PPAR$g$-induced differentiation, we conducted reporter assays to assess whether VPA interacted with the ligand binding domain (LBD) of PPAR$g$. Since TGZ and ROS had equivalent activities, these experiments were conducted using TGZ. VPA induced a weak activation of the PPAR$g$-LBD chimeric construct (PPAR-Gal4), compared to a greater than 40 fold increase by TGZ (Figure 4C). The VPA-induced fold increase in PPAR-Gal4 was similar to the increase in activation of the control plasmid (BD-Gal4) lacking the PPAR$g$-LBD. These results suggest that VPA may non-specifically increase the activation of PPAR-Gal4. VPA increases the activity of many reporter genes in transient transfection experiments and is suggested to be due to VPA acting as a HDAC inhibitor. We found that the HDAC inhibitor TSA induces a similar fold increase as VPA in BD-Gal4 and PPAR-Gal4 reporter activity (Figure 4C). These data suggest that non-specific activation of reporter genes may be due to an indirect effect of HDAC inhibitors.

PPAR$g$ partial agonists or antagonists can inhibit 3T3-L1 differentiation. Since VPA inhibits 3T3-L1 differentiation, we examined the effect of VPA on the dose response curve for TGZ-induced PPAR$g$ activity to determine whether VPA may be acting as a PPAR$g$ partial agonist or antagonist. TGZ dose-dependently induced PPAR$g$ activity with a mean fold induction of luciferase activity ranging from 23±2.5 to 35±5.2 (Figure 4D). If VPA was acting as a partial agonist or antagonist it should inhibit TGZ activity. VPA induced a mean 2.5±0.23 fold increase (Figure 4F) in TGZ-induced PPAR$g$ activity [range: 53±6.3 to 72±16.0 (Figure 4D)], however,

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2 DCL & MWN, Unpublished observations
this fold increase by VPA was similar to the increase in BD-Gal4 reporter activity (Figure 4E and F). Unlike VPA, TGZ did not induce the control BD-Gal4 reporter. TSA had similar effects as VPA in inducing an ~2 fold increase in TGZ-activated PPARγ and BD-Gal4 reporter activity. These data implies that VPA (0.5-2 mM) does not act as a PPARγ partial agonist or antagonist, most likely producing its effects through its HDAC inhibitory activity.

**VPA treatment inhibits PPARγ and SREBP1a mRNA levels**

The onset of differentiation in adipocytes involves activation of gene expression including C/EBPα/β/δ, PPARγ and SREBP1a. Northern analysis was conducted to determine whether the reduction in differentiation by VPA was accompanied by changes in gene expression. Prior to the onset of differentiation preadipocytes have undetectable levels of C/EBPα, PPARγ and SREBP1a mRNA (Figure 5A). Twenty-four hours following the initiation of differentiation a weak signal for the three transcripts was detectable. At day 1, 2 and 5 following the initiation of differentiation, VPA-treated cells had reduced levels of PPARγ and SREBP1a mRNA, and no change in C/EBPα mRNA levels compared to control cells (Figure 5A & B). We found that VPA reduced GAPDH mRNA levels at day 2 and 5 days by 33±6% and 29±8%, respectively, and therefore could not use it to normalize for equal loading. Since there was no change in C/EBPα levels at day 2 and 5 following treatment, it is unlikely that the reduction in PPARγ and SREBP1a gene expression is due to unequal loading; confirmed by 18S rRNA loading (Figure 5A).

**VPA treatment reduces PPARγ SREBP1a and C/EBPα protein levels**

In order to determine if VPA-induced a concomitant reduction in mRNA and protein levels for PPARγ total cell protein lysates were obtained from cells treated with VPA 1, 3, and 5 days

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3 DCL & MWN, Unpublished observations
after addition of the MDI medium. PPAR\textsuperscript{g} has two isoforms (1 and 2) that are undetectable in preadipocytes (day 0), are expressed at low levels one day following initiation of differentiation (day 1), and clearly detectable at all time points tested after day 3 (Figure 6A). VPA treatment caused a reduction in PPAR\textsuperscript{g} and PPAR\textsuperscript{1}, with a maximum reduction greater than 50\% on day 7 (Figure 6C). After detection of PPAR\textsuperscript{g} blots were stripped and incubated with an antibody against C/EBP\textsuperscript{a}, which detects both the 42 kDa and 30 kDa alternative translation products. While C/EBP\textsuperscript{a} proteins were undetected in preadipocytes, a signal was detected at day 1 of differentiation, and increased throughout differentiation, days 3-7 (Figure 6A). Compared to untreated cells, daily VPA treatment reduced the amount of p42 and p30 C/EBP\textsuperscript{a} protein (Figure 6C), which was surprising since no change in C/EBP\textsuperscript{a} mRNA levels were detected in the presence of VPA.

The precursor (P; 125 kDa) and the cleaved (C; 68 kDa) active form of SREBP1\textsubscript{a} were detected in differentiating adipocytes (Figure 6B). In comparison to the expression of the SREBP1\textsubscript{a} (P), which appears 1 day after inducing differentiation, SREBP1\textsubscript{a} (C) was detected at later time points, with higher amounts of expression at day 4 and 7. Daily VPA treatment did not have an effect on the amount of SREBP1\textsubscript{a} (P), but consistently decreased the SREBP1\textsubscript{a} (C) at day 4 and later time points (Figures 6B & C).

**HDAC inhibition and adipogenesis**

We sought to determine whether other HDAC inhibitors had similar effects on adipogenesis and confirm if VPA treatment in our model was associated with HDAC inhibition. Similar to VPA, daily TSA (3 nM) or 4-phenylbutyrate (4-PB; 1.5 mM) treatment inhibited 3T3-L1 differentiation (Figures 7A, B). The dose of VPA, TSA, and 4-PB was chosen based on the known HDAC IC\textsubscript{50} for these compounds (3,4,18-20). For hydroxamic acid compounds such as
TSA the in vitro HDAC IC$_{50}$ is within the nM-$\mu$M range, while short chain fatty acids such as VPA and 4-PB have HDAC IC$_{50}$ in the mM range (21,22). Since the effect of TSA and 4-PB were similar, future experiments were conducted using TSA, a more potent and commonly used HDAC inhibitor. Similar to VPA, TSA caused a dose-dependent inhibition of adipocyte differentiation (Figure 7C). In comparison to VPA, VPM (1 mM), an amide analog of VPA that does not inhibit HDAC activity, did not significantly affect 3T3-L1 differentiation (Figures 7A & B).

To examine the HDAC inhibitory activity of VPA, we treated 3T3-L1 cells with VPA (1 mM), TSA (3 nM), or VPM (1 mM) daily and assessed histone acetylation at day 3 and 7 following initiation of differentiation. Compared to untreated cells, VPA and TSA induced histone H3 acetylation (Figure 7D). Some histone H3 acetylation was observed in VPM treated cells, but the levels were consistently less than that produced by VPA or TSA (Figure 7D).

We determined whether the reduction in adipocyte differentiation by TSA affected C/EBP$a$, PPAR$g_1$, PPAR$g_2$, p42 and p30 C/EBP$a$, and the cleaved form of SREBP1a (C) but not SREBP1a (P) (Figures 8A & B). In contrast to the effects of VPA or TSA, VPM treatment, which does not inhibit differentiation, did not affect protein expression (Figure 8A). Similar to VPA treated cells, TSA treatment caused reduced levels of PPAR$g$ (average reduction 23±7%) and SREBP1a (average reduction 18±11%) mRNA levels (Figure 8C). Unlike VPA treatment, which had no effect on C/EBP$a$ mRNA levels, TSA treatment reduces C/EBP$a$ mRNA levels (average reduction 37±5%), and VPM had no effect on mRNA levels compared to UT treated cells (Figure 8C).

**Discussion**
Our results show that VPA treatment prevents mouse and human adipocyte differentiation in vitro. Pharmacologic manipulation of HDAC activity with TSA and 4PB inhibited preadipocyte differentiation, whereas VPM did not, leading to the notion that the HDAC inhibitory properties of VPA may be responsible for blocking adipogenesis. TSA treatment reduced mRNA levels for PPAR𝛾, C/EBPα, and SREBP1a, whereas VPA treatment reduced PPAR𝛾 and SREBP1a mRNA, but not C/EBPα mRNA levels. Inhibition of differentiation by VPA and TSA was accompanied by a reduction in TAG and decreased protein levels for PPAR𝛾, C/EBPα, and SREBP1a. Based on these results we hypothesize that HDAC activity is required for adipocyte differentiation.

The role of HDACs during adipocyte differentiation remains largely unknown compared to their role in the differentiation of other cell types such as skeletal muscle (21). In models of muscle cell differentiation (C2C12 skeletal muscle cells and human skeletal myoblasts) when HDAC inhibitors were added one day prior to the differentiation-inducing medium they enhanced myogenesis, however, when HDAC inhibitors were added simultaneously to the differentiation medium myogenesis was inhibited (23). Thus, augmentation or suppression of myogenesis is dependent on the time of HDAC inhibition, suggesting that HDAC activity may provide an important checkpoint to prevent precocious myogenic differentiation. Treatment of 3T3-L1 cells with VPA or TSA one day prior to the initiation of differentiation produces no abrogation in adipogenesis. Our results show that HDAC inhibition with VPA or TSA causes a dose-dependent attenuation of adipogenesis, when added once at the initiation of differentiation or chronically throughout the differentiation period. Two recent studies have examined the effect of a single dose of VPA on adipogenesis in the presence of differentiation medium (MDI). Fajas et al. (13) report that treating 3T3-L1 cells once with VPA (1.5 mM) at the onset of treatment

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4 DCL & MWN, Unpublished observations
with MDI caused a significant induction of adipocyte differentiation. By contrast Wiper-Bergeron et al. (21) illustrate that a single treatment with VPA (10 mM) at the onset of treatment with MDI did not affect differentiation. Repeating these experiments using the protocols described in these manuscripts, we found that VPA did not promote, and always inhibited, adipogenesis.

Which of the numerous HDACs may be important for adipogenesis? TSA, like most HDAC inhibitors, equally inhibits all known HDACs in a reversible fashion by displacing the requisite zinc ion within the active site (21). Unlike other known HDAC inhibitors, VPA inhibits class I HDACs with five times greater potency than class II HDACs in vitro and in vivo (4). In our experiments we tested VPA at doses of 0.5–5 mM and show that inhibition was dose dependent. Based on the published IC\textsubscript{50} for VPA [class I (HDAC2) IC\textsubscript{50} = 0.54 mM; class II (HDAC5) IC\textsubscript{50} = 2.8 mM; (4)] the lower doses of VPA used in our experiments to inhibit differentiation would inhibit class I HDACs and may not significantly affect class II HDACs. This suggests that class I HDACs may be critical for adipogenesis. Moreover, it is unlikely that the proteosome-dependent degradation of HDAC2 induced by VPA, but not TSA, is responsible for the similar effects of VPA and TSA to inhibit adipogenesis (24). Wiper-Bergeron et al. (25) demonstrated that during the initial 24 hours of 3T3-L1 cell differentiation in the presence of dexamethasone, HDAC1 protein levels are reduced by 50%, without affecting HDAC1 mRNA, when compared to cells treated without dexamethasone. We found that HDAC1 protein levels in 3T3-L1 cells were not significantly different between preadipocytes (day 0) and developing adipocytes (days 1, 3, 5) in the absence or presence of VPA, TSA, or VPM. Although HDACs may remain at a steady state level during adipogenesis this does not rule out the possibility that they may be dynamically

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regulated by their localization within the cytoplasm and nucleus. In muscle cell differentiation, the shuttling of HDAC 4, 5, and 7 between the cytoplasm and nucleus plays a critical role in myogenesis (26). In order to fully explore the role of HDACs in adipogenesis it will be necessary to identify which HDACs are present in adipocytes and determine if their localization and activity are dynamically regulated.

Three main classes of transcription factors directly influence fat cell development, PPAR\[, C/EBP, and SREBP-1 (27-29). The expression of PPAR\[ and most importantly PPAR\[, is necessary and sufficient to induce adipogenesis (30). Thiazolidinediones are synthetic PPAR\[ agonists that can induce differentiation of adipocytes, whereas PPAR\[antagonists or partial agonists reduce adipogenesis induced by treatment with either MDI or treatment with thiazolidinediones (31,32). Chronic treatment of 3T3-L1 preadipocytes with VPA was unable to induce adipogenesis and VPA blocked adipogenesis induced by either TGZ or ROS, suggesting that VPA does not act as a PPAR\[agonist. To test whether VPA can act as PPAR\[ partial agonist or antagonist we conducted reporter assays using a PPAR\[LBD chimeric receptor (PPAR-Gal4) as the activator of transcription. Lampen et al. (33) has shown that VPA (0.5-1.5 mM) can induce activation of a glucocorticoid receptor DNA binding domain-PPAR\[LBD hybrid receptor in CHO cells. Similarly, using NIH-3T3 cells expressing endogenous PPAR\[ Fajas et al. (13) demonstrated that VPA (0.5 – 1.5 mM) can activate a reporter gene (PPRE-TK-luc) driven by PPAR\[binding elements linked to a minimal thymidine kinase (TK) promoter. We have shown that VPA (1 mM) enhanced TGZ-induced reporter activation at all doses of TGZ tested (1-20 \(\mu\)M), however, the increase in luciferase activity by PPAR-Gal4 in the presence of VPA is equal to the fold increase in activation of the control protein (GAL4 DBD

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6 DCL & MWN, Unpublished observations
alone, BD-Gal4), supporting the notion that VPA is not specifically activating the PPARγ receptor. Indeed, VPA has been shown to induce a diverse number of promoters including the simian virus-40 (34), cytomegalovirus (3,35), thymidine kinase 7, 78 KD heat shock (36), and Rous sarcoma virus (3) promoters. These data suggest that VPA has a non-specific ability to indirectly induce gene transcription, likely due to its HDAC inhibitory properties (3,4). We conclude that VPA activation of PPARγ is unlikely to be due to a direct effect of VPA interaction with the PPARγ LBD, and that the effects of VPA on adipocyte differentiation are not mediated by VPA binding directly to PPARγ. Furthermore, VPA or TSA inhibition of differentiation is not simply due to prevention of PPARγ expression, since either treatment can inhibit differentiation following the removal of MDI after PPARγ expression has been upregulated. It remains possible that these drugs may be causing a direct or indirect reduction in PPARγ mRNA transcription or stability.

VPA and TSA caused a reduction in SREBP mRNA and mature protein during adipogenesis, similar to its effects on PPARγ. Surprisingly the precursor form of SREBP1a protein appears to remain at a steady state following treatment with HDAC inhibitors. In liver cells once the SREBP cleavage-activating protein senses low sterol levels the precursor SREBP translocates from the endoplasmic reticulum membrane to the Golgi where the mature SREBP is formed by a two step proteolysis via the Site-1 protease and Site-2 protease (37). In adipocytes it is unknown what signals the maturation of SREBP from its precursor. Inoue et al. (38) found that the mRNA levels for SREBP cleavage-activating protein, Site-1 protease, and Site-2 protease in adipocytes remain at a steady state throughout adipogenesis, suggesting that in adipocytes, where sterol levels are not depleted, the mechanism producing the proteolytic activation of SREBP may be

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7 DCL & MWN, Unpublished observations
unique. Similar to our findings with VPA, others using HIV protease inhibitors, which also are
known inhibitors of adipogenesis, have demonstrated reduced SREBP1a maturation despite
steady state precursor levels (39). Future studies are necessary in order to elucidate the
mechanism of SREBP1a processing during adipogenesis and how HDAC inhibitors may affect
this process.

It is possible that the down regulation of PPARγ mRNA by VPA treatment results from
VPA-induced down-regulation of C/EBPα protein. Several studies have demonstrated that
C/EBPα regulates the expression of PPARγ most notably, in vitro C/EBPα is able to directly
bind to the PPARγ promoter (40) and in vivo, mice with disrupted C/EBPα expression shown a
reduction in PPARγ levels (41). We see a striking reduction in C/EBPα protein levels with VPA
treatment despite no affect on C/EBPα mRNA levels. It remains to be determined whether VPA
decreases C/EBPα protein translation or enhances degradation. Since this effect is observed with
VPA but not TSA, it is unlikely to be mediated by HDAC inhibition. This suggests that VPA has
multiple mechanisms of action to abrogate adipogenesis.

In contrast to our work, others have suggested that HDAC inhibitors promote the
differentiation of adipocytes by enhancing the transactivation of PPARγ and C/EBPα (13,25).
Fajas et al. (13) demonstrated that treatment with HDAC inhibitors (including VPA) results in
dissociation of a PPARγ/Rb/HDAC3 complex, allowing PPARγ transactivation and stimulation
of adipocyte differentiation. Similarly, Wiper-Bergeron et al. (21) demonstrated that treatment
with HDAC inhibitors stimulated adipocyte differentiation by promoting the transcription of
C/EBPα by releasing a co-repressor complex comprised of C/EBPα/mSIN3A/HDAC1 from the
C/EBPα promoter. In agreement with these studies, we found that the mRNA and proteins for
PPARγ and C/EBPα are made in the presence of VPA and TSA, but at greatly reduced levels,
which correlates with the limited amount of adipocyte differentiation observed with treatment. It is possible that reduced levels of PPAR\(\gamma\), C/EBP, and SREBP-1 protein is due to decreased production in individual adipocytes or results from the inability of preadipocytes to turn on the differentiation program. Thus a minimal threshold of active C/EBP\(\alpha\) and PPAR\(\gamma\) may be required to promote differentiation in individual cells. The molecular mechanism underlying the ability of HDAC inhibitors to regulate C/EBP\(\alpha\) and PPAR\(\gamma\) expression and activity is unclear based on the current literature. It is likely that regulation of these transcripts is dependent on positive and/or negative factors including corepressor complexes containing the HDACs. Indeed, it has recently been shown that HDAC interaction with N-CoR can act as positive coregulators of transcriptional activation (42). Thus inhibition of HDAC activity may result in blocking transcriptional activity of genes critical for adipocyte differentiation.

We examined VPA effects on adipogenesis with the initial aim of understanding how VPA may induce weight gain in patients. Both fat cell number controlled by preadipocyte proliferation and adipogenesis, and fat cell size controlled by lipogenesis contribute to weight gain. We hypothesized that VPA would enhance adipogenesis, however, we found that VPA does the opposite. It is paradoxical that in vitro VPA inhibits adipogenesis yet in vivo induces weight gain. Weight gain is the outcome of a variety of central and peripheral inputs and we hypothesize that VPA affects numerous cell types in the central nervous system and periphery. In vivo, VPA suppression of adipogenesis may be overcome by compensatory physiological effects with the net outcome of weight gain. Lithium carbonate, another mood stabilizer that induces weight gain in vivo, inhibits adipogenesis in vitro most likely through its effects on Wnt/GSK3\(\beta\) signaling (43,44). Unlike lithium carbonate, we suggest that VPA inhibition of adipocyte differentiation is due to its HDAC inhibitory activity. VPA has recently been shown to produce some of its
neuronal effects through reduction of inositol biosynthesis, which can be blocked by supplementing cells with inositol. We found that inositol supplementation was unable to reverse the ability of VPA to inhibit adipogenesis. Moreover VPA is unlikely to mediate its effects via a direct activation of PPAR. Overall our results highlight a role for HDAC activity in adipogenesis that can be blocked by treatment with VPA.

8 DCL & MWN, Unpublished observations
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References

Figure 1. VPA inhibits differentiation of mouse 3T3-L1 preadipocytes. (A) Visualization of triacylglycerol levels by Oil Red O staining 13 days after initiation of differentiation. Cells were grown in the presence (+) or absence (-) of MDI medium (IBMX, dexamethasone, and insulin) for the first 2 days, and treated daily with VPA (0-5mM). In the absence of MDI, untreated or VPA treated cells have similar Oil Red O staining. In the presence of MDI, VPA dose-dependently reduces adipocyte differentiation. (B) High magnification (200x) picture of cells cultured using the same conditions as (A) demonstrating that few adipocytes form in the absence of MDI whether untreated (UT) or treated (1mM VPA). MDI induces adipocyte formation, which is reduced by VPA treatment. (C) Quantification of Oil Red O staining of cells cultured in MDI confirmed that VPA treatment significantly reduced formation of adipocytes. Data represents mean percent levels compared to UT (set at 100% differentiation), * p<0.001.

Figure 2. VPA inhibits differentiation of human preadipocytes. (A) Representative photomicrographs of two lots of subcutaneous preadipocytes from one individual (L091901) and cells pooled from six individuals (SL0023) stained with Oil Red O 12 days after initiation of differentiation in the presence or absence of Zen-Bio’s differentiation medium (Zen-MID), and/or daily treatment with 1 mM VPA. VPA does not induce adipocyte formation in absence of Zen-MID, and inhibited adipocyte formation in the presence of Zen-MID. (B) Quantification of TAG levels from cells described in (A) that were treated daily with VPA (0-5 mM) or 4 ng/ml of tumor necrosis factor α (TNF). Data represents mean from one experiment performed in triplicate.
Figure 3. Temporal effects of VPA treatment on adipocyte differentiation. (A) 3T3-L1 cells treated once at day 0 with 1mM VPA have significantly lower amounts of Oil Red O staining compared to untreated (UT) cells. Data represent mean levels of Oil Red O from one experiment performed in triplicate; similar findings were obtained in three independent experiments. (B) [³H] thymidine incorporation into cellular DNA measured at 1, 10, 20, and 30 hours following the addition of MDI demonstrating no significant difference in clonal expansion in the absence (UT) or presence of VPA. Representative data from one experiment performed in triplicate; similar data was obtained in two separate experiments. (C) Quantification of Oil Red O staining of cells differentiated with MDI (days 0-2), untreated (UT), treated daily with VPA (day 0-2, 3-13, 7-13, or 0-13). Data represents mean percent levels compared to UT (set at 100% differentiation). * p<0.001

Figure 4. VPA inhibits PPARγ induced differentiation in mouse and human preadipocytes, but does not act as a PPARγ ligand. (A) Quantification of Oil Red O staining of 3T3-L1 cells differentiated in the presence of a PPARγ agonist, 1 µM rosiglitazone (ROS) or 10 nM troglitazone (TGZ) demonstrating significant reduction in staining when cells were co-treated daily with VPA on days 0-13. Data represents mean percent levels compared to UT (set at 100% differentiation). * p<0.001 (B) Quantification of triacylglycerol levels from two lots of human preadipocytes, demonstrating cells differentiated with 1 µM ROS had more triacylglycerol than cells that were co-treated daily with VPA (0-5 mM). Cells treated with 4 ng/ml of TNFα at each medium change are shown. Data represent mean values from one experiment performed in triplicate. (C) Fold induction in luciferase activity in the presence of the Gal4 expression plasmid (BD-Gal4) or the Gal4 expression plasmid with the PPARγ ligand binding domain
(PPAR-Gal4) following treatment with either VPA (0.5-2 mM), TGZ (20 mM) or TSA (300 nM) in HepG2 cells. (D) Dose-response curve (1-20 µM) for TGZ-induced increase of PPAR-Gal4 activity in the absence or presence of 1 mM VPA. (E) Dose-response (1-20 µM) for TGZ on the BD-Gal4 activity in the absence or presence of 1 mM VPA. (F) Summary of VPA-induced fold increase from experiments described in (D) and (E) demonstrating that the differences in activation of BD-Gal4 vs. PPAR-Gal4 induced by VPA are not significant. Data shown from B-F are normalized with β-gal activity; similar findings were obtained in three independent experiments.

Figure 5. VPA reduces SREBP1a and PPARγ but not C/EBPα, mRNA. (A) Northern analysis of mRNA from 3T3-L1 cells prior to addition of MDI medium (day 0) and days 1, 2, and 5 after addition of MDI in the absence or presence of 1 mM VPA. (B) Mean percent reduction in mRNA levels for SREBP1a and PPARγ comparing 1 mM VPA treated to UT cells from two independent experiments, performed in duplicate at each time point.

Figure 6. VPA treatment reduces PPARγ and PPARγ, p30 and p41 C/EBPα, and SREBP1a (C) protein. (A) Western analysis of whole cell protein extracts from 3T3-L1 cells obtained prior to addition of MDI medium (day 0) and days 1, 3, and 5 after addition of MDI in the absence or presence of 1 mM VPA. (B) Western analysis of whole cell protein extracts from 3T3-L1 cells prior to (day 0) or after the addition of MDI medium (days 1, 4, and 7). Daily VPA (1 mM) treatment reduces protein levels for mature SREBP1a (C), but does not affect precursor SREBP1a (P) protein. Cells were pretreated with, and protein samples isolated in the presence of, the proteosome inhibitor N-acetyl-leucine-leucine-norleucinal (ALLN). (C) Mean percent
reduction in mature SREBP1a, PPARγ and C/EBPα protein levels at day 5 and 7 after initiation of differentiation, comparing VPA (1 mM) treated to untreated cells. Data is representative of two independent experiments performed in triplicate at each time point.

**Figure 7.** HDAC inhibition reduces adipocyte differentiation. (A) Cells stained with Oil Red O 13 days after initiation of differentiation in MDI. In comparison to untreated (UT) cells daily treatment with the HDAC inhibitors VPA (1 mM) or TSA (3 nM) inhibited differentiation, whereas valpromide (VPM 1 mM), which does not inhibit HDACs, does not affect adipocyte differentiation. (B) Quantification of Oil Red O staining of cells cultured using the same conditions as (A); TSA and VPA reduced formation of adipocytes, whereas there was no significant difference between UT and VPM treated cells. Data represents mean percent level compared to UT (set at 100% differentiation). (C) TSA dose-dependently inhibits 3T3-L1 differentiation. Data represents mean percent level compared to UT (set at 100% differentiation). (D) Western analysis of 3T3-L1 cells obtained at day 3 and 7 after the addition of MDI. Daily VPA (1 mM) and TSA (3 nM) treatment induces high levels of acetylated histone H3, compared to UT and VPM (1 mM) treated cells. ** p<0.01, *** p<0.001

**Figure 8.** VPA and TSA, but not VPM, reduce protein levels for PPARγ, SREBP1a, and C/EBPα, and differentially affect mRNA production. (A) Western analysis of 3T3-L1 cells obtained 7 days after the addition of MDI. Daily TSA (3 nM) or VPA treatment reduced PPARγ, PPARα, p30 and p42 C/EBPα, and mature SREBP1a (C) protein levels when compared to untreated cells. (B) Mean percent reduction in protein levels comparing TSA (3 nM) treated to untreated cells from two independent experiments, each performed in duplicate.
(C) Northern analysis of mRNA obtained 5 days after addition of MDI. Similar to VPA, daily TSA (3 nM) treatment reduces mRNA levels for PPARγ SREBP1a. Unlike VPA treatment, TSA-induced a reduction in C/EBPα mRNA levels. VPM does not produce a significant effect on mRNA levels.
Figure 1

A

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B

- MDI

+ MDI

UT

1mM VPA

C

![Bar graph showing Oil Red O (%) vs VPA (mM) with significant differences marked with asterisks.](image)
Figure 2

A

L091901

SL0023

Zen-MDI - - + +
1mM VPA - + - +

B

Triacylglycerol (µM glycerol)

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Figure 3
Figure 4
Figure 4
**Figure 5**

### A

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<tr>
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### B

- **SREBP1a**
  - Day 1: 35% Reduction
  - Day 2: 40% Reduction
  - Day 5: 50% Reduction

- **PPAR**
  - Day 1: 30% Reduction
  - Day 2: 45% Reduction
  - Day 5: 55% Reduction
**Figure 6**

A

Differentiation Day 0 1 3 5
1mM VPA - + - + - +

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B

Differentiation Day 0 1 4 7
1mM VPA - - + - - +

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C

![Bar chart showing % Reduction in protein for different proteins at Day 5 and Day 7](chart.png)

Legend:
- SREBP (C)
- PPAR 2
- PPAR 1
- C/EBP p42
- C/EBP p30

Day 5

Day 7
**Figure 7**

(A) 

Image showing different treatments: UT, VPA 1 mM, TSA 3 nM, 4PB 1.5 mM, and VPM 1 mM.

(B) 

Bar graph showing Oil Red O (%) with treatments UT, VPA 1 mM, TSA 3 nM, 4PB 1.5 mM, and VPM 1 mM.

(C) 

Bar graph showing Oil Red O (%) with increasing TSA concentrations (0, 0.5, 1, 3, 5, 8, 10 nM).

(D) 

Table showing differentiation day and treatment combinations for 3 and 7 days with 1 mM VPA, 3 nM TSA, 1 mM VPM, acetyl-histone H3, and Actin.
Figure 8