

# $\alpha$ -Linolenic Acid–Valproic Acid Conjugates: Toward Single-Molecule Polypharmacology for Multiple Sclerosis

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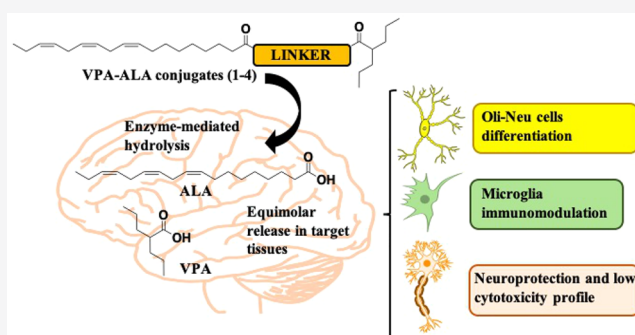
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**ABSTRACT:** Multiple sclerosis (MS) is a complex inflammatory, degenerative, and demyelinating disease of the central nervous system. Although treatments exist, MS cannot be cured by available drugs, which primarily target neuroinflammation. Thus, it is feasible that a well concerted polypharmacological approach able to act at multiple points within the intricate network of inflammation, neurodegeneration, and demyelination/remyelination pathways would succeed where other drugs have failed. Starting from reported beneficial effects of  $\alpha$ -linolenic acid (ALA) and valproic acid (VPA) in MS, and by applying a rational strategy, we developed a small set of codrugs obtained by conjugating VPA and ALA through proper linkers. A cellular profiling identified **1** as a polypharmacological tool able not only to modulate microglia polarization, but also to counteract neurodegeneration and demyelination and induce oligodendrocyte precursor cell differentiation, by acting on multiple biochemical and epigenetic pathways.

**KEYWORDS:** Polypharmacology, codrugs,  $\alpha$ -linolenic acid, valproic acid



Multiple sclerosis (MS) is a chronic complex malady of the central nervous system (CNS) characterized by inflammation, demyelination, and neurodegeneration, resulting in a progressively increasing disability.<sup>1</sup> With the only exceptions of ocrelizumab and siponimod, which have a moderate effect on disease progression, none of the 17 currently available drugs are able to halt or at least slow the relentless neuronal disability.<sup>2</sup> Indeed, current therapies, focused primarily on pathological immune responses (i.e., immunomodulation and immunosuppression), are generally less effective in the progressive forms of the disease than in the relapsing remitting ones.<sup>2</sup> Therefore, curing demyelination and the concomitant neurodegeneration, in addition to immunomodulation, needs to be addressed for a truly curative effect in all patients.<sup>3</sup>

Developing such treatment is not an easy task, as it involves targeting complex pathological cascades in different brain cell types. Therefore, while monotherapies may be inappropriate, multipronged approaches will likely be needed to support the wide range of CNS functions and minimize injury and toxicity involving neurons, oligodendrocytes, and microglia.<sup>4</sup> On this basis, we were motivated to develop a polypharmacological treatment able not only to modulate immunomodulatory/inflammatory aspects of the disease, but also to counteract neurodegeneration and demyelination and to sustain remyelination.

We have contributed to the implementation of polypharmacology in the field of neurodegenerative drug discovery.<sup>5,6</sup> In this project, we aimed to harness this knowledge to develop new polypharmacological tools that deliberately hit multiple biological targets (i.e., inflammation, neurodegeneration, demyelination/remyelination) in different brain cell types. To note, although combination therapy has been advocated and tested in the clinics for MS,<sup>4</sup> to the best of our knowledge, no polypharmacological approach based on a single pharmaceutical ingredient, has been reported so far.

To do so, we turned our attention to the development of versatile polypharmacological tools, i.e., codrugs.<sup>7</sup> “Codrugs” or “mutual prodrugs” are single molecules obtained by the conjugation of two therapeutic compounds with synergistic activity, via a cleavable linker.<sup>8,9</sup> A marketed example is the antibacterial sultamicillin, an ampicillin/sulbactam codrug (Figure S1). The fact that the two starting compounds, following metabolic transformation, have the potential to be released in the same target cells and at the same time is a peculiar feature of codrugs with respect to combinations (two

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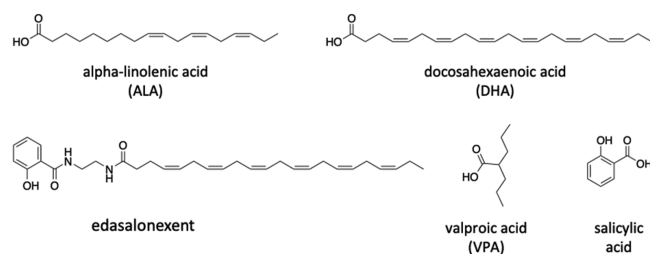
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single compounds, each one with an individual pharmacokinetic profile).<sup>9,10</sup> This potentially concomitant delivery appears particularly advantageous from a polypharmacology perspective.<sup>9,10</sup> On the other hand, it should be mentioned that the codrug approach is applicable only to starting compounds carrying functional groups suitable for conjugation.<sup>8</sup>

We focused on the development of codrugs between valproic acid (VPA) and  $\alpha$ -linolenic acid (ALA), both amenable to linking/conjugation (Figure 1). We reasoned



**Figure 1.** Chemical structures of  $\alpha$ -linolenic acid, docosahexaenoic acid, edasalonexent, valproic acid, and salicylic acid.

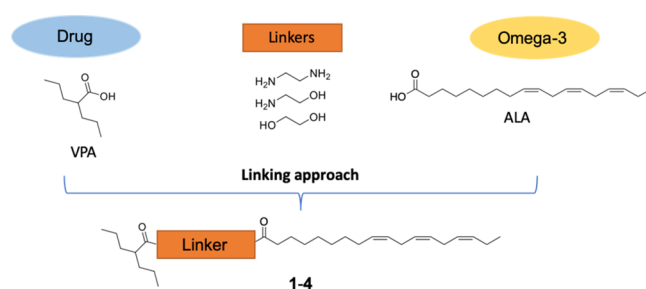
that starting from a marketed drug (VPA) and an omega-3 polyunsaturated fatty acid food supplement (ALA) could mitigate the risk of toxicity for use in humans, thereby potentially expediting the clinical translation of eventual candidates.

Our strategy was founded upon the reported beneficial effects of ALA and VPA in MS. Several studies have demonstrated that ALA supplementation delays onset and reduces demyelination and cognitive dysfunction in the experimental autoimmune encephalomyelitis (EAE) model. These positive effects are accompanied by a shift in microglial polarization toward the beneficial M2 phenotype.<sup>11</sup> In addition, higher levels of ALA were associated with lower disease activity.<sup>12</sup>

Similarly, VPA has been shown to be effective in animal models of the disease.<sup>13</sup> VPA, used for the treatment of neurological disorders for more than 40 years, exerts its therapeutic effects through multiple mechanisms. Among them, which include GABA, glutamate receptor and sodium and calcium voltage-gated channel modulation, its activity as a histone deacetylase (HDAC) inhibitor has recently come to light.<sup>14</sup> As an HDAC inhibitor, VPA is reported to be neuroprotective and neuroregenerative in various neurological diseases,<sup>14</sup> including MS (although some controversy does exist).<sup>15</sup>

Thus, we embarked on the development of ALA-VPA codrugs for MS. We deemed that such a concerted, simultaneous modulation of multiple critical pathways by VPA and ALA can result in a truly immunomodulatory, neuroprotective, and neurorestorative effect.

**Design.** The codrug strategy was based on a set of linkers (ethylene glycol, ethanolamine, ethylenediamine) that allowed VPA and ALA to be covalently joined via their carboxylic acid functions. This linker strategy has been recently validated by the development of edasalonexent,<sup>9</sup> a conjugate of salicylic acid and docosahexaenoic acid (DHA, Figure 1), currently clinically evaluated to treat Duchenne muscular dystrophy.<sup>16</sup> The linking strategy allowed the formation of ester or amide bonds between the acid functions of VPA and ALA, obtaining conjugates 1–4 (Figure 2).



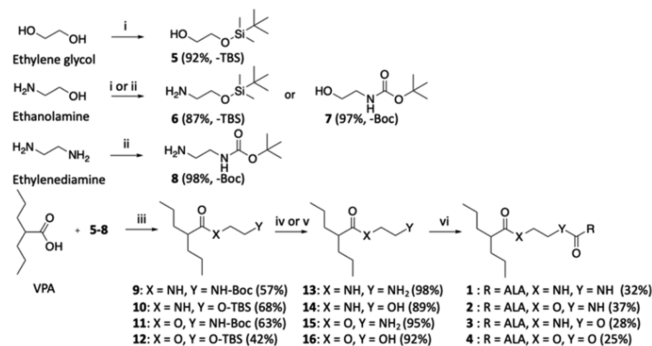
**Figure 2.** Design of ALA-VPA conjugates 1–4.

On the basis of previous studies,<sup>9,17</sup> we expected 1–4 to be stable in the circulation at different extents, depending on the nature of the formed bonds (amide vs ester). Then, hydrolysis should occur in the brain tissues where specific enzymes able to hydrolyze endogenous fatty acid conjugates are present. As reported for edasalonexent<sup>9</sup> and a fatty acid cysteamine conjugate,<sup>17</sup> the metabolism of 1–4 might be mediated by endocannabinoid metabolic enzymes, including fatty acid amide hydrolase (FAAH), monoacylglycerol lipase, and *N*-acyl ethanolamine acid amidase. Then, each released starting framework (VPA or ALA) should maintain the ability to recognize its specific targets and, collectively, to produce multiple, synergistic pharmacological effects. In principle, the same effects could be also reached by a combination treatment of VPA and ALA. However, according to the previous reports,<sup>9,17</sup> the herein proposed single-molecule conjugates might have advantages with respect to a classical drug combination: VPA and omega-3 FAs have the potential to be delivered in the target cells at the same time and in equimolar concentrations. Since multiple biological pathways in multiple brain cell types might be simultaneously hit, it is feasible to hypothesize that the resulting polypharmacology of 1–4 could be peculiar and not be replicated by administering VPA and ALA in combination, where each one has an individual ADME (absorption, distribution, metabolism and excretion) profile.

In the following, the synthesis, preliminary pharmacokinetic evaluation, and biological characterization of 1–4 at a cellular level are reported. We believe that, at this initial stage, cell-based systems maintain a reasonable screening efficiency while preserving network interactions, critical in a polypharmacology context.

**Chemistry.** For the synthesis of conjugates 1–4, a linear synthetic strategy based on 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDC)/4-dimethylaminopyridine (DMAP) coupling and protection/deprotection steps was followed (Scheme 1). First, the three bifunctional linkers, i.e., ethylene glycol, ethanolamine, and ethylenediamine, were monoprotected with the suitable protecting group to allow selective reaction on the proper function.

Standard reactions with di-*tert*-butyl decarbonate (Boc<sub>2</sub>O) or *tert*-butyldimethylsilyl chloride (TBSCl) provided the *N*-Boc- or the *O*-TBS-protected linkers 5–8 in very good yields (87–98%). Monoprotected 5–8 were then coupled with VPA after activation with EDC/DMAP to provide ester or amide intermediates 9–12, in moderate to good yields (42–68%). Treatment with trifluoroacetic acid (TFA) or tetrabutylammonium fluoride (TBAF) removed the Boc- or TBS-protecting groups, respectively, providing intermediates 13–16 (89–98%). Both deprotection reactions were conducted under anhydrous conditions and at 0 °C, in order to avoid

Scheme 1. Synthesis of PUFA–VPA Conjugates 1–4<sup>a</sup>

<sup>a</sup>Reagents and conditions: (i) TBSCl, imidazole, DCM, r.t., 24 h; (ii) Boc<sub>2</sub>O, DCM, r.t., 24 h. (iii) EDC, DMAP, DCM, r.t., 8 h, N<sub>2</sub>; (iv) TFA, DCM, r.t., 2 h; (v) TBAF, r.t., 24 h; (vi) ALA, EDC, DMAP, DCM, r.t., 8 h, N<sub>2</sub>.

concomitant VPA hydrolysis. Finally, targets 1–4 were synthesized from ALA and the respective VPA-functionalized linker (13–16), using the previous EDC/DMAP protocol. However, in this case, the reaction was carried out under nitrogen atmosphere and in the dark, in order to minimize oxidation side-reactions.

**Neuro- and Hepatotoxicity Assays.** A preliminary cytotoxicity screening was performed on conjugates 1–4 using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Tables 1 and S1). Increasing

**Table 1. Effect of 1–4 and Parent Compounds VPA and ALA on Cell Survival/Death in CGNs, As Determined by MTT Assay after 24 h Treatment**

compd	% mean ± SE <sup>a</sup>				
	0 [μM]	5 [μM]	10 [μM]	25 [μM]	50 [μM]
VPA	100 ± 6	100 ± 15	106 ± 3	85 ± 14	97 ± 17
ALA	100 ± 6	93 ± 13	97 ± 3	79 ± 12	45 ± 5*** <sup>b</sup>
1	100 ± 6	98 ± 18	107 ± 3	89 ± 14	99 ± 15
2	100 ± 6	97 ± 16	110 ± 3	96 ± 15	85 ± 14
3	100 ± 6	119 ± 17	116 ± 3	99 ± 17	41 ± 6*** <sup>b</sup>
4	100 ± 6	118 ± 16	114 ± 3	71 ± 8	45 ± 8*** <sup>b</sup>

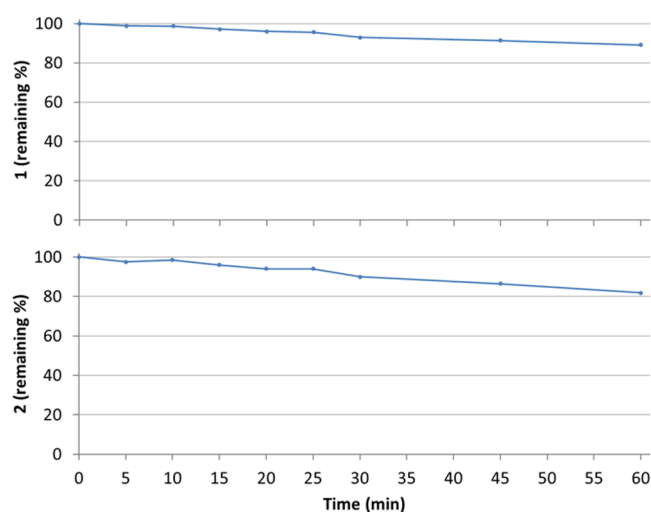
<sup>a</sup>Results are expressed as percentages of controls and are the mean ± SE of at least 3 independent experiments, each run in triplicate. <sup>b</sup>\*\*\**p* < 0.001 compared to control conditions (0 μM), Student's *t* test.

concentrations (5, 10, 25, 50 μM) of each conjugate and parent compound (VPA and ALA) were tested for 24 h in primary rat cerebellar granule neurons (CGNs) and the human liver carcinoma immortalized cell line (HepG2). Drug toxicity is one of the significant shortcomings in CNS drug discovery.<sup>18</sup> Furthermore, drug-induced liver injury has been associated with a number of MS drugs, including disease-modifying and symptomatic therapies.<sup>19</sup>

From Table 1, it is evident that diamide 1 and ethanolamide 2 display a low neurotoxicity profile, and also at the higher tested concentrations (viability >85% at 50 μM). They are less toxic compared to the inverse ethanolamide (3, 41% viability) and diester (4, 45%) analogues and also to reference compounds. Notably, while the CNS drug VPA shows negligible neurotoxicity, ALA is neurotoxic at 50 μM (45%). In addition, both diamide 1 and ethanolamide 2 showed a low hepatotoxicity, even at the higher tested concentrations

(viability > 80% at 25 μM; Table S1). Hence, we selected 1 and 2 for further studies, because they are representative of two different linker types and are the less toxic ones among the synthesized set, especially at the more therapeutically relevant concentrations (5 and 10 μM).

**Plasma Stability Assay.** Our aim was to develop plasma-stable conjugates that would be hydrolyzed in the brain. Selective localized hydrolysis of conjugates 1 and 2 would permit a CNS-targeted profile and less side effects. Thus, plasma stability is an important pharmacokinetic prerequisite for 1 and 2. As reported by stability assays performed by LC-DAD-MS/MS analysis (Figure 3), conjugates 1 and 2 are



**Figure 3.** Rat plasma stability of conjugates 1 and 2 at 37 °C: percentage remaining upon incubation, assessed by means of LC-DAD-MS/MS. Analyses were performed in triplicate.

stable for more than 85% after 1 h of incubation at 37 °C in rat plasma. Specifically, the percentage of diamide 1 remaining upon incubation is higher than that of ester/amide 2 (89% for 1 and 85% for 2). The mean plasma remaining fraction of the two conjugates after 1 h of incubation was compared by means of *t* test and a statistically relevant difference (*p* < 0.01) was observed.

**In Vitro BBB Permeation Assay.** In parallel, we tested 1 and 2 in the blood-brain barrier (BBB) specific parallel artificial membrane permeability assay (PAMPA-BBB) (Table 2). Both compounds were predicted to be BBB-permeable. Conjugate 1 had an effective permeability (*P<sub>e</sub>*) of 27.98, which placed it in

**Table 2. PAMPA Effective Permeability (*P<sub>e</sub>*) Values with Related Predictive BBB Penetrations of Commercial Drugs, 1 and 2**

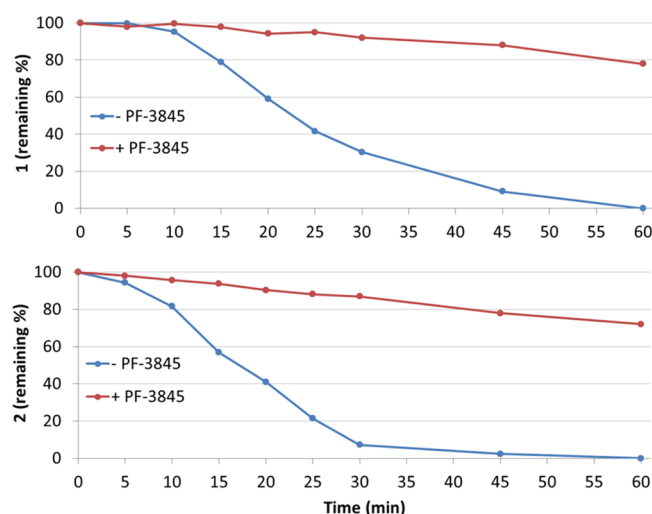
compd	BBB penetration estimation	
	<i>P<sub>e</sub></i> ± SEM (× 10 <sup>-6</sup> cm s <sup>-1</sup> ) <sup>a</sup>	CNS (+/-)
1	27.98 ± 5.30	CNS +
2	14.59 ± 1.61	CNS +
furosemide	0.19 ± 0.07	CNS -
ranitidine	0.35 ± 0.31	CNS -
donepezil	21.93 ± 2.06	CNS +
tacrine	5.96 ± 0.59	CNS +

<sup>a</sup>*P<sub>e</sub>* ± SEM (*n* = 3). Each compound was assessed in quadruplicate.

the high BBB permeability category (Table 2), higher than that of two CNS drugs (donepezil and tacrine).

**Brain Stability Assay.** We anticipated that, once they entered into the brain, conjugates would be hydrolyzed to the individual components by intracellular metabolic enzymes processing fatty acid derivatives (e.g., FAAH).<sup>9,17</sup> Thus, the stability of **1** and **2** was evaluated by LC-DAD-MS/MS analysis of rat brain homogenate incubated with the conjugates, which was sampled at regular intervals over 1 h. To obtain initial clues on the implication of FAAH enzymes in hydrolysis, the assays were performed in the absence and presence of 5  $\mu$ M FAAH inhibitor PF-3845.<sup>9</sup>

As shown in Figure 4, both conjugates undergo metabolism (i.e., total disappearance of starting compound) within 1 h of



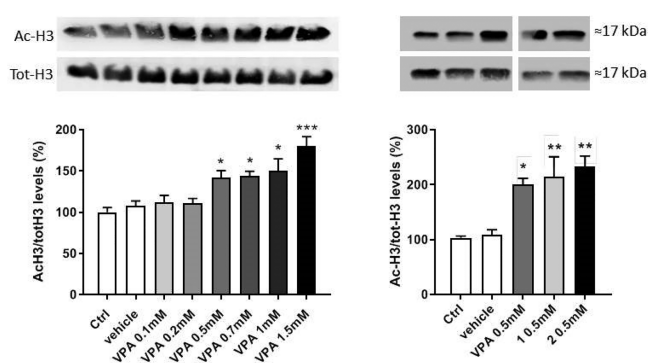
**Figure 4.** Hydrolysis kinetics in rat brain lysate (37 °C). Percentages of **1** or **2** remaining over time upon incubation in the absence and presence of FAAH inhibitor PF-3845, assessed by means of LC-DAD-MS/MS. Analyses were performed in triplicate.

incubation, with a more marked decrease rate with regard to ethanolamide **2** (half-life of about 16 min) compared to diamide **1** (half-life of about 23 min). Moreover, we can infer that the observed hydrolysis is FAAH-dependent, as the addition of PF-3845 blocks the hydrolysis of both **1** and **2** to a large extent. To note, although it appears that the plasma stability of these codrugs is higher than that in rat brain homogenates, we cannot exclude that hydrolytic reactions might occur in tissues like the liver.

After getting a preliminary indication that conjugates **1** and **2** would be metabolized to afford the parent compounds selectively in brain target cells, we investigated the biological effects that they could induce.

**HDAC Assays.** Several studies have shown that long-term administration of VPA results in regulation of inflammatory processes, neuroprotection, and cell differentiation through HDAC inhibition.<sup>20,21</sup> Thus, we preliminarily verified whether **1** and **2** would retain the HDAC inhibitory capacity of the parent VPA in human medulloblastoma cell line DAOY (Figure 5). VPA is a relatively weak HDAC inhibitor, with millimolar activity (a clinically achievable concentration).<sup>22</sup> Thus, we tested it in the 0.1–1.5 mM range.

As expected, VPA treatment induced a strong inhibition of HDAC, shown by a significant, concentration-dependent increment in acetylation levels of histone H3 (Figure 5, left



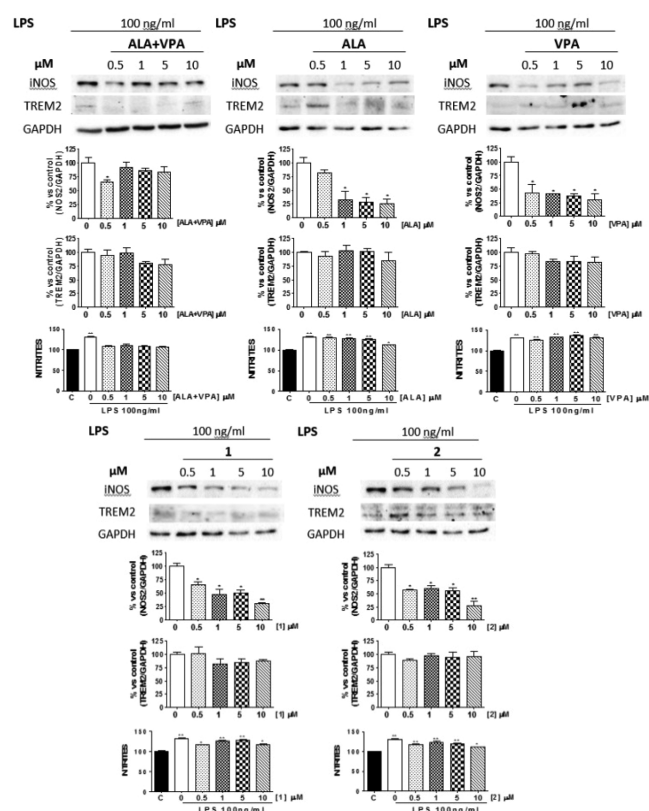
**Figure 5.** Effect of **1**, **2**, and VPA on HDAC inhibition, evaluated through analysis of acetylated histone levels in medulloblastoma (DAOY) cells. Densitometric analysis of the bands is shown (mean  $\pm$  SD of three independent experiments); the amount of AcH3 is normalized to that of totH3. \* $p$  < 0.05; \*\* $p$  < 0.01; \*\*\* $p$  < 0.001 vs vehicle (Newman–Keuls test after ANOVA).

panel). Remarkably, a similar increase of H3 acetylation was detected in neuronal cells exposed to conjugates **1** and **2** at 0.5 mM (Figure 5, right panel).

**Immunomodulation Assays.** Microglia, essential regulators of neuroinflammatory processes, can be polarized to two distinct activation states: M1 and M2. M1 microglia have proinflammatory, neurotoxic properties and are modeled in vitro through lipopolysaccharides (LPS) activation. Alternatively, M2 microglia are involved in anti-inflammatory processes, contributing to trophic support of neurons and neuroprotective functions. A marked microglial activation with high expression levels of proinflammatory genes is found in MS lesions.<sup>23</sup> Conversely, the neuroprotective effects of the MS drug glatiramer are mediated by activated M2 microglia.<sup>24</sup> To this end, we evaluated whether **1** and **2** modulate microglial shift from an M1 neurotoxic phenotype to an M2 neuroprotective one. Therefore, N9 microglial cells were treated with 100 ng/mL LPS in the presence or absence of increasing concentrations (0.5, 1.5, 10  $\mu$ M) of **1**, **2**, parent compounds VPA and ALA, as well as and their 1:1 combination (ALA+VPA, Figure 6). After pretreatment for 6 h and administration for a further 24 h, the microglial phenotype was evaluated through Western blot analysis of the M1-iNOS (inducible Nitric Oxide Synthase) and M2-TREM2 (Triggering Receptor Expressed on Myeloid cells 2) markers (Figure 6). We also assessed nitrite production, due to iNOS induction, in the incubation media.

As reported in Figure 6, compounds **1** and **2**, even at low concentration (0.5  $\mu$ M), significantly decrease microglia production of the M1 proinflammatory marker iNOS, at an extent similar to that of parent compounds. Remarkably, they were more effective than the ALA+VPA combination. Interestingly, **1** and **2** display immunomodulatory properties, as revealed by the unchanged expression of TREM2 (M2 marker). This is important, as TREM2 is reported to invoke microglia to phagocytose myelin debris in MS lesions<sup>25</sup> and its blockage results in an amplified demyelination in EAE.<sup>26</sup> Regarding the levels of nitrites, **1** and **2** reduced their concentration more effectively than VPA and similarly to the ALA+VPA combination.

**Oli-Neu Cell Cytotoxicity Assays.** Oligodendrocytes are the myelin-forming cells. They are the end product of a cell lineage which, following finely tuned cycles of proliferation, migration, and differentiation, finally produces myelin.<sup>27</sup> The



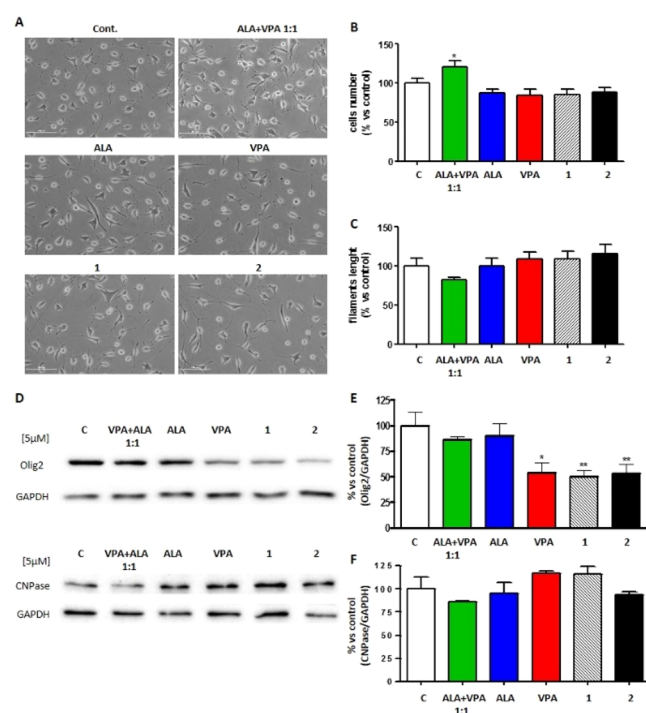
**Figure 6.** Immunomodulatory effects of conjugates **1** and **2**, ALA, VPA, and their 1:1 equimolar combination (ALA+VPA) in microglial cells evaluated through Western blot analysis of iNOS and TREM2 expression, as well as the indirect extent of released NO through nitrite measurement in the medium. GAPDH was used as loading control. Results are expressed as percentage of controls and are the mean  $\pm$  SE of at least two independent experiments.  $**p < 0.01$ ;  $*p < 0.05$  compared to control conditions ( $0 \mu\text{M}$ ), one way-ANOVA followed by Bonferroni's posthoc test.

inhibitory microenvironment in MS lesions abolishes the expansion and differentiation of resident oligodendrocyte precursor cells (OPCs) into mature myelin-forming oligodendrocytes. Loss of myelin, in turn, accounts for motor and cognitive deficits in MS patients. Thus, chemical manipulation of OPC fate may open new avenues for regenerative therapies in MS.<sup>28</sup>

Here, we used Oli-neu cells, an oligodendroglial precursor cell line, as an accepted in vitro model to screen the remyelination potential of our molecules.<sup>29</sup> First, we tested the toxicity of our conjugates and parent compounds against Oli-Neu. After treatment with increasing concentrations (1–10  $\mu\text{M}$ ) for 24 h, the MTT assay shows that, while VPA and ALA

decrease cell viability already at 1  $\mu\text{M}$ , conjugates **1** and **2** show no toxicity even at the higher concentrations (10  $\mu\text{M}$ ). Intriguingly, cells treated with ethanolamide **2** revealed a significantly higher metabolic activity (MTT readout) compared with control cells (Table 3).

**Oli-Neu Cell Proliferation and Differentiation Assay.** OPC proliferation and migration to the CNS injury site as well as their differentiation are critical steps toward remyelination in MS. HDAC activity plays a crucial role in OPC proliferation/differentiation; therefore, HDAC modulation is widely considered a promising therapeutic target for MS.<sup>30</sup> Accordingly, VPA has been shown to increase myelin repair in the EAE model, by acting on OPC recruitment.<sup>31</sup> Therefore, we tested the effect of our compounds on OPC proliferation and differentiation, through cell counting, measure of filaments, and analysis of proliferation/differentiation markers (Figure 7).



**Figure 7.** Effect of conjugates **1** and **2**, ALA, VPA, and their 1:1 equimolar combination (ALA+VPA) on proliferation and differentiation of Oli-Neu cells at 5  $\mu\text{M}$  through cell counting (A, B) measure of filaments length (A, C) at 48 h, as well as expression of Olig2 at 48h (D, E) and CNPase (D, F) at 72 h. Results are expressed as percentage of controls and are the mean  $\pm$  SE of at least three different experiments.  $**p < 0.01$ ;  $*p < 0.05$  compared to control conditions ( $0 \mu\text{M}$ ), one way-ANOVA followed by Bonferroni's posthoc test.

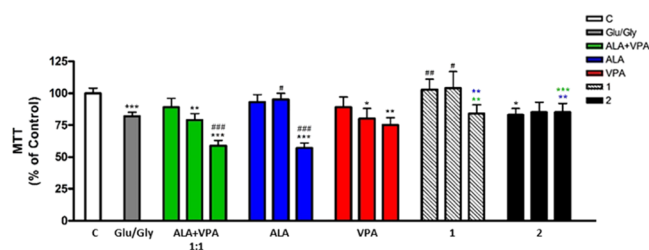
**Table 3.** Effect of **1** and **2** on Cell Survival/Death through MTT Assay in Oli-Neu Cells (Immortalized Mouse Oligodendrocyte Precursor Cells), in Comparison with Parent Compounds ALA and VPA

compd	% mean $\pm$ SE <sup>a</sup>				
	0 [ $\mu\text{M}$ ]	0.5 [ $\mu\text{M}$ ]	1 [ $\mu\text{M}$ ]	5 [ $\mu\text{M}$ ]	10 [ $\mu\text{M}$ ]
VPA	100 $\pm$ 6	75 $\pm$ 7 <sup>**b</sup>	72 $\pm$ 6 <sup>**b</sup>	74 $\pm$ 9 <sup>**b</sup>	76 $\pm$ 7 <sup>**b</sup>
ALA	100 $\pm$ 6	78 $\pm$ 5 <sup>**b</sup>	75 $\pm$ 9	71 $\pm$ 8 <sup>**b</sup>	68 $\pm$ 6 <sup>**b</sup>
<b>1</b>	100 $\pm$ 6	89 $\pm$ 9	95 $\pm$ 8	88 $\pm$ 5 <sup>c</sup>	93 $\pm$ 6 <sup>**b</sup>
<b>2</b>	100 $\pm$ 6	110 $\pm$ 4	115 $\pm$ 7 <sup>**b</sup>	105 $\pm$ 8	101 $\pm$ 8

<sup>a</sup>Results are expressed as percentages of controls and are the mean  $\pm$  SE of at least 2 independent experiments, each run in triplicate. <sup>b</sup> $**p < 0.01$ . <sup>c</sup> $*p < 0.05$  compared to control conditions ( $0 \mu\text{M}$ ), one way-ANOVA followed by Bonferroni's posthoc test.

While Oli-Neu cells treated with VPA+ALA show a significant increase in the cell number, with a parallel decrease in the filament length, compound **1** and **2**, similarly to VPA, decrease cell number with a parallel increase in filament length. This suggests that **1** and **2** might induce OPCs differentiation to oligodendrocytes. To further support this hypothesis, Western blot analysis showed a marked decrease in the expression of Olig2, a marker of OPCs proliferation, for **1**, **2** and VPA, but not for ALA and ALA+VPA. This profile was paralleled by a significant increase in the expression of CNPase, a marker of differentiation, for both **1** and VPA. Collectively, these data point to an induction of OPC differentiation toward oligodendrocytes by **1** and **2**, which, similarly to VPA, can perform myelination/remyelination. Importantly, this behavior is not shown by ALA and neither by ALA+VPA combination but is peculiar to conjugate **1**.

**Neuroprotection Assay.** Several lines of evidence point to MS as not only an inflammatory but also a neurodegenerative disease. Thus, neuroprotective agents may hold promise for MS therapy.<sup>32</sup> VPA has been reported to exert neuroprotective effects and to reduce glutamatergic excitatory neurotransmission.<sup>33</sup> Thus, to test the neuroprotective capability of **1** and **2**, we used a glutamate excitotoxicity model of primary cultural neurons. At day 8 in vitro (8 DIV), CGNs were pretreated with the compounds for 6 h, before adding 100  $\mu$ M glutamate/10  $\mu$ M glycine insult for further 24 h. As illustrated in Figure 8,



**Figure 8.** Neuroprotective effect of **1** and **2**, ALA, VPA, and their 1:1 equimolar combination (ALA+VPA) on glutamate/glycine excitotoxicity through MTT assay in differentiated CGNs (8 DIV) pretreated for 6 h and cotreated for a further 24 h at 5, 10, and 25  $\mu$ M. Results are expressed as percentage of controls and are the mean  $\pm$  SE of at least four different experiments, each run in triplicate. \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001 vs control, # $p$  < 0.05, ## $p$  < 0.01, ### $p$  < 0.001 vs Glu/Gly, green asterisk \*\* $p$  < 0.01, green asterisk \*\*\* $p$  < 0.001 vs ALA+VPA 1:1, blue asterisk \*\* $p$  < 0.01 vs ALA, one way-ANOVA followed by Bonferroni's posthoc test.

pretreatment with **1** resulted a more neuroprotective against glutamatergic excitotoxicity when compared with VPA, ALA VPA+ALA 1:1, and **2**, especially at lower concentrations (5 and 10  $\mu$ M). Furthermore, while reference compounds (alone and in combination) increase glutamate/glycine excitotoxicity at higher doses, **1** was not toxic even at the highest concentration tested (25  $\mu$ M).

## CONCLUSIONS

Although significant progress has been made in developing immunomodulatory treatments, there is a lack of therapeutic options that address the multiple, concomitant pathophysiological aspects of MS. This study, albeit very preliminary, demonstrates for the first time the potential of VPA/ALA codrugs to achieve polypharmacology in MS. Particularly, diamide conjugate **1** displays an overall immunomodulatory, remyelinating, and neuroprotective profile that is superior to

that of the parent compounds, as well as to their equimolar combination. The collected results, the experience gained from the use of VPA in humans for over 40 years, and the fact that ALA is a natural product present in many foods might warrant further investigation into this series. In parallel, the fact that a recent study has highlighted a dampened remyelination and a decreased oligodendrocyte cell count for VPA<sup>34</sup> and its use in MS therapy is still controversial<sup>15</sup> suggests that other conjugations of promising compounds should be explored.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsmchemlett.0c00375>.

Experimental details for chemistry and biological assays, cell toxicity plots, NMR spectra, LC-MS/MS chromatograms and MS spectra for compounds **1** and **2** (PDF)

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### Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

### Author Contributions

<sup>§</sup>M.R. and S.P. equally contributed to this work.

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## Notes

The authors declare no competing financial interest.

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## ABBREVIATIONS

ALA,  $\alpha$ -linolenic acid; BBB, blood-brain barrier; CGNs, cerebellar granule neurons; CNS, central nervous system; DHA, acid docosahexaenoic acid; DMAP, 4-dimethylaminopyridine; Eight DIV, day 8 in vitro; EAE, experimental autoimmune encephalomyelitis; EDC, 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide; FAAH, fatty acid amide hydrolase; HDAC, histone deacetylase; iNOS, inducible nitric oxide synthase; MS, multiple sclerosis; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OPCs, oligodendrocyte precursor cells; PAMPA, parallel artificial membrane permeability assay; TBAF, tetrabutylammonium fluoride; TFA, trifluoroacetic acid; TREM2, triggering receptor expressed on myeloid cells 2; VPA, valproic acid.

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