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Effects of valproic acid derivatives on inositol trisphosphate depletion, teratogenicity, GSK-3β inhibition and viral replication - A screening approach for new bipolar disorder drugs based on the valproic acid core structure[¶]

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Running title: Distinguishing $InsP_3$-depletion and other effects of VPA

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Abbreviations: GABA, gamma-aminobutyric acid; DMEM, Dulbecco's modified Eagle medium; FCS, foetal calf serum; GFP; green fluorescent protein; GSK-3β, glycogen synthase kinase 3β; HIV, human immunodeficiency virus; InsP, inositol phosphate; $InsP_3$, inositol (1,4,5) trisphosphate; NGF, nerve growth factor; PIP_2, phosphatidyl-inositol (4,5) bisphosphate; PO, prolyl oligopeptidase; VPA, valproic acid; VPD, valpromide.

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Abstract:

Inositol trisphosphate ($InsP_3$) depletion has been implicated in the therapeutic action of bipolar disorder drugs including valproic acid (VPA). It is not currently known if the effect of VPA on $InsP_3$ depletion is related to the deleterious effects of teratogenicity or elevated viral replication or if it occurs via putative inhibitory effects on Glycogen Synthase Kinase-3β (GSK-3β). In addition, the structural requirements of VPA-related compounds to cause $InsP_3$ depletion are unknown. In the current study we have selected a set of 10 VPA congeners to examine their effects on $InsP_3$ depletion, *in vivo* teratogenic potency and their effects on HIV replication and GSK-3β activity *in vitro*. We found four compounds that function to deplete $InsP_3$ in the model eukaryote *Dictyostelium discoideum* and these drugs all cause growth cone enlargement in mammalian primary neurons, consistent with the effect of $InsP_3$ depletion. No relationship was found between $InsP_3$ depletion and teratogenic or elevated viral replication effects and none of the VPA congeners were found to affect GSK-3β activity. Structural requirements of VPA congers to maintain $InsP_3$ depletion efficacy greater than that of lithium are a carboxylic acid function without dependence on side chain length, branching or saturation. Noteworthy is the enantiomeric differentiation if a chiral centre exists, suggesting that $InsP_3$ depletion is mediated by a stereoselective mode of action. Thus the effect of $InsP_3$ depletion can be separated from that of teratogenic potency and elevated viral replication effect. We have used this to identify two VPA derivatives which share the common $InsP_3$-depleting action of VPA, lithium and carbamazepine but do not show the side effects of VPA, thus providing promising novel candidates for bipolar disorder treatment.

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Introduction

There are three commonly used drugs for the treatment of bipolar disorder: lithium, valproic acid (VPA) carbamazepine, and lamotrigine, all of which were found accidentally to be therapeutically effective. The latter three are also anti-epileptic drugs and are commonly used for the prophylaxis of bipolar disorder. The serendipitous nature of these drug discoveries betrays the lack of understanding of how these drugs function, and has so far precluded the design and development of novel bipolar disorder drugs. Given the importance of this disorder — judged by the World Health Organisation to be ranked sixth highest in years of life lost to death or suicide (Murray and Lopez, 1996) — it is essential to develop new and more effective treatments for this disorder.

The first theory of mood disorder drug action (Berridge et al., 1989) — the 'inositol depletion theory' — proposed that lithium works by 'dampening down' an over-active inositol (1,4,5) trisphosphate ($InsP_3$) signalling cascade. The effect of bipolar disorder treatments on $InsP_3$ signalling is becoming increasingly apparent. Lithium acts as an uncompetitive inhibitor of a family of phosphatases that includes inositol monophosphatase (Leech et al., 1993) and polyphosphatase (York et al., 1995), two enzymes involved in the breakdown and recycling of $InsP_3$. Both VPA and lithium decrease the amount of inositol (O'Donnell et al., 2003) and attenuate $InsP_3$ signalling (Li et al., 1993) in the rat brain. In addition, they change membrane lipid concentration, a process directly linked to $InsP_3$ signalling (Ding and Greenberg, 2003). Indeed, a recent study in bipolar disorder patients suggested that altered $InsP_3$ signalling may be corrected by both VPA and lithium (Silverstone et al., 2002).

This inositol depletion theory of bipolar disorder drug action has been strengthened by two papers describing the effects of these treatments initially in *Dictyostelium discoideum* and

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later in primary rat neurons (Williams *et al.*, 1999;Williams *et al.*, 2002). Inhibition of the *D. discoideum* enzyme prolyl oligopeptidase (PO) gave rise to lithium resistance via the elevation of basal $InsP_3$ levels, thus overcoming the drug-induced $InsP_3$ depletion effect through an unknown mechanism. This work identified lithium, VPA and carbamazepine as acting via $InsP_3$ depletion in primary mammalian neurons, a mechanism also controlled by PO activity in astroglioma cell lines (Schulz et al., 2002). Interestingly, bipolar disorder patients show altered activity of PO (Breen et al., 2004), suggesting altered $InsP_3$ signalling in this disorder. Finally, all three drugs alter inositol uptake in human astrocytoma cells (Wolfson et al., 2000). Despite these indications, both the primary target for VPA and carbamazepine in $InsP_3$ depletion and the structural requirements of VPA for this effect remain unknown and no links exist between VPA side effects (such as teratogenicity) and inositol depletion.

Subsequent to the inositol depletion theory, a second target has been proposed for bipolar disorder drugs, the enzyme Glycogen Synthase Kinase 3β (GSK-3β). This enzyme has been widely shown to be targeted by lithium (Klein and Melton, 1996;Stambolic *et al.*, 1996) and it has also been reported to be directly inhibited by VPA (Chen et al., 1999) despite subsequent reports not finding this inhibition (Hall *et al.*, 2002;Phiel *et al.*, 2001). GSK-3β is still currently proposed to be involved in the therapeutic action of lithium in bipolar disorder treatment.

Although VPA is fast becoming the first choice treatment for bipolar disorder worldwide — newly diagnosed patients are twice as likely to be prescribed VPA than lithium in the US (Goodwin et al., 2003) — it also has some rare but severe side effects. These include

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teratogenicity (Nau et al., 1991), whereby mothers taking VPA during the first trimester of pregnancy have an increased chance of embryonic malformations (Loscher, 1999). Recent data suggests that this teratogenicity is due to histone deacetylase inhibition (Phiel et al., 2001), giving rise to elevated histone acetylation and altered gene transcription. This effect has also been implicated in altering expression of the inositol biosynthetic enzyme, *ino*1 (Kadosh and Struhl, 1997), suggesting a possible link of teratogenic potency, histone deacteylase inhibition and $InsP_3$ depletion. The mechanism by which VPA has been shown to increase viral load in HIV positive patients (Jennings and Romanelli, 1999;Maggi and Halman, 2001) may also be mediated through its effects on promoter acetylation (Ylisastigui et al., 2004). Finally, an effect of VPA on GSK-3 activity, as seen for lithium (Stambolic et al., 1996), could also cause abnormal development. A correlation between the effect of both teratogenicity and viral amplification caused by VPA and its ability to cause $InsP_3$ depletion remains unknown. In this paper we use 10 compounds based on the core structure of VPA to compare $InsP_3$, teratogenicity, viral amplification and GSK-3β inhibitory effects and find these adverse effect are discrete to that that of $InsP_3$ depletion.

Methods

Materials: All chemicals used were of analytical grade if not stated otherwise. Lithium chloride, valproic acid (VPA), myo-inositol, Trichostatin A, dimethylsulfoxide and vigabatrin was supplied by Sigma-Aldrich UK Co. Ltd. 2-methyl-2-pentenoic acid (IX) was provided by Avocado Ltd. and VPD was kindly supplied by Katwijk Chemie bv, The Netherlands. Prolyl oligopeptidase inhibitor Z-Pro-Pro-aldehyde-dimethyl acetal was provided by Bachem, UK. Valproic acid derivatives were synthesised according to methods described elsewhere (Bojic *et al.*, 1996;Bojic *et al.*, 1998;Gravemann, 2002;Hauck *et al.*,

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1991;Levi *et al.*, 1997). Standard GC-MS purity analysis procedures demonstrate a chemical purity of the derivatives ≥ 98 %, and after suitable derivatisation an enantiomeric purity of ≥ 95 %ee (enantiomeric access) of the chiral compounds. All VPA derivatives used in the *in vitro* experiments were dissolved in dimethylsulfoxide to result stock solutions of 1 M. Recombinant mammalian GSK-3β was purchase from Upstate Laboratories, USA (Inc Ltd).

***Dictyostelium* cell culture and $InsP_3$ analysis:** Wild type *Dictyostelium* cells (Ax2g) were grown for 20 hours in axenic media at $1x10^6$ cells per ml in the presence of drugs at indicated concentrations or with vehicle only control dimethylsulfoxide. Cells were washed and re-suspended in 1 ml of phosphate buffer and aerated for 10 minutes in the presence of the drug. Afterwards $InsP_3$ levels were measured by isotope dilution as previously reported (Williams et al., 1999). Protein was measured by Bradford assay (BioRad Laboratories Gmbh).

Teratogenic potency assay: The exencephaly rate as a model for teratogenic effects was measured within the NMRI-exencephaly-mouse model (Nau *et al.*, 1981) at one or more concentrations of the substances (Bojic *et al.*, 1996;Bojic *et al.*, 1998;Hauck *et al.*, 1991;Volland, 2002) before being transformed to the arbitrary scale of teratogenic potency grading of 0 indicating no teratogenic potency to +++++ for compounds showing very high teratogenic potency (Table 1).

Dorsal root ganglion explant culture: DRG explants from E18 rat embryos were plated onto poly-L-Lysine (20 μg/ml) and laminin (20 μg/ml) coated glass coverslips, and cultures were incubated for 24 hours in DMEM/10 % FCS/1 % penicillin/streptomycin, supplemented with 20 ng/ml NGF in the presence of specified drugs, before fixation in 4 %

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paraformaldhyde. DRG explants were washed twice with PBS, permeablized in PBS/1 % Triton X-100 and blocked in PBS/0.5 % Triton/2 % BSA. Cultures were stained with Alexa595 conjugated Phalloidin (Molecular Probes Inc USA) and anti-tubulin antibody (Sigma UK, Ltd). Growth cone sizes were determined in SimplePCI and statistical analysis was carried out using a student T test.

GSK-3β kinase assay: GSK-3β specific activity was determined by measuring the transfer of 32P from [32P]-γ-ATP to the GSK-specific peptide substrate, GSM as previously described (Ryves et al., 1998). The final concentration of each assay component was as follows: 40 mM Tris (pH 7.5), 12.5 mM MgCl2, 2 mM dithiothreitol, 400 µM GSM, 100 µM ATP and 40,000 cpm/µl of [32P]-α-ATP. Phosphate incorporation was linear with up to 200 units of kinase per assay for at least 10 minutes at room temperature (1 unit = 1 picomole of phosphate transferred to GSM peptide in 10 minutes). All experiments used 25-50 units of activity which produced 12-15,000 cpm per assay under these conditions. Assays were conducted in triplicate and activity was expressed as percentage of no vehicle dimethylsulfoxide control with error bars showing standard deviation.

HIV-1 infection assay

HIV-1 vectors encoding GFP were pseudotyped with the vesicular stomatitis G envelope protein as previously described (Besnier et al., 2002). Briefly 293T cells were transfected with 3 plasmids; p8.91, encoding HIV-1 gag-pol (HIV-1 structural and enzymatic proteins); pCSGW encoding an HIV-1 RNA including the GFP gene; and pMDG encoding the vesicular stomatitis G envelope protein. 48 hours later supernatant containing HIV-1 particles was collected and used to infect TE671 cells (ATCC Manassa VA USA), plated at 10^5

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cells/well in 6 well plates, in the presence or absence of drug. 48 hours later infected cells were enumerated by fluorescence activated cell sorting (FACS) (Beckton Dickenson UK) and percentage infections determined. Infections in the presence and absence of drug were compared (Fig. 5). Viral doses were chosen in order to infect between 0.5 and 5 % of the target cells to ensure linearity of the assay.

Results

Preliminary screening for $InsP_3$ depleting VPA analogues

We have used *D. discoideum* to examine the effect of a set of VPA analogues (denoted I to IX) on $InsP_3$ levels (Fig. 1). Analogues were chosen by broad category including branched and non-branched side chain, saturated and unsaturated derivatives, R- and S-enantiomeric pairs, as well as analogues with a derivatised carboxylic acid function like valpromide (VPD) and hydroxamates. We included both lithium and VPA as reference substances. Cells were exposed for 20 hours to drugs at a concentration of 0.5 mM, which is within the therapeutic range found in patient plasma undergoing VPA treatment. $InsP_3$ levels were measured using a direct $InsP_3$-binding protein assay. The results of this experiment clearly show that both VPA and lithium lower $InsP_3$ levels (Fig. 2A,B), and some VPA analogues also exhibit this effect. To our knowledge, this represents the first direct assay for screening $InsP_3$ depletion efficacy of potentially new bipolar disorder drugs.

Structural comparison of analogues causing a strong $InsP_3$ reduction significantly below lithium treatment (Fig. 2B, compound I, III, VIII and IX) shows that these drugs have variable main and side chain length and contain both saturated and unsaturated bonds. All active compounds contain an acid group whereas VPA derivatives containing modified acid groups like the amide or the hydroxamic acid function (Fig. 2A, B; compound V, VI, VII and

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VPD) showed less $InsP_3$ reduction. It is also noteworthy that two pairs of enantiomers with acid function showed opposite effects on $InsP_3$ reduction (compare compound I and II, III and IV) with both the S-enantiomers being the more potent derivative, although the enantiomers of the corresponding hydroxamic acid (compound V and VI) do not show this effect. These results are in accordance to Pfieffers' rule, which states that the greater the difference in the pharmacological effect of two enantiomers the greater is the specificity of the active isomer for the response of the system under test, our results suggest a stereoselective mode of receptor interaction of valproic acid derivatives for $InsP_3$ depletion.

Teratogenic potency of VPA acid derivatives

We have measured the teratogenic potency of the VPA analogues based on the NMRI-exencephaly mouse model (Nau et al., 1981) and transformed the previously measured exencephaly rates into an arbitrary scale of teratogenic potency rating from non-teratogenic (0) to highly teratogenic (+++++), where VPA is considered intermediate (+++)(Fig. 2C). Data is not available in this scale for compound IX although it is known to be non-teratogenic (Phiel et al., 2001). Valpromide (VPD) has been suggested to be converted to VPA *in vivo*, and therefore shows teratogenicity (Radatz et al., 1998), but it is highly likely that it does not posses an intrinsic teratogenic potency. Although some of the teratogenic structural requirements like carbonic acid function and the distinction of enantiomers are common to the observed requirements for $InsP_3$ depletion, there seems to be no direct correlation between $InsP_3$ depletion and teratogenic potency (Fig. 1, 2A, B). This data therefore provides the opportunity to select VPA derivatives that deplete $InsP_3$ without the potency for teratogenic side effects. As the teratogenic effects of valproic acid may be caused by the inhibition of histone deacetylases (Gurvich et al., 2004) and taking into account the relative

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small number of VPA derivatives in this first set of analogues these results also suggest no correlation between histone deacetylase inhibition and $InsP_3$ depletion.

Defining VPA analogues with a common mode of action to current bipolar disorder treatments

The common increase in growth cone size of primary rat dorsal root ganglia neurons (DRGs) has been suggested to be involved in the therapeutic effect of lithium, VPA and carbamazepine (Williams et al., 2002). We therefore compared the effects of VPA, the four VPA analogues showing the most acute reduction in $InsP_3$ levels in *Dictyostelium* (I, III, VIII, IX) and one analogue showing no $InsP_3$ depletion (VII) on the growth cone size of rat DRGs neurons. Cells were treated with these drugs for one day, fixed and stained with phalloidin, and growth cone areas were measured. An indication of growth cone morphology is shown (Fig. 3A), following staining with phalloidin and anti-tubulin antibody to visualise growth cone morphology. Both VPA and the four $InsP_3$ depleting drugs showed a significant two-fold increase in growth cone size ($p<0.05$), consistent with earlier reports of an 81% increase caused by VPA (Williams et al., 2002) in these cells (Fig. 3B). No significant difference in growth cone enlargement was seen between highly teratogenic (compound I and III) and non-teratogenic compounds (VIII and IX). Similar growth cone enlargement effects were also seen using DRG neurons derived from mice or embryonic chick embryos (data not shown).

To confirm that these effects occurred through the modification of inositol-based signalling, cells were also treated in the presence of an inhibitor of the enzyme Prolyl Oligopeptidase (133 μM), whose inhibition leads to the elevation of intracellular $InsP_3$ levels and increased resistance to the effects of bipolar disorder treatments (Schulz *et al.*, 2002;Williams *et al.*,

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1999;Williams *et al.*, 2002) or myo-inositol (2 mM). The increase in growth cone size caused by the VPA analogues was completely reversed by the addition of either PO inhibitor or myo-inositol (Fig. 3B), thus confirming that the effect of these drugs is through the modification of inositol-based signalling pathways within the mammalian growth cone. Therefore, these VPA analogues share the same mode of action seen with the three most commonly used bipolar disorder treatments; lithium, VPA and carbamazepine.

Effect of VPA analogues on GSK-3β activity

The first published report on the inhibitory effect of VPA on GSK-3β activity showed direct inhibition at therapeutically relevant concentrations (Chen et al., 1999). Although this result was not found in subsequent reports (Hall *et al.*, 2002;Phiel *et al.*, 2001;Williams *et al.*, 2002) it still remains possible that modification of VPA *in vivo* may lead to a GSK-3β inhibitory compound. To look for effects of the VPA congeners on GSK-3β activity we directly assayed purified mammalian GSK-3β activity in the presence of VPA and its congeners at 3 mM (Fig. 4). No changes were observed in GSK-3β activity with VPA or any congener tested. These assays were carried out at optimal magnesium concentration which were still found to lead to direct GSK-3β inhibition in earlier experiments (Chen et al., 1999).

Effect of VPA analogues on Human Immunodeficiency Virus infection

Recent data analysing the treatment of HIV positive patients with bipolar disorder have been conflicting, with some data suggests that VPA treatment might increase viral loads by an undetermined mechanism — leading to worsening disease (Jennings and Romanelli, 1999;Maggi and Halman, 2001) — whilst other data suggests that VPA might be protective against neuronal AIDS symptoms (Dou et al., 2003). We have therefore examined a series of

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VPA analogues for effects on *in vitro* HIV-1 infection (Fig. 4). In concordance with previous reports (Jennings and Romanelli, 1999;Maggi and Halman, 2001) we find that VPA increased HIV-1 vector infectivity at high (3 mM) concentrations (Fig. 4B), but had little effect at low drug concentration (0.5 mM). Three compounds (I, V, VI) increased HIV-1 vector infectivity two-fold at low concentrations. All three of these compounds contained a seven carbon backbone and a three carbon side chain with a terminal triple bond and either carboxylic or hydroxamic acid groups. All three caused significant cell death at high (3 mM) concentrations as indicated by a large reduction in fold HIV infection, indicating cytotoxicity. Five of these analogues had no significant effect on HIV-1 infectivity in this assay (II, IV, VIII, IX and VPD). This effect was not common to $InsP_3$ depleting drugs including lithium (Fig. 2A), was not caused by an inhibitor of GABA transaminase and was only partially caused by histone deacetylase inhibition. Furthermore, we could not reproduce the elevated infectivity shown by VPA and related compounds using a range of Trichostatin A concentration (Fig. 4C). Similar results were also found using murine leukaemia virus (data not shown).

Discussion

We have examined the effect of a set of ten VPA congeners on $InsP_3$ depletion, using the cellular slime mould *D. discoideum* (Fig. 1,2A). This had enabled the first partial characterisation of the structural requirements of compounds, based upon the core structure of VPA, to deplete $InsP_3$. We report four valproic acid derivatives that deplete $InsP_3$ more strongly than lithium (Fig. 2B). These four compounds contained a carboxylic acid group, whereas the analogues with amide or hydroxamic acid function were less potent – although a recent report by Shaltiel *et al.* (2004) shows high levels of inositol depleting activity of a carboxamide VPA derivative. The active compounds varied in side chain length, composition

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and degree of saturation, but it is noteworthy that two pairs of enantiomers showed different potency in $InsP_3$ depletion (compound I, III and II, IV) with the corresponding S-enantiomer being more potent. This observation suggests a stereoselective mode of receptor interaction. Unlike teratogenic rating, a hydrogen on the second carbon is not necessary for $InsP_3$ depletion (compound IX). Although these results provide the first indication of the structural requirements for $InsP_3$ depletion efficacy, a much larger cohort of VPA analogues must be analyses for a complete structural definition of efficacy.

Previous investigation of a variety of valproic acid derivatives in an exencephaly model of a NMRI-mice-strain (Nau *et al.*, 1981;Spiegelstein *et al.*, 2003) revealed that the intrinsic structural requirements for the teratogenic potency are: (A) A carboxylic acid group; (B) A hydrogen atom at the second carbon atom; (C) A branching at the second carbon atom with two side chains containing at least three carbon atoms at each side chain. Furthermore unsaturated derivatives with one double or triple bond are found to exhibit a higher teratogenic potency as are R-enantiomers of an enantiomeric pair at the second carbon atom suggesting a stereogenic mode of action for the teratogenic effects of valproic acid derivatives (Bojic *et al.*, 1996;Bojic *et al.*, 1998;Hauck *et al.*, 1991). Teratogenic potential of the currently analysed drugs are consistent with these findings (Table 1).

Comparison of $InsP_3$ depletion efficacy with the teratogenic potency of the VPA analogues showed no relationship between these two effects, as S-2-pentyl-4-pentynoic acid (I) depleted $InsP_3$ (Fig. 2A and 3B) and was highly teratogenic (Fig. 2C) whereas 2-ethyl-4-methyl-pentanoic acid (VIII) and 2-methyl-2-pentenoic acid (IX) showed a similar $InsP_3$ depletion effect but were not teratogenic (Fig. 2C). These results suggest that it is possible to isolate VPA derivatives with putative bipolar disorder efficacy without teratogenic side effects, and

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as a correlation between histone deacetylase inhibition and the teratogenic potency of VPA derivatives has been suggested, these results infer that there is no correlation of histone deacteylase inhibition and $InsP_3$ depletion. It is interesting to note that O'Loinsigh and coworkers (O'Loinsigh et al., 2004) recently studied the enantiomeric forms of 2-pentyl-4-pentynoic acid (compounds I and II) and defined the R-enantiomer to show cognition enhancement in water maze tests whereas the S-enantiomer showed anti-proliferative and pro-differentiative effects. This suggests that these latter actions are involved in either teratogenic, viral replication or $InsP_3$ depletion effects whereas the R-enantiomer may function through other means.

It is possible that analysis of $InsP_3$ depleting drugs in a simple model system, such as *D. discoideum* will not yield similar results to that found in primary mammalian neurons. To examine this, we tested a non-$InsP_3$ depleting compound (VII) and all four compounds that strongly deplete $InsP_3$ (I, III, VIII and IX) on rat DRG neurons. We found that all drugs which depleted $InsP_3$ in *D. discoideum* also caused an effect consistent with $InsP_3$ depletion in mammalian neurons (Williams et al., 2002). This effect, seen as the doubling of the growth cone size, was not seen for a non-$InsP_3$ depletion compound (VII). These results suggest that this growth cone enlargement effect, shared by the commonly used bipolar disorder treatments (Williams et al., 2002), can now be extended to defined VPA analogues. These results also confirm *D. discoideum* as a good model system for testing bipolar disorder drugs. The reversal of these effects using either myo-inositol or inhibitors of prolyl oligopeptidase is consistent with the drugs working through $InsP_3$ depletion, as shown for lithium, VPA and carbamazepine, and that the teratogenic effect of VPA is independent of its $InsP_3$ depletion action.

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The inhibition of GSK-3β by VPA remains a contentious issue, as the first report concerning this issue showed the direct inhibition of GSK-3β *in vitro* at physiological levels of VPA (Chen et al., 1999), but this result has yet to be confirmed (Hall *et al.*, 2002;Phiel *et al.*, 2001;Williams *et al.*, 2002) and it is still currently considered to be a direct inhibitor of GSK-3β. Here we show that VPA does not caused a direct inhibition of GSK-3β. These results do not however, exclude the possibility of modified VPA structures, produced through *in vivo* metabolic processes, playing a role in its action. Indeed, VPA has also been shown to be metabolised (Granneman et al., 1984) with structurally related products causing altered *in vivo* effects. Although we have not eliminated all structural changes possible by biotransformation, we have shown no direct inhibition of GSK-3β by any VPA-related compound tested. Subsequent to the first reported inhibition of GSK-3β by VPA (Chen et al., 1999), *in vivo* studies have suggested that VPA may function to mimic the inhibition of GSK-3β by elevating the expression of β-catenin, a GSK-3β target which is degraded upon phosphorylation (Phiel et al., 2001). However, this effect was shown to correspond to VPA's teratogenic action due to its histone deacteylase inhibitory effect. As we have determined which of these congeners are teratogenic, the teratogenically-induced effect upon GSK-3β can be eliminated by the choice of non-teratogenic VPA derivatives.

In addition to teratogenicity, we have examined the possibility that the $InsP_3$ depletion effect may be related to increase HIV-1 infectivity. HIV is widespread, with up to 46 million people infected, many with AIDS. Recent data analysing the treatment of HIV positive patients with bipolar disorder have been conflicting. Some data suggests that VPA treatment might increase viral loads by an undetermined mechanism — leading to worsening disease (Jennings and Romanelli, 1999;Maggi and Halman, 2001) — whilst other data suggests that

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VPA might be protective against neuronal AIDS symptoms (Dou et al., 2003). To examine this we exposed cells to combined drug and HIV-1 vector for 48 hours after which GFP-expressing infected cells were enumerated by fluorescence activated cell sorting (FACS), thus measuring the effect of a drug on the ability of HIV-1 to infect human cells in culture. The HIV-1 vectors are non-replicative and therefore this assay measures the effect of the drugs on the ability of HIV-1 to infect the target cells, reverse transcribe its RNA to DNA, deliver its genome to the nucleus and integrate it into the host chromosome. We therefore employed this assay to measure the effects of bipolar disorder treatments and VPA analogues on HIV infectivity. In addition, we used an inhibitor of GABA transaminase (Vigabatrin) to mimic the proposed anti-epileptic mechanism of drug action (Loscher, 1999) and Trichostatin A — which mimics the histone deacetylase inhibition caused by VPA (Phiel et al., 2001) — to examine the effects of VPA in this assay.

In concordance with previous HIV virus data (Jennings and Romanelli, 1999;Maggi and Halman, 2001) we implicate VPA as being able to increase HIV-1 infectivity *in vitro*. No correlation was found between $InsP_3$ depleting efficacy and HIV-1 infectivity suggesting an unrelated mechanism. In support of this, no increase in viral load has been found in patients using other $InsP_3$ depleting bipolar disorder treatments. Comparison of the teratogenic ratings of compounds with effects on HIV-1 infection shows that broadly, the more teratogenic compounds show the highest increase in HIV infectivity (compounds I, III, V, VI and VPA, Fig. 2C, 3B), as reported for other viral activities (Michaelis et al., 2004). This suggests that teratogenicity and viral infectivity may be linked, although only a small increase in HIV infectivity was found using the teratogenic inhibitor to histone deacetylase, Trichostatin A. No significant increase in viral infectivity is produced by compounds VIII and IX or an

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inhibitor to GABA transaminase (vigabatrin) suggesting this effect is not mediated through altered GABA signalling.

We have screened 10 VPA congeners for their ability to depleted $InsP_3$, to cause *in vivo* teratogenicity and *in vitro* effects on viral replication. We find no relationship between these VPA-side effects and the $InsP_3$ depletion efficacy. We also find no indication of direct GSK-3β inhibition by VPA or any of the tested congeners. Instead, we find some correlation between teratogenicity and effects on HIV replication. This approach has allowed the first preliminary definition of changes in VPA structure which do not cause a reduction in the $InsP_3$ depleting ability below that of lithium. $InsP_3$ depleting activity is greatest in the presence of the carboxylic acid function but is not reliant on side chain length, branching or saturation. Interestingly the $InsP_3$ depleting activity is enantiosensitive, suggesting a stereoselective mode of receptor interaction. This study has also identified two VPA congeners — 2-ethyl-4-methyl-pentanoic acid (compound VIII) and 2-methyl-2-pentenoic acid (compound IX) which show the same effects as lithium and carbamazepine in primary mammalian neurons and do not possess a teratogenic potency or a HIV replication effect of VPA. These drugs thus provide promising novel compounds to test for bipolar disorder control.

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Footnotes:

a) Reprint requests to:

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Figures:

Figure 1: The structure of Valproic acid and its congeners. Compounds are referred to by roman numeral prefix (I-IX) except VPA and its amide derivative VPD. Compounds were chosen by broad category to result in a test set with highest structural diversity in branching (VIII), saturation (I-IV, IX), side-chain length, derivatisation of the carboxylic acid function and chiral aspects (R- and S–enantiomers: I and II, III and IV, V and VI).

Figure 2: Characterization of inositol trisphosphate ($InsP_3$) depletion efficacy and teratogenicity of VPA analogues and current bipolar disorder treatments. *Dictyostelium discoideum* cells were treated with lithium, VPA or compounds based upon the chemical structure of VPA (Fig. 1). A: Cells were grown overnight in complete medium in the presence of VPA or one of its analogues at 0.5 mM, a concentration found in patient plasma undergoing VPA treatment, or with lithium at 10 mM. Changes in $InsP_3$ levels were subsequently measured by isotope dilution assay (Amersham Inc, see methods), and compared with vehicle-only (black) control or currently used bipolar disorder treatments (grey). Results represent four experiments assayed in triplicate ($\pm$SEM). (B) Compounds were tested for teratogenic rating in an *in vivo* model. Arbitrary scale of teratogenic ratings for these drugs, from not teratogenic (0) to highly teratogenic (+++++) as described in methods (*inferred from Gurvich *et al.*, 2004;Phiel *et al.*, 2001). RM= racemic mixture. ND=not determined. (C) Comparison of relative efficacy of VPA congeners to cause $InsP_3$ depletion expressed as a percentage of that found for VPA or lithium (underline indicates increased $InsP_3$ levels).

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Figure 3: Enlargement of growth cones from Dorsal Root Ganglia (DRG) neurons following treatment with $InsP_3$ depleting drugs. Rat DRGs were cultured for 24 hours in the presence of 0.5 mM VPA, analogues showing $InsP_3$ depletion in *Dictyostelium* or one analogue showing no effect on $InsP_3$ levels in *Dictyostelium* (Fig. 2A) and afterwards cells were fixed and stained prior to growth cone size quantification. A; Representative growth cones are shown for vehicle-only control (top left), 0.5 mM VPA (top right), or VPA with myo-inositol (2mM)(bottom left) or prolyl oligopeptidase inhibitor (133 μM) (bottom right). B; Quantification of growth cone size for five VPA derivatives and VPA following treatment (black) with the indicated drug or with the drug plus inositol (grey) or PO inhibitor (white). Data represents two to four independent experiments containing approximately 25 growth cones per experiment (± SEM). (*= $P<0.05$).

Figure 4: Direct inhibition studies of glycogen synthase kinase-3β (GSK-3) activity by VPA and its 10 congeners. Drugs (3 mM) were tested for inhibitory effect on mammalian GSK-3β with vector only control (dimethylsulfoxide, -). Results are expressed as percentage of activity in the absence of drug, +/- SD.

Figure 5: Characterisation of bipolar disorder treatments, VPA analogues and other treatments on HIV replication. Human 293T cells were infected with GFP labelled HIV particles modified to block cell lysis, in the presence of the indicated drugs. Cells were analysed for the presence of green fluorescent protein (GFP) labelled HIV after 48 hours using FACS analysis (see methods). A: Cell infection rates were quantified by measuring GFP-labelled cells, shown for control (1.1%), compound IX (1.1%) and VPA (2.5%) treatments with increased FL1 levels (grey shaded area, control). Quantification of HIV

infection is provided as increase in infection rates over control (vehicle only) for B: the indicated compounds at low (0.5 mM) and high concentrations (3mM) or lithium (3 and 10 mM) or an inhibitor of GABA transaminases (Vigabatrin: VGB, 30 and 100 µM). C: The inhibition of histone deacetylases by VPA was mimicked using a range of concentrations of Trichostatin A (TSA). Decreased infection rates reflect toxicity of the compounds on target cells at high drug concentrations. Data represents FACS analysis of at least two independent experiments (+/- SEM).

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Table 1: Rating criteria for teratogenic potency

Teratogenic potency grading	Dose range (mmol/kg)	Exencephaly rate (%)	Description
0	> 3	0	No teratogenic potency
+	2 – 3	1 – 5	Low teratogenic potency
++	2 – 3	5 – 25	Lower teratogenic potency than VPA
+++	2 – 3	25 – 60	Equal teratogenic potency to VPA
++++	1 – 2	40 – 60	Higher teratogenic potency than VPA
+++++	0.25 – 1	40 – 60	Very high teratogenic potency

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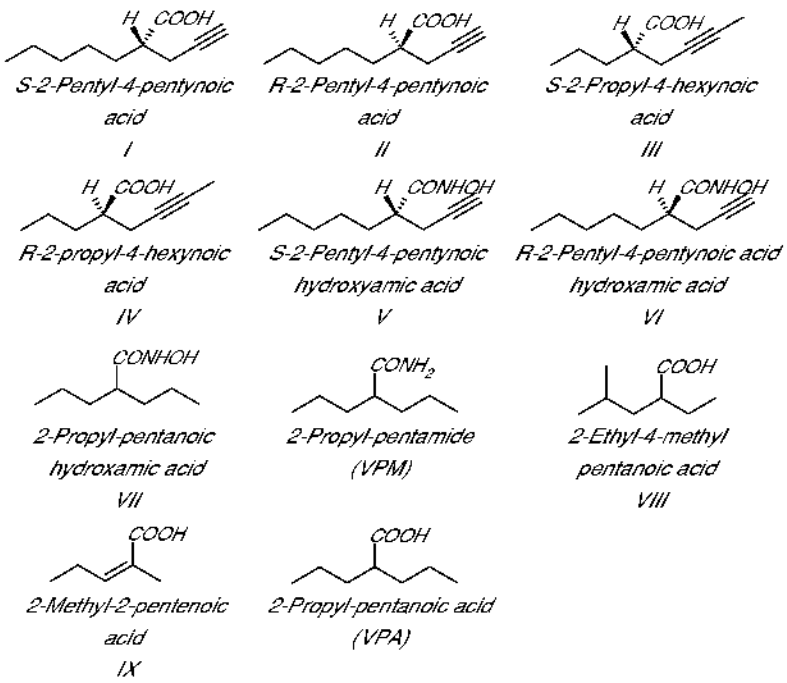
Figure 1



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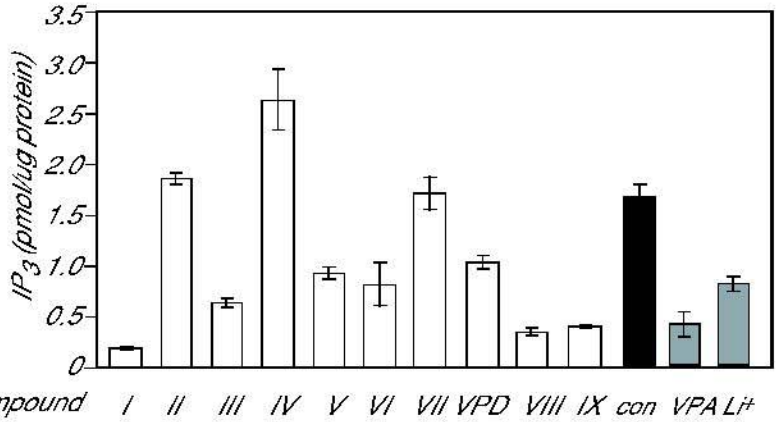
Eickholt et al Figure 2

A



B

	I	II	III	IV	V	VI	VII	VPD	VIII	IX	con	VPA	Li+
VPA	117	13	82	74	59	68	2	51	113	100	-	100	70
Li+	168	19	117	106	84	97	3	73	162	143	-	144	100

C

I	II	III	IV	V	VI	VII	VPD	VIII	IX	con	VPA	Li+
+++++	+++	+++	+	+ rm	+	0	0	0	0*	0	+++	ND

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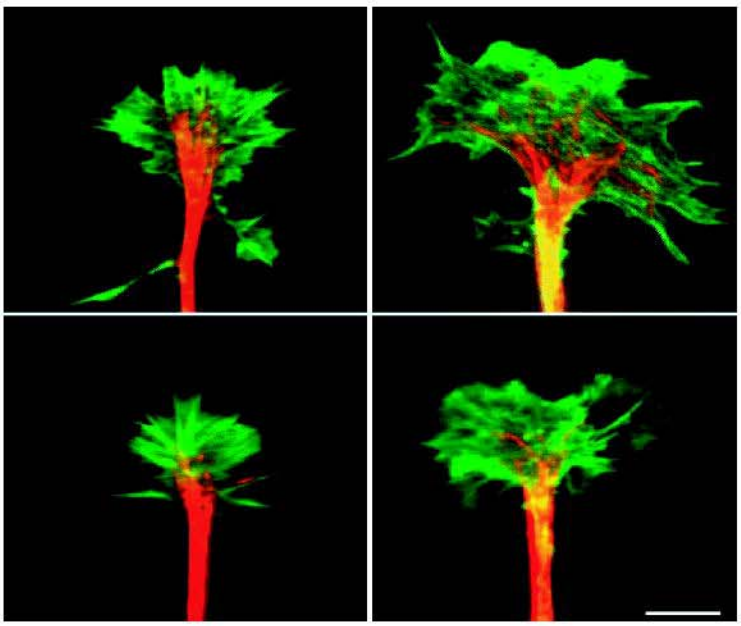
Figure 3

A



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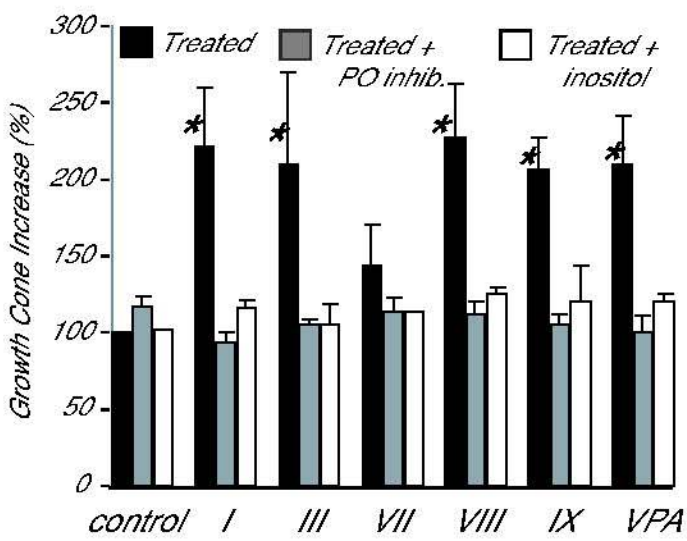

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Figure 4

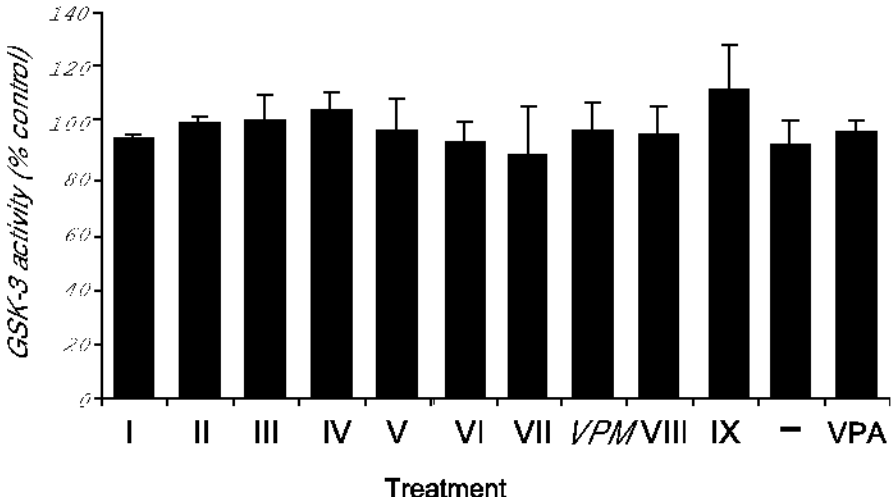

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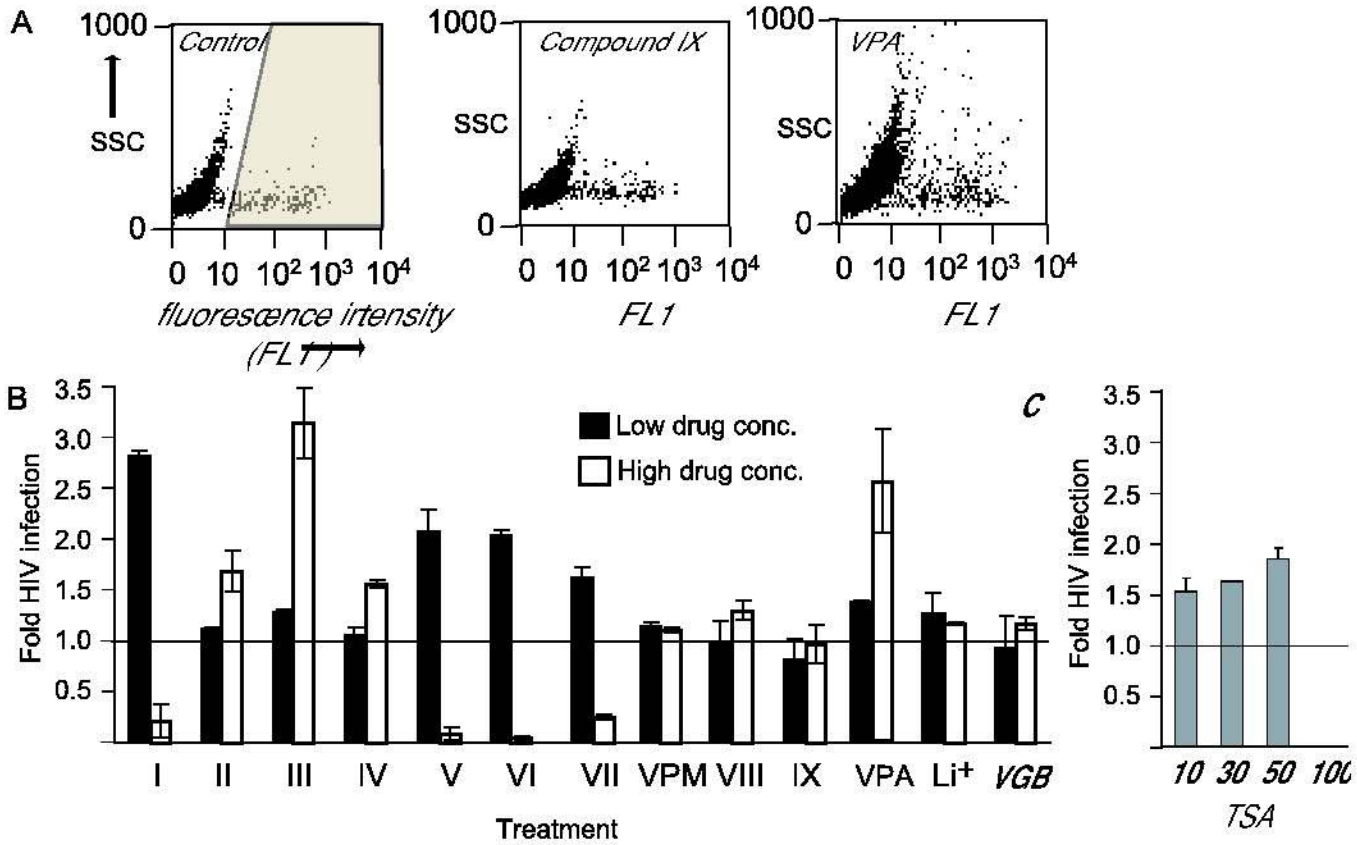
Figure 5



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