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Conserved valproic-acid-induced lipid droplet formation in *Dictyostelium* and human hepatocytes identifies structurally active compounds

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SUMMARY

Lipid droplet formation and subsequent steatosis (the abnormal retention of lipids within a cell) has been reported to contribute to hepatotoxicity and is an adverse effect of many pharmacological agents including the antiepileptic drug valproic acid (VPA). In this study, we have developed a simple model system (*Dictyostelium discoideum*) to investigate the effects of VPA and related compounds in lipid droplet formation. In mammalian hepatocytes, VPA increases lipid droplet accumulation over a 24-hour period, giving rise to liver cell damage, and we show a similar effect in *Dictyostelium* following 30 minutes of VPA treatment. Using ^{3}H-labelled polyunsaturated (arachidonic) or saturated (palmitic) fatty acids, we shown that VPA treatment of *Dictyostelium* gives rise to an increased accumulation of both types of fatty acids in phosphatidylcholine, phosphatidylethanolamine and non-polar lipids in this time period, with a similar trend observed in human hepatocytes (Huh7 cells) labelled with [^{3}H]arachidonic acid. In addition, pharmacological inhibition of β-oxidation in *Dictyostelium* phenocopies fatty acid accumulation, in agreement with data reported in mammalian systems. Using *Dictyostelium*, we then screened a range of VPA-related compounds to identify those with high and low lipid-accumulation potential, and validated these activities for effects on lipid droplet formation by using human hepatocytes. Structure-activity relationships for these VPA-related compounds suggest that lipid accumulation is independent of VPA-catalysed teratogenicity and inositol depletion. These results suggest that *Dictyostelium* could provide both a novel model system for the analysis of lipid droplet formation in human hepatocytes and a rapid method for identifying VPA-related compounds that show liver toxicology.

INTRODUCTION

Valproic acid (VPA) was first identified as an antiepileptic in 1963 (Meunier et al., 1963), and since then it has become a commonly used treatment for epilepsy, bipolar disorder and migraine (Lagace et al., 2005; Terbach and Williams, 2009). In trying to understand the therapeutic role of VPA, a range of cellular effects have been identified, including inositol depletion (associated with bipolar disorder treatment) (Eickholt et al., 2005; Shimshoni et al., 2007; Williams, 2005; Williams et al., 2002) and histone deacetylase (HDAC) inhibition (associated with teratogenicity) (Gottlicher et al., 2001; Phiel et al., 2001). In addition, VPA is associated with a range of adverse effects, including hepatotoxicity, tremors, alopecia and drowsiness (Lagace et al., 2005). Hepatotoxicity is more severe in those patients on multiple prescriptions; however, the related condition of non-alcoholic fatty liver disease or steatosis (abnormal lipid accumulation) is also frequent in patients taking VPA, alone or in combination with other agents (Luef et al., 2009; Verrotti et al., 2011a). Thus, the analysis of hepatotoxicity and steatosis in relation to VPA treatment, and the development of model systems for this research, are important priorities because they will enable the development of novel therapeutics with improved risk:benefit ratios.

Within mammalian cells, fatty acids such as the polyunsaturated fatty acid arachidonic acid (AA) (Svennerholm, 1968) can be incorporated into phospholipids directly or stored as non-polar lipids such as diacyl- and triacylglycerols (DAGs and TAGs, respectively) prior to reincorporation or metabolism. Release of the fatty acid from these phospholipids or other lipid classes occurs mainly through lipase-catalysed catabolism, such as that involving phospholipase A2 (PLA_2) (Rapoport, 2008). Once released, free (non-esterified) fatty acid species can then be reincorporated or transported to the mitochondria to be metabolised by β-oxidation. VPA treatment has been shown to act as a PLA_2-like inhibitor (Bosetti et al., 2003; Rapoport and Bosetti, 2002), reducing expression of defined isoforms of PLA_2 (Chang et al., 2001) while also disrupting fatty acid β-oxidation (Aires et al., 2011; Silva et al., 2008). A range of in vitro mammalian models has been used to show VPA-induced hepatotoxicity and steatosis effects (Eadie et al., 1988), with increased lipid droplet accumulation being observed in hepatocytes (Fujimura et al., 2009) and skeletal muscle (Melegh and Trombitas, 1997). Although this VPA-catalysed effect is likely to cause liver damage to individuals undergoing treatment, it remains possible that these effects are disassociated from the therapeutic mechanisms; thus, a better understanding of

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compounds causing this effect is of interest in the design of novel therapeutics.

Structure-activity relationship (SAR) studies have previously been employed to delineate the potential targets of VPA (Bialer et al., 2010; Eickholt et al., 2005; Eikel et al., 2006; Eyal et al., 2005; Shimshoni et al., 2007). In this approach, the structural characteristics of VPA-related compounds can be used to isolate and characterise the molecular mechanism of individual effects, which can then be used to differentiate between distinct mechanisms of action. SAR studies have been used to examine the teratogenic nature of VPA, which is thought to be due to inhibition of histone deacetylase function (Eikel et al., 2006; Phiel et al., 2001; Spiegelstein et al., 2000). Similarly, the inhibition of inositol phosphate signalling by VPA has also been examined in SAR studies in both *Dictyostelium* cells and mammalian neurons (Eickholt et al., 2005; Shimshoni et al., 2007; Williams et al., 2002). These previous studies have clearly identified distinct structural characteristics of various VPA-related compounds that are responsible for these effects; therefore, these processes are likely to have different mechanisms of action. It remains unclear whether either of these effects is related to lipid accumulation, which is the aim of the current investigation.

In this study, we examined VPA-induced lipid accumulation in *Dictyostelium discoideum*. We have previously used this model to characterise the effect of VPA in inositol trisphosphate [$InsP_3$; also known as Ins(1,4,5)P_3] (Eickholt et al., 2005; Shimshoni et al., 2007; Williams et al., 2002) and phospholipid (Chang et al., 2012; Xu et al., 2007) signalling, with subsequent translation in mammalian systems. Here we show that, in *Dictyostelium*, pharmacologically relevant concentrations of VPA acutely enhance the uptake of fluorescently labelled and radiolabelled fatty acids {both polyunsaturated fatty acid [^{3}H]AA and the saturated fatty acid [^{3}H]PaA (palmitic acid)}, and that the increase in fatty acid uptake occurs concurrently with a decrease in fatty acid release. Analysis of the cellular distribution of incorporated, radiolabelled fatty acids by two-dimensional (2D) thin-layer chromatography (TLC) revealed that VPA-induced fatty acid accumulation increased the overall accumulation in all phospholipids and non-polar lipids examined but did not alter the overall distribution within lipids, and an equivalent trend is shown using human hepatocytes (Huh7 cells) following [^{3}H]AA labelling. In *Dictyostelium*, a similar effect of lipid accumulation was shown by pharmacological inhibition of β-oxidation. Analysis of series of VPA-related compounds in *Dictyostelium* identified a broad range of activities, tightly defined by structure, and a selection of these compounds was then used in Huh7 cells to show corresponding lipid accumulation. Finally, using a range of compounds with known teratogenic or inositol-depleting activity, we show that the biological effect of VPA-induced fatty acid accumulation might be independent of inositol depletion and HDAC inhibition and/or teratogenicity, indicating the potential for identifying VPA-based therapeutics with reduced hepatotoxic liability.

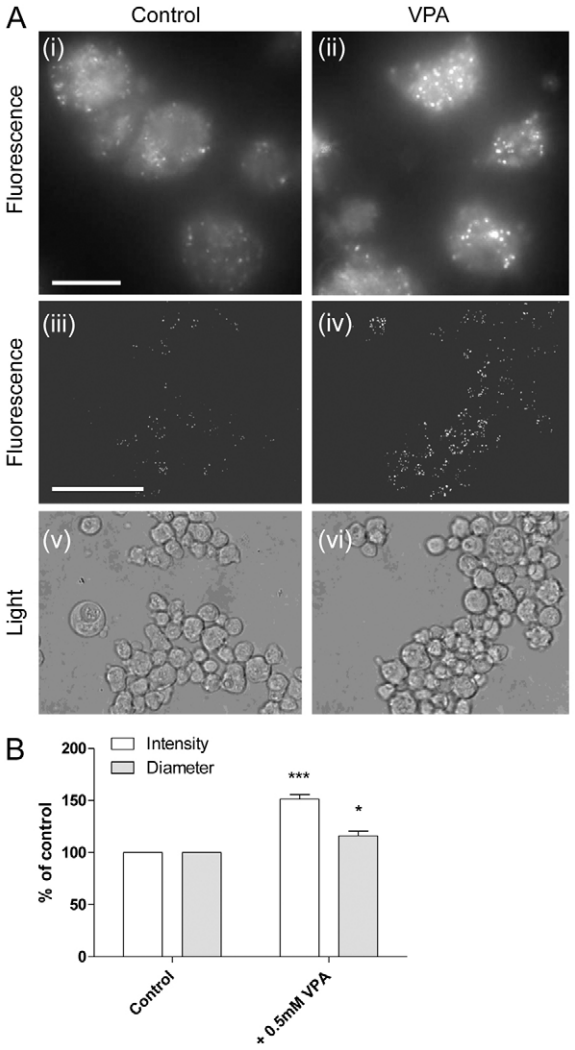

Fig. 1. VPA induces lipid droplet accumulation of fluorescent C_1-BODIPY-C_{12} in *Dictyostelium*. (A) Cells were incubated for 30 minutes with the fluorescent fatty acid C_1-BODIPY-C_{12} in the absence (i, iii, v) or presence (ii, iv, vi) of VPA (0.5 mM). Analysis of lipid droplets by fluorescent imaging in (i) untreated and (ii) VPA-treated cells shows increased droplet size and intensity upon treatment. Fluorescent visualisation of cell populations for (iii) untreated and (iv) VPA-treated samples, compared with corresponding transmission images (v and vi, respectively), also shows this increase. (B) Quantification of individual droplet intensity and average diameter compared with control, as measured by ImagePro software (*t*-test: **P*<0.05, ****P*<0.001). Cell images contained approximately 12,500 pixels per cell. Scale bars: 10 μm (for i and ii; shown in i); 50 μm (for iii-vi; shown in iii).

RESULTS

VPA increases fatty acid accumulation

To investigate the effectiveness of using *Dictyostelium* to analyse lipid droplet formation following VPA treatment, we visualised VPA-induced fatty acid accumulation with a compound containing a 12-carbon fatty acid chain linked to a fluorescent head group (C_1-BODIPY-C_{12}) (von Lohneysen et al., 2003; Worsfold et al., 2004). Upon incubation of cells with C_1-BODIPY-C_{12}-labelled lipid, VPA (0.5 mM) caused a large increase in the intensity and a small but significant increase in diameter of fluorescent lipid droplets within cells compared with untreated cells (Fig. 1A,B). These changes were observed after just 30 minutes of treatment with VPA and are comparable to the lipid accumulation observed in hepatocytes after 24 hours (Fujimura et al., 2009).

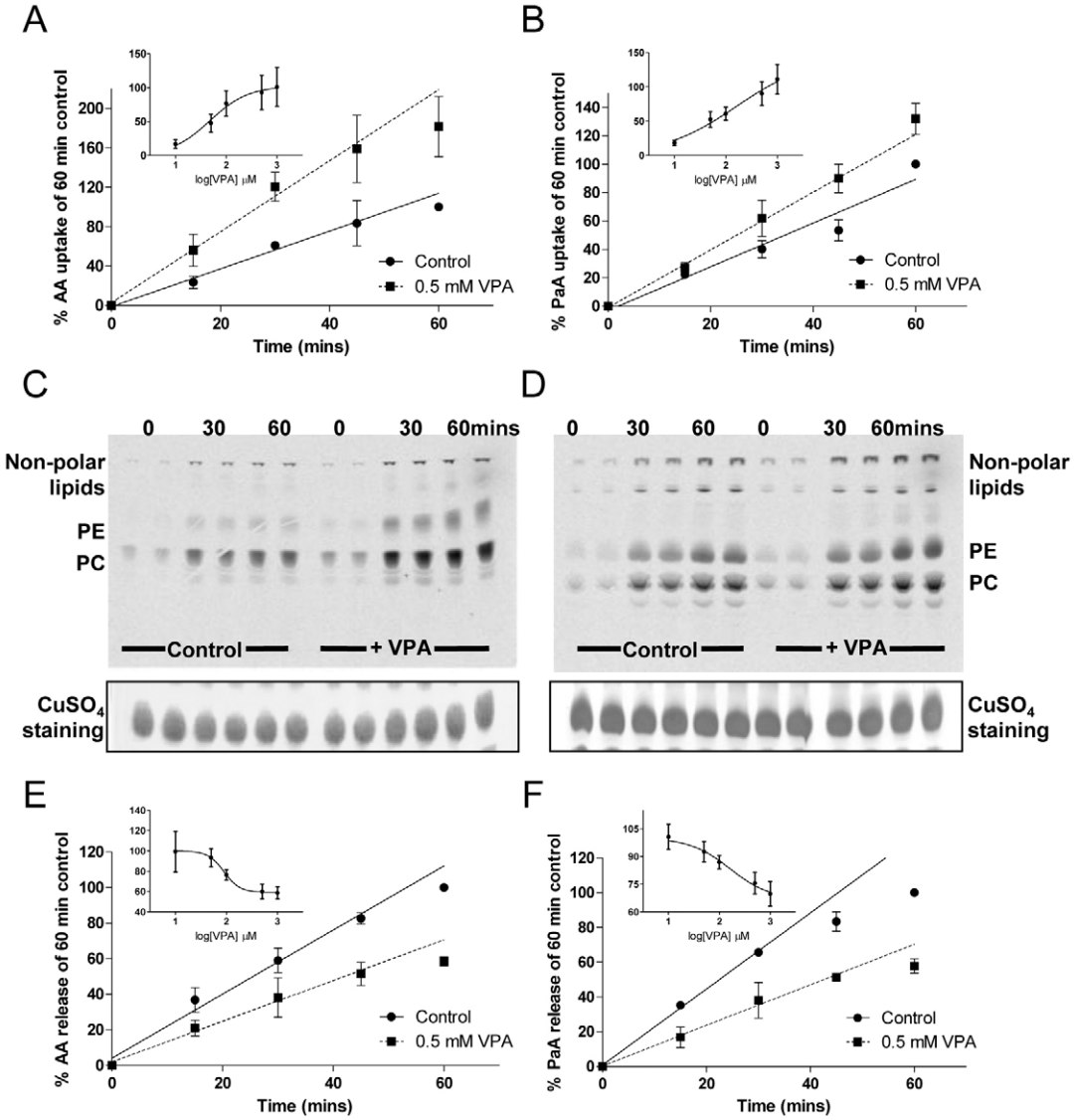

Fig. 2. VPA induces changes in fatty acid uptake and release in a time- and concentration-dependent manner. (A,B) *Dictyostelium* wild-type cells (Ax2) were used to monitor the uptake of [^{3}H]AA (A) or [^{3}H]PaA (B) into cells in the absence (circles) or presence (squares) of VPA (0.5 mM) and quantified by scintillation counting. (C,D) This effect was also monitored following TLC separation of the cellular lipids for (C) [^{3}H]AA and (D) [^{3}H]PaA. Total lipid loading is shown following copper sulphate ($CuSO_4$) staining. (E,F) Cells were pre-incubated with (E) [^{3}H]AA or (F) [^{3}H]PaA for 1 hour before removing excess label and scintillation quantification of release of ^{3}H into external buffer in the absence (circles) or presence (squares) of VPA (0.5 mM). Results from scintillation assays (A,B,E,F) are expressed as the percentage of ^{3}H in cpm obtained from control samples at 60 minutes. Insets show dose response curves at 30 minutes, expressed as a percentage of ^{3}H uptake/release in the absence of VPA. Statistics and dose response curves were calculated using GraphPad Prism software with linear regression for line fitting based upon 0-30 minute data. All data are replicates of at least three independent experiments and show means ± s.e.m. PC, phosphatidylcholine; PE, phosphatidylethanolamine.

To quantify this effect of increase lipid accumulation in *Dictyostelium*, we measured the uptake of [^{3}H]AA fatty acid into cells over time, to show linear incorporation over a 30-minute period (Fig. 2A). VPA caused a dose-dependent increase in [^{3}H]AA fatty acid incorporation, with a half maximal effective concentration (EC50) of 47 μM (Fig. 2A inset). This effect is not fatty acid specific, and similar results were also seen using [^{3}H]PaA, with an EC50 value of 160 μM VPA (Fig. 2B and inset). For both fatty acids, increased uptake was significant following a 30-minute treatment (P<0.05). These results were confirmed following lipid extraction and one-dimensional (1D) TLC separation with duplicate samples following 30 and 60 minutes of VPA (0.5 mM) treatment (Fig. 2C,D).

We also assessed the release of radiolabel into medium from cells labelled with either [^{3}H]AA or [^{3}H]PaA. In both cases, the release of radiolabel was linear over a 30-minute period (Fig. 2E,F). Inhibition of this release by VPA (0.5 mM) was acute and dose dependent, with a half maximal inhibitory concentration (IC50) of 89 μM and 163 μM for [^{3}H]AA and [^{3}H]PaA, respectively (Fig. 2E,F insets), with both [^{3}H]AA- and [^{3}H]PaA-labelled cells showing a significant reduction in ^{3}H release after 30 minutes exposure (0.5

mM) (P<0.05). This provided a fast and easy assay for determining the deregulation of fatty acid accumulation by VPA in *Dictyostelium*.

We then examined the specific phospholipid classes involved in increased fatty acid incorporation regulated by VPA. In these experiments, *Dictyostelium* cells were labelled for 60 minutes with [^{3}H]AA or [^{3}H]PaA in the absence or presence of VPA (1 mM), and then lipids were extracted and separated by 2D TLC prior to quantification by scintillation counting. Equal lipid loading was validated by UV visualisation of 2D separated lipids (Fig. 3A,B). VPA treatment caused a strong and significant increase in incorporation of both [^{3}H]AA and [^{3}H]PaA into phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Fig. 3C,D). VPA did not alter the preference of phospholipid species retaining each fatty acid, suggesting that the mechanism of action is not restricted to a specific class of lipid. As well as phospholipid incorporation, TLC analysis revealed a significant increase in fatty acid accumulation in non-polar lipids (Fig. 3C,D), which correlates with the observed lipid droplet accumulation in *Dictyostelium* and hepatocytes. Repeating the [^{3}H]AA labelling experiments in a human hepatocyte cell line (Huh7) showed a similar trend of increased incorporation into phospholipids and non-polar lipids (Fig. 3E).

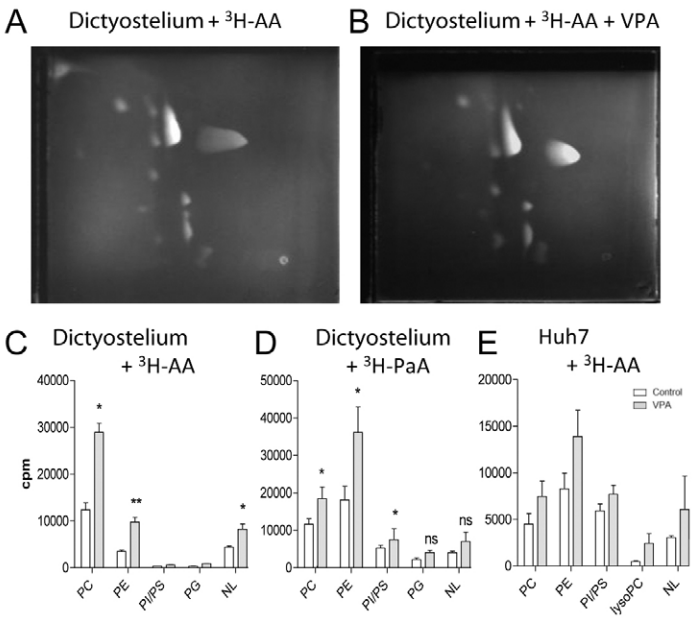

Fig. 3. 2D TLC analysis of radiolabelled lipids in *Dictyostelium* and Huh7 cells indicates their general accumulation into phospholipids and neutral lipids following VPA treatment. (A,B) In *Dictyostelium*, radiolabelling cells with [^{3}H]AA under (A) control conditions or (B) during exposure to VPA (60 minutes; 1.0 mM), followed by lipid extraction, 2D separation and visualisation by phosphorimage analysis, enabled the identification of phospholipid species that accumulated the radiolabelled fatty acid. (C,D) Individual phospholipid species were then quantified by scintillation counting following (C) [^{3}H]AA (D) or [^{3}H]PaA labelling in the presence or absence of VPA. *P<0.05; **P<0.01; ns, not significant. (E) Quantification of phospholipid species labelling in Huh7 cells, incubated in the presence of [^{3}H]AA with or without VPA for 24 hours prior to lipid harvesting. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PG phosphatidylglycerol; NL, non-polar (neutral) lipids.

Inhibition of β-oxidation partially phenocopies VPA regulation of fatty acid turnover

To investigate the mechanism of VPA-induced lipid accumulation, we assessed the role of inhibiting various lipid-turnover-associated enzymes on [^{3}H]AA in *Dictyostelium* using a pharmacological approach (Fig. 4A). Because VPA has been associated with PLA_2-inhibitory-like effects (Bosetti et al., 2003; Rapoport and Bosetti, 2002), we first used a combination of PLA_2 class-specific inhibitors {bromoenol lactone (BEL) [80 μM], a Ca^{2+}-independent PLA_2 inhibitor (Ackermann et al., 1995); methyl arachidonyl fluorophosphonate (MAFP) [50 μM], an inhibitor of Ca^{2+}-dependent and Ca^{2+}-independent cytosolic PLA_2 (Balsinde and Dennis 1996; Lio et al., 1996); and bromophenacyl bromide (BPB) [20 μM], a general PLA_2 inhibitor (Mitchell et al., 1977)}. This PLA_2-inhibitor cocktail gave the opposite effect to VPA, showing reduced fatty acid accumulation (Fig. 4B). Similarly, the reduction of DAG acyltransferase activity, responsible for conversion of DAG to TAG using an acyl-coenzyme A (acyl-CoA) substrate (with the inhibitor xanthohumol; 50 μM), also inhibited fatty acid accumulation. However, the use of 2-mercaptoacetate (2-MA; 15 mM), previously shown to inhibit long-chain acyl-CoA dehydrogenase in the β-oxidation of fatty acids (Bauche et al., 1983), phenocopied the effect of VPA by causing an increase in fatty acid uptake (Fig. 4B). Combining VPA and 2-MA (Fig. 4C) gave rise to a significant increase in uptake (P=0.04) above VPA alone, and similar to that of 2-MA alone, suggesting that VPA might function in this effect by weakly inhibiting the β-oxidation of fatty acids.

Structure-activity study of fatty acid accumulation in *Dictyostelium*

To examine the structural features of VPA that affect fatty acid regulation, we employed a range of compounds related to VPA with varying carbon backbone and side chain length, head group, and saturation. Radiolabel release from ^{3}H-fatty-acid-labelled cells following 60 minutes of treatment (0.5 mM) was measured (Fig. 5). The compounds tested showed a range of inhibitory activity, from stronger than VPA to no change from control. Compounds showing high inhibitory activity were carboxylic acids, branched at the second carbon, with the strongest compound containing an isopropyl group. Branched compounds were generally stronger than corresponding straight chains, with a preference for longer side chains (propyl groups showing stronger inhibition than methyl side chains).

Correlating *Dictyostelium* fatty acid effects and steatosis

Because our data suggest a spectrum of fatty-acid-regulating activities for the VPA-related compounds tested, we then examined the effects of a set of these compounds (showing a wide range of activities) on lipid droplet accumulation in human hepatocytes (Huh7 cells). In these experiments, Huh7 cells were exposed to VPA or these related compounds for 24 hours prior to staining of lipid droplets and image recording (Fig. 6). Compounds showing little attenuation of fatty acid release in *Dictyostelium* did not produce lipid droplets in hepatocytes (Fig. 6A,C,E,F), and compounds showing a VPA-like inhibitory effect on fatty acid release in *Dictyostelium* had a VPA-like effect on lipid droplet formation in the human cells (Fig. 6B,D,G). Finally, compounds showing a stronger attenuation of fatty acid release in *Dictyostelium* compared

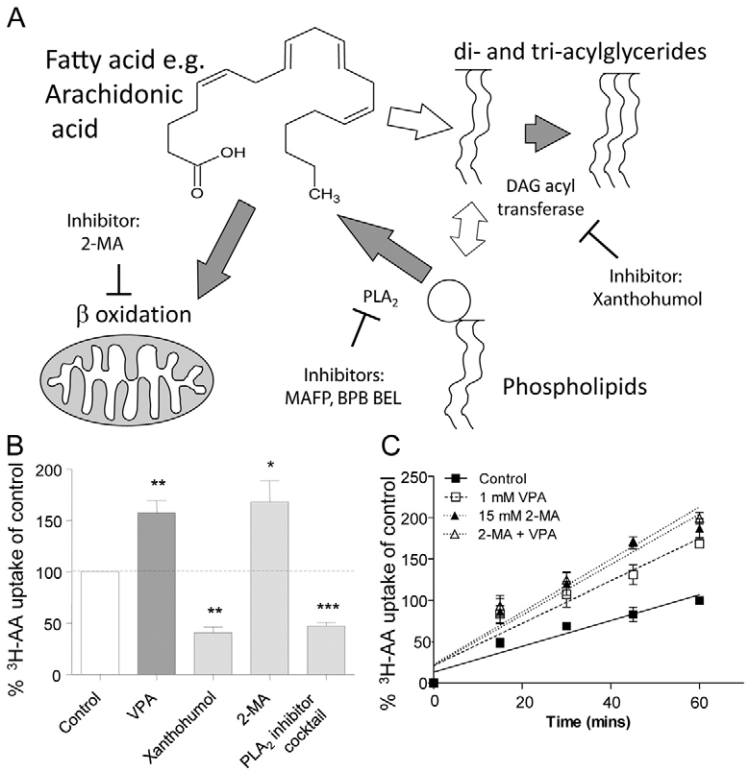

Fig. 4. Pharmacological evaluation of lipid accumulation. (A) Schematic representation of the pharmacological approach taken to investigate VPA-mediated fatty acid regulation. PLA_2 activity gives rise to the release of fatty acids from phospholipids in the membrane, whereas DAG acyl transferase catalyses the interconversion of neutral lipids, both of which are effects that are potentially responsible for regulating fatty acid levels, and thus might be affected by an inhibition of β-oxidation of fatty acids. (B) *Dictyostelium* were labelled with [^{3}H]AA in the presence of various drugs: 1 mM VPA, 50 μM xanthohumol (DAG acyl transferase inhibitor), PLA_2 inhibitor cocktail (BEL [80 μM], a Ca^{2+}-independent PLA_2 inhibitor; MAFP [50 μM], an inhibitor of Ca^{2+}-dependent and Ca^{2+}-independent cytosolic PLA_2; and BPB [20 μM], a general phospholipase A_2 inhibitor) or 2-mercaptoacetate (2-MA, 15 mM, an inhibitor of long-chain acyl-CoA dehydrogenase). Statistical analysis was performed by GraphPad Prism software using a Student's *t*-test: **P*<0.05, ***P*<0.005 and ****P*<0.001 versus control. (C) Uptake of [^{3}H]AA over time in the absence (control) or presence of VPA (0.5 mM), 2-MA (15 mM) or combined VPA and 2-MA.

with VPA also showed enhanced lipid droplet formation in hepatocytes (Fig. 6H,I).

Distinguishing fatty acid regulation, inositol depletion and teratogenic effects

VPA and related compounds have been widely shown to cause the potentially therapeutic effect of inositol depletion, and the side effect of teratogenicity (Terbach and Williams, 2009). We thus compared activities for compounds of known efficacy in inositol depletion, as previously shown in both *Dictyostelium* $InsP_3$ depletion and mammalian dorsal root ganglia (DRG) neuronal explant experiments, and in teratogenicity (Table 1) with effects shown here on fatty acid regulation (in *Dictyostelium*) and lipid droplet formation in Huh7 cells. No relationship was found between inositol depletion efficacy and regulation of fatty acids. For example, 2-methyl-2-pentenoic acid reduces $InsP_3$ levels in *Dictyostelium* and elicits an inositol-dependent increase in growth cone size in primary rat DRG (Eickholt et al., 2005), and we show here that it did not affect fatty acid regulation in *Dictyostelium.* By contrast, 2-ethyl-4-methylpentanoic acid had a strong (VPA-like) effect on release from fatty-acid-labelled cells and was strongly effective in inositol depletion in both *Dictyostelium* and DRG experiments. Similarly, with regards to teratogenicity (Table 1), no correlation was shown for compounds when comparing lipid regulation and teratogenic activities (Eickholt et al., 2005; Riebeling et al., 2011).

DISCUSSION

Lipid droplet formation in the liver, steatosis, has been linked to hepatotoxicity (Stephens and Levy, 1992), and is commonly associated with VPA treatment, although the mechanism giving rise to this effect remains unclear (Lagace et al., 2005). Understanding the molecular mechanism for this effect has been difficult because VPA gives rise to numerous molecular effects, many of which have not yet been fully elucidated, nor have the effects and pathways been correlated (Terbach and Williams, 2009). We have thus developed a simple biomedical model, *Dictyostelium*, to unravel the multiple biochemical effects of VPA (Adley et al., 2005; Boeckeler et al., 2006; Chang et al., 2012; Eickholt et al., 2005; Ludtmann et al., 2011; Terbach et al., 2011; Williams et al., 2002; Xu et al., 2007). Subsequent to identifying molecular effects of VPA in *Dictyostelium*, we applied this knowledge to mammalian systems to validate the model. Here we investigate the effect of VPA in lipid accumulation and correlate effects shown in *Dictyostelium* to human liver cells. In this process we have identified a rapid method for examining lipid accumulation using *Dictyostelium* and successfully translated results from this model to a mammalian cell line to identify VPA-related compounds exhibiting reduced lipid accumulation. We also show that this effect is likely to be unrelated to other VPA-mediated effects of teratogenicity and inositol depletion. The identification of analogues with reduced hepatotoxic potential is, therefore, of particular importance in the development of novel therapeutics.

VPA increases the accumulation of lipids in *Dictyostelium* as observed using both fluorescently labelled and radiolabelled fatty acids (Figs 1, 2). In the radiolabelled uptake studies, we employed both a polyunsaturated fatty acid, AA, and a saturated fatty acid, PaA; both the fatty acids showed enhanced accumulation and a decreased release of radiolabelled product over time. PaA is a naturally occurring fatty acid in *Dictyostelium* and, although AA is not normally found in the model, it is both incorporated into *Dictyostelium* phospholipids (Weeks, 1976) and triggers the

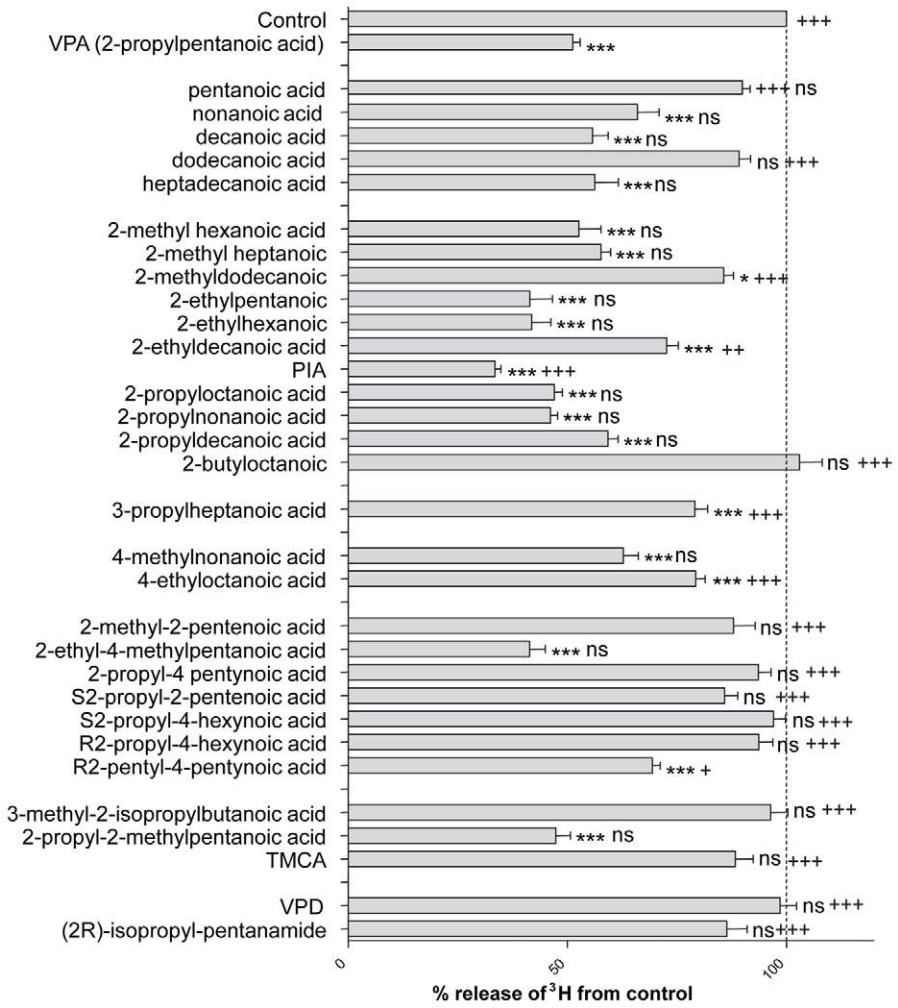

Fig. 5. Structural requirements for VPA-related compounds to inhibit fatty acid release in *Dictyostelium*. Effects of a wide range of VPA-related compounds were tested by measuring ^{3}H release from [^{3}H]AA-labelled cells. Compounds are grouped into straight chain analogues, 2-branched, 3-branched, 4-branched, unsaturated VPA analogues or other VPA-related compounds and amides. This approach provided a rapid mechanism for screening VPA-related compounds that could regulate fatty acids in vivo. TMCA, 2,2,3,3-tetramethyl cyclopropanecarboxylic acid; PIA, 2-isopropyl-pentanoic acid; VPD, valpromide. Statistical analysis was performed using GraphPad Prism software using a one-way ANOVA with a Tukey post hoc. ***P<0.001 and *P<0.05 vs control; +++ P<0.01, ++ P<0.01 and + P<0.05 vs VPA; ns, not significant.

complex cellular and biochemical processes involved in *Dictyostelium* cell movement (Schaloske et al., 2007). The conserved nature of this VPA-induced lipid accumulation, observed with both polyunsaturated and saturated fatty acids, suggests a generalised molecular mechanism for VPA in the accumulation of fatty acids within *Dictyostelium* cells.

The increased accumulation of fatty acids caused by VPA is independent of phospholipid species, because the distribution of radiolabelled fatty acids into phospholipid classes did not change with treatment (Fig. 3). The common trend for an increased fatty acid uptake and incorporation into phospholipids between *Dictyostelium* and human hepatocytes suggests a relevance of this simple model. VPA also caused an increased accumulation of non-polar lipids in both systems (Figs 2, 3), consistent with an increase in lipid droplet formation, because non-polar and/or neutral lipids typically consist of DAG and TAG species and are stored in these droplets (Kalantari et al., 2010). These results therefore suggest that *Dictyostelium* could provide a suitable model for liver fatty acid uptake and steatosis research, and will enable a combined genetic and biochemical approach to be used in the future to better understand this effect.

Although we have shown a linked VPA-induced effect of increased fatty acid uptake and reduced release, the use of a cell biological system for this discovery (rather than purified enzymes in biochemical approaches) makes identifying the cause of these two effects difficult. However, we note that increased lipid droplet formation occurs in the presence of only trace quantities of C_1-BODIPY-C_{12} lipid label – a quantity that is too small to give rise to the substantial increase in lipid droplets upon VPA treatment, thus suggesting a VPA-dependent regulation of existing cellular lipids to increase droplet size (rather than increased uptake) (Fig. 1). This observation would support a role for VPA and related structures (identified in Figs 5, 6 and Table 1) in regulating cellular fatty acids, giving rise to increased incorporation into lipid droplets and reduced release.

To investigate the mechanism of VPA-induced fatty acid accumulation in *Dictyostelium*, we employed a range of pharmacological inhibitors of fatty-acid-turnover enzymes to attempt to phenocopy the effect of VPA. Because PLA_2 catalyses the release of fatty acids, and VPA has previously been suggested to regulate PLA_2-dependent signalling (Chang et al., 2001), we employed a cocktail of PLA_2 pharmacological inhibitors in our cell-based lipid-accumulation assay. The effect of PLA_2 inhibition was to reduce fatty acid accumulation (Fig. 4B). Similarly, inhibition of DAG acyltransferase also resulted in decreased fatty acid accumulation. These results are consistent with both inhibitors

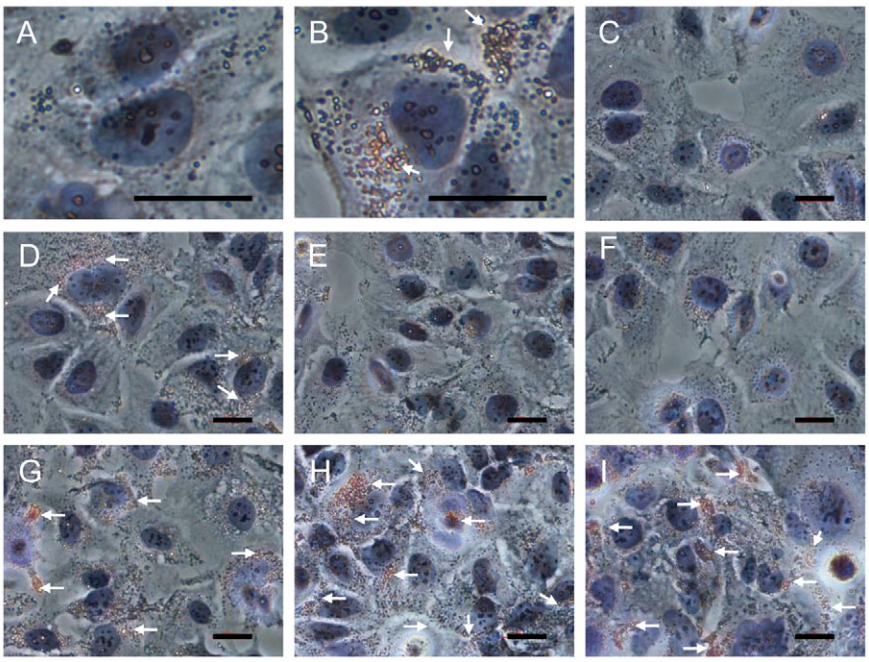

Fig. 6. Lipid droplet accumulation caused by VPA-related compounds in human hepatocytes correlates with inhibitory activity for fatty acid release in *Dictyostelium*. Also see Fig. 5. (A-D) Lipid droplets, visualised as patches of small circular white/pink spots, do not commonly appear in untreated human hepatocytes (Huh7 cells) (A,C), but are evident following 24 hours of VPA treatment (B,D; arrows). (E,F) VPA-related compounds showing no inhibitory effect on radiolabel release from ^{3}[^{3}H]AA-labelled *Dictyostelium* cells did not induce lipid droplet formation in hepatocytes. (E) Pentanoic acid; (F) TMCA. (G) By contrast, compounds shown to cause a VPA-like effect on *Dictyostelium* fatty acid release (4-methylnonanoic acid) showed similar efficacy in causing increased lipid droplet accumulation. (H,I) Compounds shown to be more strongly active than VPA in *Dictyostelium* also gave rise to increased lipid droplet formation over VPA treatment in hepatocytes. (H) Nonanoic acid; (I) 2-propyloctanoic acid. Arrows indicate oil-red-O-stained lipid droplets. Scale bars: 20 μm.

reducing the availability of unesterified positions on the glycerol of phospholipids, thus reducing the incorporation of new fatty acid into these lipids. However, because the effect of β-oxidation inhibition was to reproduce fatty acid accumulation in *Dictyostelium*, our data are consistent with a VPA-induced block in β-oxidation causing steatosis, although this mechanism is unlikely to be due to inhibition of long-chain acyl-CoA dehydrogenases because the effect is additive for each drug.

To define the structural prerequisites for VPA-related compounds in inhibiting fatty acid release in *Dictyostelium*, we carried out a SAR study. This study identified a range of activities from no inhibition to being more active than VPA. Although no clear structural constrains are evident from the compounds tested here, this study provided a rapid means for testing potential lipid regulatory effects of VPA congeners. Using a selection of inactive and highly active compounds identified in the *Dictyostelium* study, similar efficacies were shown in lipid droplet formation in human hepatocytes (Huh7 cells). In addition, this study enabled the investigation of a potential overlap between this effect and VPA-induced inositol signalling inhibition and teratogenicity. Here, we compared the activity of a range of VPA-related compounds (Fig. 5) with varying backbone length, side chains, and side chain length and position for inhibitory activity in fatty acid release, teratogenicity (HDAC inhibition) and inositol depletion strengths (Eickholt et al., 2005; Eikel et al., 2006; Shimshoni et al., 2007). The structural characteristics of compounds inhibiting fatty acid release are different to those causing inositol depletion and teratogenicity, because a range of compounds showing enhanced or reduced inositol depletion or teratogenicity do not show corresponding activity for this fatty-acid-related effect (Table 1). Fatty acid regulation, teratogenicity and inositol depletion might therefore provide three independent mechanisms of action for VPA in *Dictyostelium*. This study also provides the first description of VPA-related compounds for the regulation of fatty acid accumulation.

Continued research to understand the molecular mechanisms of lipid droplet formation and subsequent steatosis could help in the development of novel therapeutic treatments with improved risk:benefit ratios. It should be noted that it is not currently clear whether the observed lipid droplet accumulation has only detrimental consequences. For example, an increase in free AA has been shown in the brain following seizures (Basselin et al., 2003; Bazan et al., 2002; Rintala et al., 1999), and a reduction in AA signalling has also been observed in both epilepsy and bipolar disorder patient populations following treatment (Basselin et al., 2003; Bazinet et al., 2005). Our identification of a simple model system to study these effects and the identification of a structural specificity for an effect on fatty acid regulation might provide a potential mechanism for selection of novel therapeutics that lack the current side effects of weight gain during VPA treatment (Masuccio et al., 2010; Verrotti et al., 2011b; Wirrell, 2003).

In conclusion, we describe the analysis of a VPA-induced change in fatty acid uptake and release in the simple model *Dictyostelium*. We demonstrate an acute VPA-induced incorporation of fatty acids into complex glycerolipids, consistent with the observed lipid droplet accumulation in hepatocytes. This effect occurs with both a polyunsaturated and a saturated fatty acid through incorporation into different phospholipids and non-polar lipids in *Dictyostelium* (and for AA labelling in hepatocytes) and seems, at least in part, to be due to a block in β-oxidation caused by VPA. We have also identified a range of novel compounds with varying strengths of inhibitory effects on fatty acid release, identifying a range of compounds with either enhanced or reduced activity (compared with VPA) and we have validated this using a human hepatocyte cell line. Finally, this data suggest that the VPA-induced fatty acid regulation is independent of teratogenic or inositol depletion efficacy.

Table 1. Structure-activity relationship of VPA and related structures for a variety of biological endpoints

Compound	FA release (% control) (Dicty)	Lipid droplets (Huh7)	Teratogenicity	$InsP_3$ depleting (Dicty)	DRG-inositol-dependent effect (rat)	Reference
VPA (2-propylpentanoic acid)	52±10	3	+++	xxx	Yes	Eickholt et al., 2005; Williams et al., 2002
Valpromide (2-propyl pentamide; VPD)	99±11	0	0	x	No	Eickholt et al., 2005; Shimshoni et al., 2007; Eikel et al., 2006
Pentanoic acid	91±8	1	0	–	–	–
2-methyl-2-pentenoic acid	88±13	–	0	xx	Yes	Eickholt et al., 2005
2-propyl-4-pentynoic acid	90±8	0	+++	–	–	Eikel et al., 2006
2-ethyl-4-methylpentanoic acid	36±5	–	0	xx	Yes	Eickholt et al., 2005; Riebeling et al., 2011
2-isopropyl pentanoic acid (PIA)	34±4	4	0	xxx	Yes	Eyal et al., 2005; Shimshoni et al., 2007
Di-isopropylacetic acid (DIA)	96±11	0	0	0	Yes	Eyal et al., 2005; Shimshoni et al., 2007
R2-pentyl-4-pentynoic acid	69±5	–	+++	0	–	Eickholt et al., 2005; Riebeling et al., 2011
S2-propyl-4-hexynoic acid	94±12	–	+++	xx	Yes	Eickholt et al., 2005
R2-propyl-4-hexynoic acid	92±12	–	+	0	No	Eickholt et al., 2005; Eikel et al., 2006
TMCA	88±11	0	0	x	No	Shimshoni et al., 2007; Eyal et al., 2005
2-propyloctanoic	45±4	4	+++	–	–	Eikel et al., 2006
Nonanoic acid	66±13	4	–	–	–	–
4-methylnonanoic	56±6	4	–	–	–	–

Comparison of VPA-related compound activity in the inhibition of *Dictyostelium* (Dicty) fatty acid (FA) release and the formation of human (Huh7) liver cell lipid droplet (shown here), teratogenicity and inositol-signalling-based effects in *Dictyostelium* and rat primary dorsal root ganglia (DRG) neurons. Compound activity for inhibition of FA release in *Dictyostelium* is given as a percentage of control (no added compound) activity. Lipid droplet formation in Huh7 cells was rated on an arbitrary scale: (0) no effect; (1) low inhibition; up to 25% inhibition; (2) between 25% and VPA-like activity; (3) inhibition similar to that of VPA; (4) inhibition greater than VPA. Teratogenic rating is shown on an arbitrary scale, as defined previously (Shimshoni et al., 2007; Eyal et al., 2005): (0) not teratogenic; (+) low teratogenicity; (++) intermediate teratogenicity; (+++) VPA-liketeratogenicity or higher. Inositol trisphosphate ($InsP_3$)-depletion effects, using *Dictyostelium* as a model, is also shown on an arbitrary scale: (0) no $InsP_3$ depletion effect; (x) low $InsP_3$ reduction; (xx) intermediate reduction; (xxx) high reduction similar to VPA. Rat DRG inositol-dependent increase in growth cone area, as shown previously (Eickholt et al., 2005), was also rated as 'yes' or 'no'. TMCA, 2,2,3,3-tetramethyl cyclopropanecarboxylic acid. –, not determined.

METHODS

Reagents

[^{3}H]AA was purchased from Hartmann Analytic (Germany); [^{3}H]PaA was from Perkin Elmer (Cambridge, UK). VPA-related compounds were provided by H.N., Sigma Aldrich UK, Alfa Aesar, ChemSampCo, TCI Europe, Avocado, ACC Corporation, Katwijk Chemie and VWR. *Dictyostelium* media was supplied by Formedium (Norfolk, UK). Isoproyl-pentanoic acid (PIA), (2R)-isopropyl-pentamamide (PID), 2,2,3,3-tetramethyl cyclopropanecarboxylic acid (TMCA), 3-methyl-2-isopropylbutanoic acid and 4-methylpentanoic acid were generously provided by Meir Bialer (The Hebrew University of Jerusalem, Israel). All other reagents were supplied by Sigma Aldrich (Poole, UK).

Cells and development

Dictyostelium cells were grown in axenic medium or on Sussman's agar plates in association with *Raoultella planticola* (Drancourt et al., 2001). *Dictyostelium* cells were artificially developed by placing in phosphate buffer (16.5 mM KH_2PO_4, 3.8 mM K_2HPO_4) and pulsing with 25 nM cAMP every 6 minutes for 4 hours at 20°C. Cell labelling was carried out by the addition of radiolabelled lipid to pulsing cells for a further 60 minutes (Boeckeler et al., 2006).

The Huh7 human hepatocellular carcinoma cell line (Nakabayashi et al., 1982) was a kind gift from Steve Hood (GlaxoSmithKline, Ware, UK). All cells were routinely cultured in 75 cm^2 vented tissue culture flasks (Nunc, UK) using minimal essential medium with Earle's salts supplemented with 1% non-essential amino acids, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% foetal bovine serum. In order to maintain phenotypic consistency, Huh7 cells were only used for 3 weeks (approximately five passages) following recovery from liquid nitrogen. For imaging analysis, 1×10^5 cells were seeded into six-well plates containing sterile coverslips or 2.5×10^4 cells seeded into 24-well plates. Cells were allowed to adhere at 37°C for >2 hours prior to treatment as indicated in the figure legends.

Lipid droplet analysis

Oil red-O staining was performed on cells fixed overnight in 10% formalin (3.7% formaldehyde) in PBS before washing twice with 70% ethanol and water. Cells were stained with 0.18% oil red-O for

10 minutes, and washed with ethanol and water prior to hematoxylin counterstain.

Fatty acid uptake and release

To investigate fatty acid uptake, *Dictyostelium* cells were pulsed with cAMP (as above) for 4 hours in shaking liquid culture at 2.5×10^6 cells/ml prior to the addition of 0.25 μCi of ^{3}H-labelled fatty acid added in 0.5% fatty-acid-free BSA, at which point drugs were also added at the indicated concentrations. Samples were taken at the indicated times (by removing about 7.5×10^5 cells for scintillation counting and 3×10^7 cells for TLC analysis and washing once in phosphate buffer).

For fatty acid release experiments, cells were pulsed (as above) for 4 hours at 1.5×10^6 cells/ml. Cells were then labelled by resuspending in phosphate buffer with 0.5% fatty-acid-free BSA and 0.25 μCi of ^{3}H-labelled fatty acid for 1 hour. Unincorporated ^{3}H was removed by washing twice in phosphate buffer. Cells were finally resuspended in phosphate buffer/BSA at 5×10^6 cells/ml and 300 μl samples (5%) removed at the indicated time points. Samples were briefly centrifuged to remove cells and 250 μl of supernatant was removed for scintillation counting.

C_1-BODIPY-C_{12} labelling used 4-hour-pulsed cells, incubated with fluorescent fatty acid (Invitrogen) for 30 minutes in the presence or absence of VPA (0.5 mM); images were recorded on an Olympus IX 71 inverted fluorescence microscope with Retiga EXi Fast 1394 camera and analysed by ImagePro software. Data were obtained from three independent experiments (totalling 161 images each) for control and VPA treatment.

1D TLC analysis was performed as previously described (Pawolleck and Williams, 2009). Briefly, lipids were extracted from cells with 2:1 chloroform:methanol, dried and separated using the TLC solvent 40:15:13:12:7 chloroform:acetone:methanol:acetic acid:distilled water. 2D TLC analysis was performed on lipids extracted as previously described (Garbus et al., 1963). Lipids were separated first in a basic solvent (chloroform:methanol:ammonium hydroxide, 65:25:4, v/v/v) followed, in the second dimension, by an acidic solvent (n-butanol:acetic acid:water, 90:20:20, v/v/v). Plates were sprayed with 0.2% (wt/vol) 8-anilino-4-naphthosulphonic acid (ANSA) in methanol prior to visualisation under UV fluorescence. Quantification of spots was performed by phosphorimaging or scraping of spots and scintillation counting.

Statistical analysis

Graphical and statistical analysis was carried out using GraphPad Prism software. Error bars show ± standard error of mean (s.e.m.) and statistical significance was carried out using Student's *t*-test. All data represent at least $n\geq3$. Fatty acid uptake and release data are expressed as a % cpm of control.

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COMPETING INTERESTS

The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS

L.M.E., N.P. and I.A.G. carried out most of the *Dictyostelium* and lipid work. L.C. initiated the SAR study, and D.E. and H.N. provided critical compounds and structural advice, and participated in writing the manuscript. N.J.P. provided a major contribution to the Huh7 experiments and J.L.H. provided key lipid analysis direction, and both contributed to manuscript preparation. R.S.B.W. conceived and supervised the work and wrote the manuscript.

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TRANSLATIONAL IMPACT

Clinical issue

Valproic acid (VPA) is commonly prescribed for epilepsy and bipolar disorder, as well as for migraine prophylaxis. However, despite more than 45 years of research, its mechanisms of action are still unclear. Also, VPA has several significant side effects, including hepatotoxicity and non-alcoholic fatty liver disease (also known as hepatic steatosis), suggesting that VPA has adverse effects on lipid metabolism in the liver. In addition, the formation of lipid droplets following VPA treatment is seen in human hepatocytes in vitro, but the mechanism of this effect is unclear. The development of model systems to investigate VPA-related compounds with reduced hepatotoxic potential will aid the development of safer therapies for indications that are treated with this drug.

Results

In this paper, the authors use a simple model system, the social amoeba *Dictyostelium discoideum*, to analyse the adverse effects of VPA on fatty acid metabolism, and specifically its role in inducing the formation of intracellular lipid droplets. As previously observed in human hepatocytes, the authors show that pharmacologically relevant amounts of VPA also cause lipid accumulation in *Dictyostelium*. Following VPA treatment, fatty acids are taken up and incorporated into specific phospholipids, with a concurrent reduction in fatty acid release, an effect caused at least in part by inhibiting β-oxidation of fatty acids. The authors then use structure-activity analysis to screen a panel of VPA-related compounds for this inhibitory effect, and show that the magnitude of inhibition depends on chemical structural features of the compounds. Testing a selection of these compounds in human hepatocytes indicates one high correlation: compounds that inhibit fatty acid release in *Dictyostelium* cause lipid droplet formation in human hepatocytes, whereas compounds with minimal effect on fatty acid release in *Dictyostelium* do not induce lipid droplet formation in hepatocytes.

Implications and future directions

This study suggests that *Dictyostelium* can be used as a non-animal model for analysing the adverse effects of VPA and related compounds on lipid droplet formation. It also identifies novel compounds (and a system to identify more) that have mechanisms similar to VPA but that are unlikely to effect lipid droplet formation in hepatocytes, and could therefore help to identify safer treatments for epilepsy, bipolar disorder and migraine.

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