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Effects of Combining Biofactors on Bioenergetic Parameters, A β Levels and Survival in Alzheimer Model Organisms

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Abstract: Increased amyloid beta (A β) levels and mitochondrial dysfunction (MD) in the human brain characterize Alzheimer disease (AD). Folic acid, magnesium and vitamin B6 are essential micronutrients that may provide neuroprotection. Bioenergetic parameters and amyloid precursor protein (APP) processing products were investigated in vitro in human neuroblastoma SH-SY5Y-APP695 cells, expressing neuronal APP, and in vivo, in the invertebrate *Caenorhabditis elegans* (CL2006 & GMC101) expressing muscular APP. Model organisms were incubated with either folic acid and magnesium-orotate (ID63) or folic acid, magnesium-orotate and vitamin B6 (ID64) in different concentrations. ID63 and ID64 reduced A β , soluble alpha APP (sAPP α), and lactate levels in SH-SY5Y-APP695 cells. The latter might be explained by enhanced expression of lactate dehydrogenase (LDHA). Micronutrient combinations had no effects on mitochondrial parameters in SH-SY5Y-APP695 cells. ID64 showed a significant life-prolonging effect in *C. elegans* CL2006. Incubation of GMC101 with ID63 significantly lowered A β aggregation. Both combinations significantly reduced paralysis and thus improved the phenotype in GMC101. Thus, the combinations of the tested biofactors are effective in pre-clinical models of AD by interfering with A β related pathways and glycolysis.



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Keywords: Alzheimer disease; mitochondria; mitochondria dysfunction; folic acid; vitamin B6; magnesium-orotate; amyloid beta; *C. elegans*; biofactor

1. Introduction

At present, 50 million people are suffering from Alzheimer's disease (AD) and this number will rise to approximately 152 million in 2050 [1]. Unfortunately, there is no cure for AD yet. Approved drugs only treat symptoms [2]. There are several hypotheses regarding the etiology of Alzheimer's disease, but the causes of the disease are unknown. Previous research has focused on amyloid and tau, which has not yet led to major breakthroughs. Therefore, there is a trend towards multifactorial treatments and, among other things, energy metabolism with regard to mitochondrial functions. Two of the hallmarks of AD are mitochondrial dysfunction (MD) [3] and overproduction of beta-amyloid (A β) [4]. The first signs of beginning MD are a reduction of glucose consumption [5] and a reduced activity of key enzymes of the oxidative metabolism [6,7]. Almost all mitochondrial functions are impaired in AD [8,9]. The limited function of the electron transport chain (ETC) is the reason for the decrease in complexes IV and I. This results in a decreased mitochondrial membrane potential (MMP) and ATP production [10]. Another important characteristic of AD is that A β is cleaved from a much larger amyloid precursor protein (APP) [11]. APP is cleaved via two pathways, a non-amyloid and an amyloidogenic pathway. The APP is spliced by the different types of protease, namely α -, β - and γ -secretase [11,12]. Depending on which protease cleaves the APP, A β peptides are produced. The α -protease cleaves the APP closer to the membrane, resulting in a shorter fragment in the membrane, which is then further cleaved by the γ -protease to a non-amyloidogenic product. However, when β -protease cleaves the protein, larger fragments are produced, which are then cleaved by

γ -protease to form different large A β proteins [12] including A β_{1-40} and A β_{1-42} which may be the main triggers for AD [2,11,12]. It appears that A β_{1-42} has the greater neurotoxic potential compared to the A β_{1-40} form. Furthermore, A β_{1-42} tends to aggregate more, which can lead to plaque formation. In addition, the relationship between A β_{1-40} and A β_{1-42} is important, as the two influence each other [13–15]. There is evidence that sAPP α and sAPP β share some properties [16], where sAPP α can be neuroprotective, whereas sAPP β lacks most of the neuroprotective properties and has a rather negative effect [17]. Another early sign of AD is impaired glucose metabolism which leads to MD and an increase in oxidant production [18,19]. The glycolysis pathway represents a way to ensure sufficient energy production and bypass A β induced impairment of mitochondria [20,21]. The enzymes pyruvate dehydrogenase kinase 1 (PDK1) and LDHA are of interest here. PDK1 phosphorylates pyruvate dehydrogenase and inactivates it. LDHA converts pyruvate into lactate. Both are markers of aerobic glycolysis. If a change occurs here, conclusions can be drawn about energy production [22,23].

There is evidence that specific biofactors [24], which are defined as substances required by the body for its normal physiological functioning and/ or with health-beneficial and/ or disease-preventive biological activities, may interfere with pathophysiological processes leading to AD [25–27]. A cocktail containing some of our tested compounds had a positive effect on AD symptoms in a TgF344-AD rat model. This could raise the mitochondrial function of the transgenic rats to the level of the wild-type rats [28]. Here, folic acid, magnesium-orotate and vitamin B6 in different combinations were tested in cellular and an invertebrate model of AD. The synthetically produced water-soluble folic acid, which consists of pterin, p-aminobenzoic acid and L-glutamic acid, belongs to the vitamin B complexes. Folate (also known as vitamin B9) is used as an umbrella term for the various derivatives of tetrahydrofolate (THF), with the synthetically produced form being referred to as folic acid [29,30]. Folate deficiency impairs DNA as well as mtDNA synthesis and stability and causes oxidative stress in the form of ROS, which, as already listed, is also associated with AD pathogenesis. In this context, neuronal impairment and increased cell death occur in AD. In addition, a deficiency of folate leads to a decrease in the methylation of enzymes and promoter regions of genes that are presumably also involved in AD pathogenesis [31,32]. Magnesium-orotate (MgOr), which is very poorly soluble in water, is the magnesium salt of orotic acid. As a source of magnesium (Mg), MgOr is used for the oral treatment of Mg deficiency. Orotic acid is a key intermediate in the biosynthetic pathway of pyrimidines and improves energy status by stimulating, among other things, the synthesis of glycogen and ATP [33]. Mg²⁺ is the fourth most abundant mineral as well as the second most abundant intracellular divalent cation in the human body and acts as a cofactor [34–36]. Mg is involved in protein synthesis, cellular energy production and storage, reproduction, DNA and RNA synthesis, and mitochondrial membrane potential [35]. Mg is also involved in the maintenance of physiological nerve and muscle function, cardiac excitability, and neuromuscular conduction [35,36]. Several pathological mechanisms in AD are discussed on which Mg might have a positive influence. Mg appears to inhibit the activity of γ -secretase and the proinflammatory TNF- α (tumor necrosis factor α) produced by microglia. Mg also inhibited IL-1 β (interleukin-1 β) and A β -induced, which all together induced inflammation. In addition, Mg has been reported to decrease the influx of A β across the blood-brain barrier [34]. High Mg concentrations have been shown to promote APP processing towards α -secretase due to the upregulation of transcription factors such as CREB [37]. Mg deficiency may be a risk factor for ADs and that possible supplementation may be a potentially valuable adjunct treatment for AD [38]. The water soluble vitamin B6 (Vit B6) is used as an umbrella term for various derivatives from which pyridoxine is the most common form. It is an enzymatic cofactor required for more than 140 biochemical reactions, including transaminations, α -decarboxylations and replacement reactions [39]. Through the application of Vit B6, the oxidative stress induced by A β could be inhibited [40]. Furthermore, Vit B6 reduces the plasma levels of A β [41] and prevents the grey matter atrophy related to AD [42].

The present work investigated the effects of different B vitamins and MgOr on MD and the processing of APP in SH-SY5Y-APP₆₉₅ cells a cellular model of early AD. Furthermore, the effects of the substances were tested in CL2006 and GMC101, both invertebrate models of AD.

2. Results

2.1. General Overview of Tests and Results

In Table 1 below, all tests and results are listed to provide a general overview of the subsequent tests and results. Here, the substance under investigation is shown against the control. For more detailed insights, the results are described in the respective chapters.

Table 1. General overview of all tests and results of all biofactors. Combinations ID63 and ID64 and the single substances compared to the control.

	ID63 vs. CTR	ID64 vs. CTR	MgOr vs. CTR	Fol vs. CTR	Vit B6 vs. CTR
A β ₁₋₄₀	Significant lower	Significant lower	Significant lower	Significant lower	No significant change
A β ₁₋₄₂	No significant change	Significant lower	Significant lower	Significant lower	Significant lower
sAPP α	Significant lower	Significant lower	Not tested	Not tested	Not tested
sAPP β	No significant change	No significant change	Not tested	Not tested	Not tested
ATP level	No significant change	No significant change	Not tested	Not tested	Not tested
MMP level	No significant change	No significant change	Not tested	Not tested	Not tested
Respiration	No significant change	No significant change	Not tested	Not tested	Not tested
Citrate synthase activity	No significant change	No significant change	Not tested	Not tested	Not tested
Lactate level	Significant lower	Significant lower	Not tested	Not tested	Not tested
Pyruvate level	No significant change	No significant change	Not tested	Not tested	Not tested
Lactate/Pyruvate Ratio	No significant change	Significant lower	Not tested	Not tested	Not tested
Gen expression PDK1	No significant change	No significant change	Not tested	Not tested	Not tested
Gen expression LDHA	No significant change	Significant higher	Not tested	Not tested	Not tested
Lifespan <i>C. elegans</i> in %	No significant change	Significant higher	Significant higher	Significant higher	Significant higher
Mean survival <i>C. elegans</i>	Significant higher	Significant higher	Significant higher	Significant higher	Significant higher
Paralysis <i>C. elegans</i>	Significant lower	Significant lower	Not tested	Not tested	Not tested
A β ₁₋₄₂ <i>C. elegans</i>	No significant change	No significant change	Not tested	Not tested	Not tested
A β ₁₋₄₂ aggregation <i>C. elegans</i>	Significant lower	No significant change	Not tested	Not tested	Not tested

2.2. A β ₁₋₄₀ Production

First, we tested different concentrations of biofactors on A β ₁₋₄₀ production in SH-SY5Y-APP₆₉₅ cells. Cells were incubated for 24 h with zinc orotate (ZnO), magnesium-orotate (MgO), benfotiamine (vitamin B1), folic acid (Fol), cholecalciferol (Vit D3), cobalamin (Vit B12), and pyridoxine (Vit B6) to select possible hit substances for further experiments (data not shown). Potential hit substances were identified, from which finally Fol 10 μ M, MgOr 200 μ M and Vit B6 100 nM turned out to be the most promising ones, which we applied in two different combinations.

To investigate the effect on the A β ₁₋₄₀ production, SH-SY5Y-APP₆₉₅ cells were incubated with both combinations (MgOr 200 μ M & Fol 10 μ M = ID63 / /MgOr 200 μ M & Fol 10 μ M & Vit B6 100 nM = ID64) and the single compounds for 24 h (Figure 1). ID63 had a significant lowering effect on the A β ₁₋₄₀ level ($p > 0.0001$). The ID63 combination even had an over additive effect compared to the single substances MgOr ($p = 0.0028$) and Fol ($p > 0.0001$). The ID64 combination had a significantly decreasing effect on the A β

levels compared to the control ($p > 0.0001$). Furthermore, ID64 had a significantly reducing effect in comparison to the single substances MgOr ($p = 0.0497$), Fol ($p > 0.0001$) and Vit B6 ($p > 0.0001$).

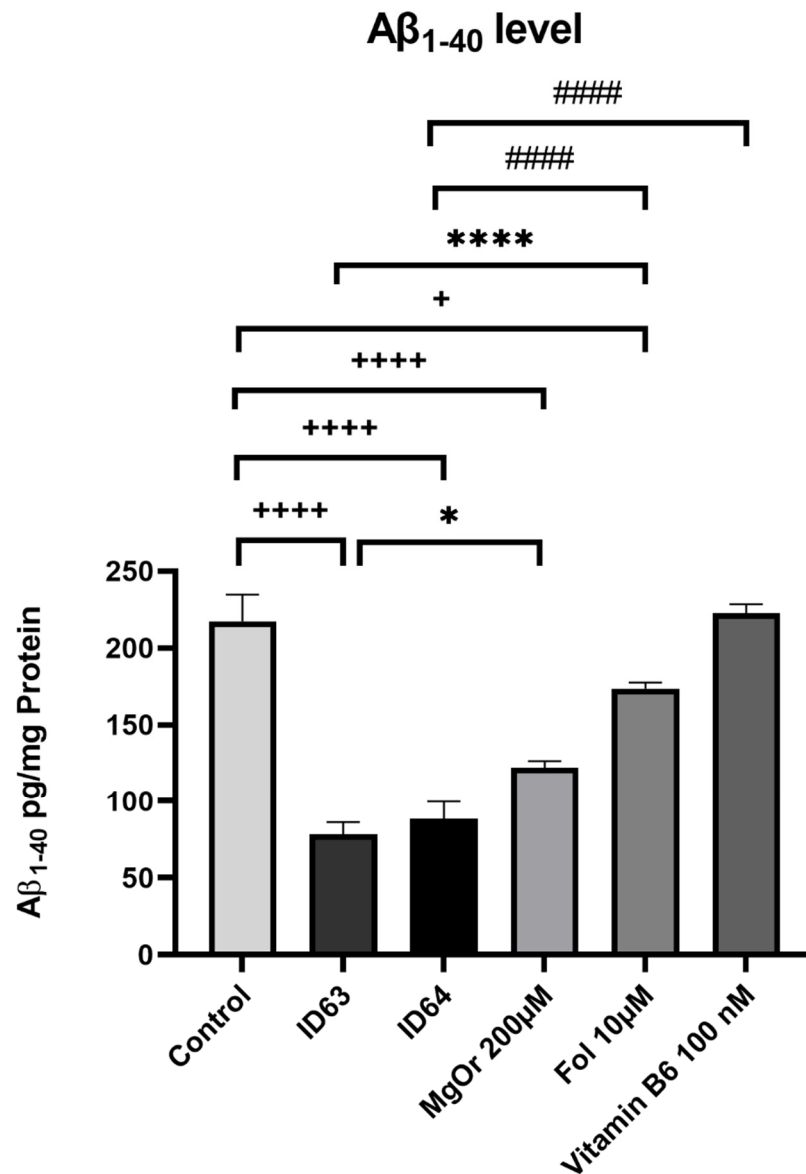


Figure 1. Effect of ID63 and ID64 in SH-SY5Y₆₉₅ cells compared to the control or their single substances on the Aβ₁₋₄₀ level after 24 h incubation. N = 6. Aβ₁₋₄₀ levels were adjusted to the protein content. Significance was determined by Student's unpaired *t*-test and one-way ANOVA. + significant against control; * significant against ID63; # significant against ID64. * $p < 0.05$, *** $p < 0.0001$, #### $p < 0.0001$ and + $p < 0.05$, ++++ $p < 0.0001$. Data are displayed as the mean \pm SEM. ID63 = 200 μ M MgOr and 10 μ M Fol; ID64 = 200 μ M MgOr, 10 μ M Fol and 100 nM Vit B6.

2.3. Aβ₁₋₄₂ Production

To study the production of Aβ₁₋₄₂, SH-SY5Y-APP₆₉₅ cells were incubated for 24 h with ID63, ID64 or the single compounds (Figure 2). In comparison to the control, the single compounds MgOr ($p = 0.0024$) and folic acid ($p = 0.0004$), as well as the combination ID 64 ($p = 0.0039$) had a significant lowering effect on Aβ₁₋₄₂ levels, while the combination ID63 had a slight reducing effect on the Aβ₁₋₄₂ levels, although not a significant one. ID64 also showed significantly lower Aβ₁₋₄₂ levels in comparison to B6 ($p = 0.0009$). However, folic acid alone, reduced the levels to a higher extent than any combinations.

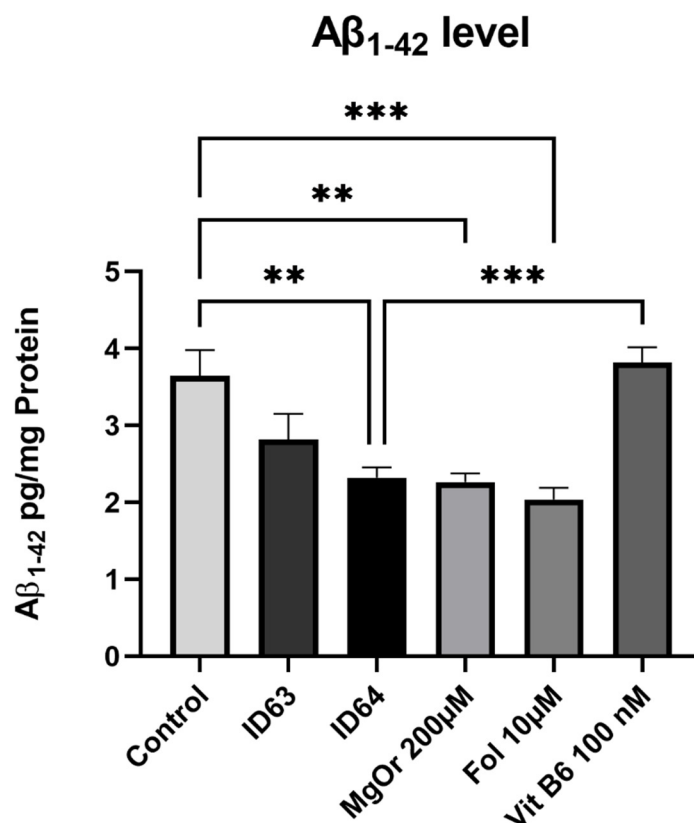


Figure 2. Effect of ID63 and ID64 in SH-SY5Y₆₉₅ cells compared to the control or their single substances on the Aβ₁₋₄₂ level after 24 h incubation. N = 6. Aβ₁₋₄₂ levels were adjusted to the protein content. Significance was determined by Student's unpaired *t*-test and one-way ANOVA. ** *p* < 0.01 and *** *p* < 0.001. Data are displayed as the mean ± SEM. ID63 = 200 µM MgOr and 10 µM Fol; ID64 = 200 µM MgOr, 10 µM Fol and 100 nM Vit B6.

2.4. sAPPα and sAPPβ Level

The α- and β-secretase cleaving products of APP, sAPPα (Figure 3A) and sAPPβ (Figure 3B), respectively, were determined after incubation with either ID63 or ID64 for 24 h. Figure 4A shows that ID63 had a significantly lowering effect on the sAPPα (*p* = 0.0214) compared to the control. ID64 had an even greater effect on the reduction of sAPPα fragments (*p* > 0.0001). In contrast to the sAPPα fragment production, the sAPPβ fragments (Figure 3B) were lowered compared to the control though not significantly. ID64 had a greater effect than ID63. It should be noted that basal levels of sAPPβ were approximately one hundredfold lower compared with sAPPα (Figure 3).

2.5. Effect on the Mitochondrial Function

To investigate the effect of ID63 and ID64 on mitochondrial function, we incubated SH-SY5Y-APP₆₉₅ cells for 24 h with ID63 or ID64. Respiration under O₂ consumption through the respiratory chain builds up the mitochondrial membrane potential, which allows ATP to be generated with the help of ATP synthase. First, we measured the ATP level after incubation with ID63 or ID64. Afterwards, the MMP was examined as well as the O₂ consumption and citrate synthase activity (Figure 4).

Neither ID63 nor ID64 had an increased effect on the ATP level (Figure 5A,B) or an effect on the MMP level (Figure 4C,D). ID63 had a slightly increasing effect on the complex activity of complex I, II and IV compared to the control (Figure 4E). In contrast, ID64 had a slightly decreasing effect on the complex activity of complex II and IV (Figure 4F). Whereas ID63 had no effect on the citrate synthase activity compared with the control (Figure 4G),

and ID64 even had a slightly decreasing effect compared with the control (Figure 4H). However, none of these effects is statistically significant.

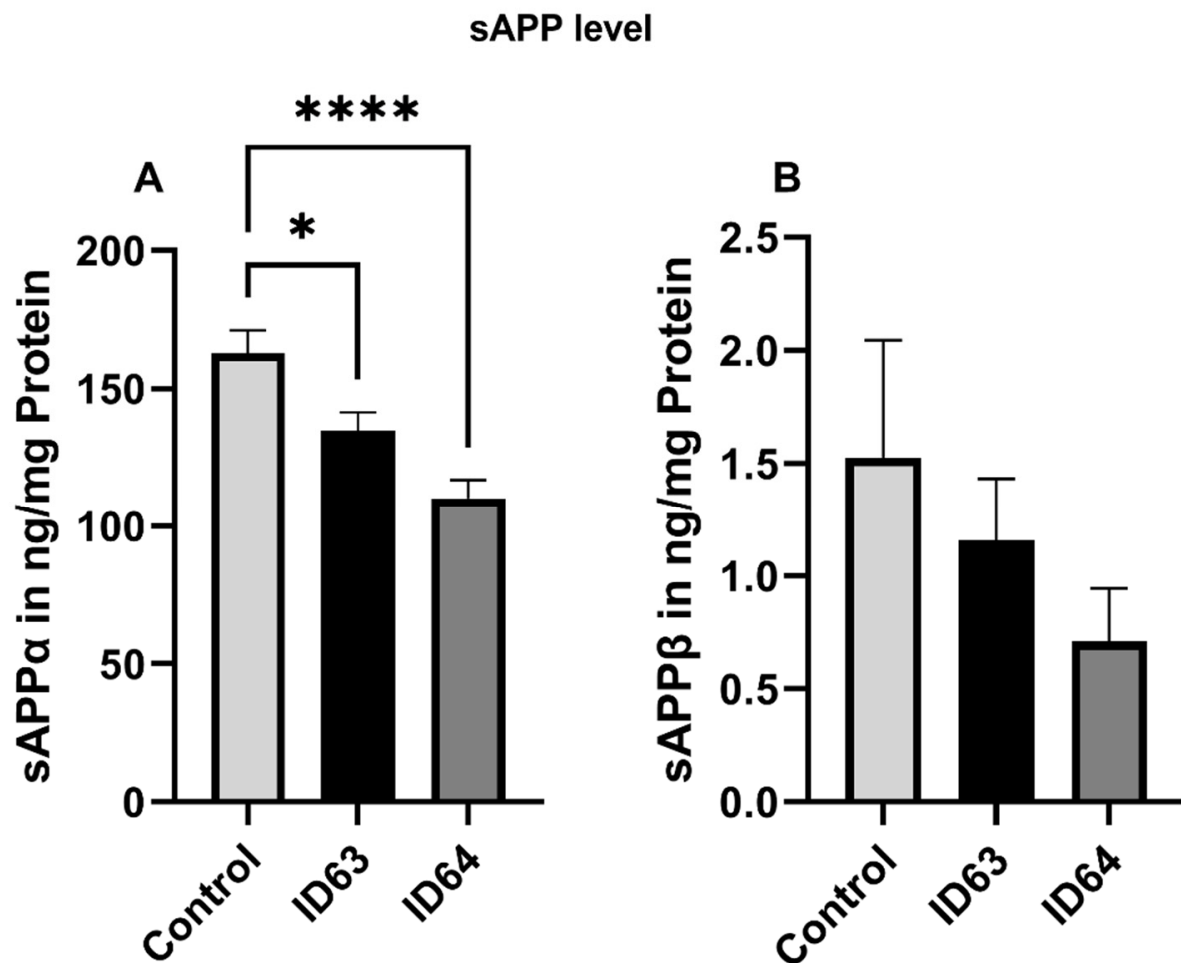


Figure 3. Effect of the incubation with ID63 or ID64 on the human soluble amyloid precursor protein α (sAPP α) and β (sAPP β) after 24 h of incubation. (A) sAPP α level of SH-SY5Y₆₉₅ cells after the incubation with ID63 or ID64 compared to the control. (B) sAPP β level of SH-SY5Y₆₉₅ cells after the incubation with ID63 or ID64 compared to the control. N = 6. sAPP levels were adjusted to the protein content. Significance was determined by one-way ANOVA. * $p < 0.05$ and **** $p < 0.0001$. Data are displayed as the mean \pm SEM. ID63 = 200 μ M MgOr and 10 μ M Fol; ID64 = 200 μ M MgOr, 10 μ M Fol and 100 nM Vit B6.

2.6. Lactate and Pyruvate Level

To investigate if the glycolysis is affected by ID63 or ID64, the lactate and pyruvate levels were measured. As seen in Figure 6, only lactate is significantly reduced by ID63 ($p = 0.0328$) and ID64 ($p > 0.0001$) compared to the control while pyruvate levels were not influenced. The ratio of lactate/pyruvate was significantly affected by ID64 ($p = 0.0057$) (Figure 5C).

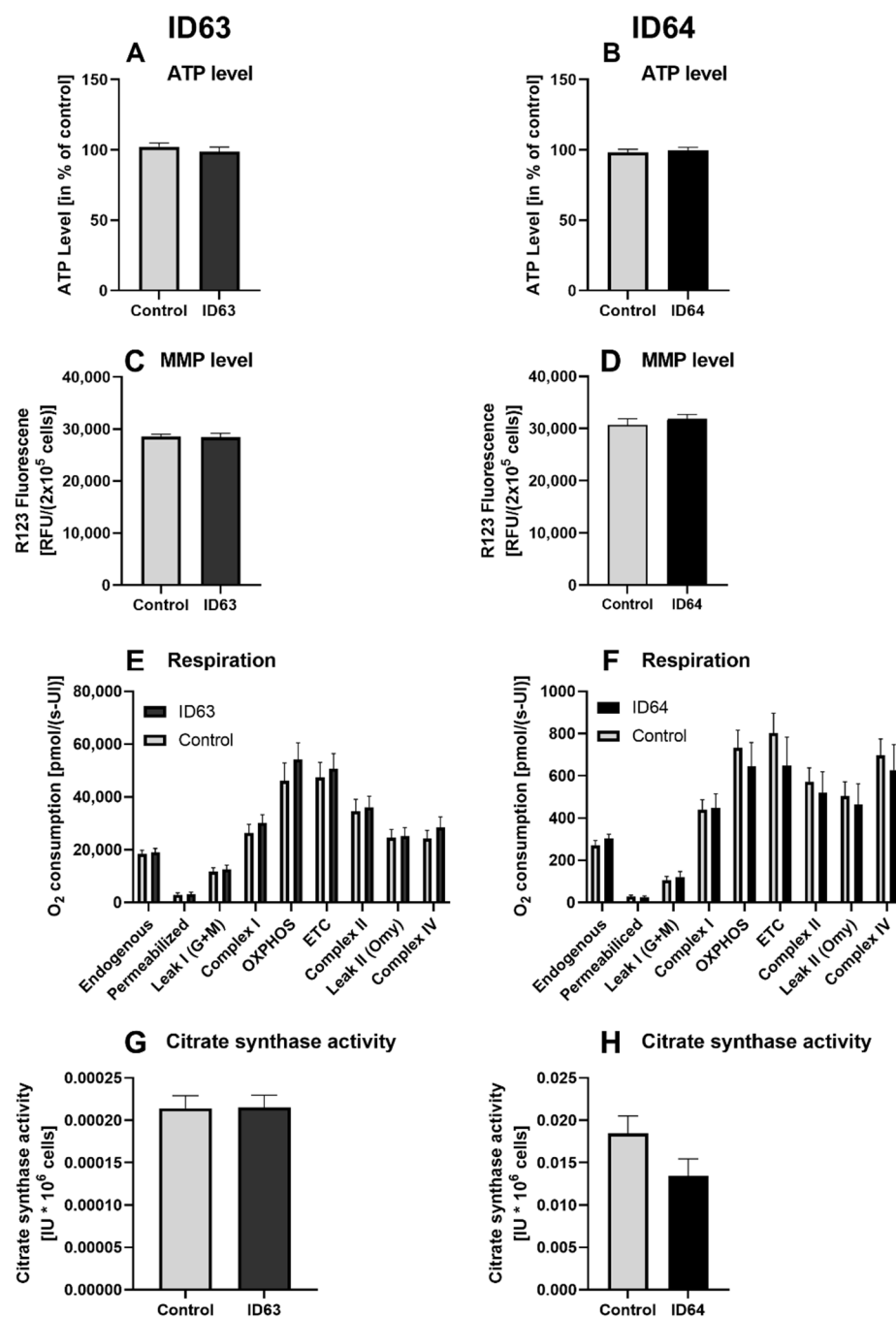


Figure 4. ATP level, MMP level, respiration and citrate synthase activity of SH-SY5Y-APP₆₉₅ cells incubated for 24 h with ID63 or ID64. (A) ATP level of SH-SY5Y-APP₆₉₅ cells incubated with ID63 and (B) ATP level of incubation with ID64 compared to the control. Cells treated with cell culture medium served as control (100%). N = 12. (C) MMP level of 2 × 10⁵ SH-SY5Y-APP₆₉₅ cells incubated with ID63 and (D) MMP level of incubation with ID64 compared to the control. N = 16. (E) Respiration of SH-SY5Y-APP₆₉₅ cells incubated with ID63 and (F) incubation with ID64 compared to the control. SH-SY5Y-APP₆₉₅ cells adjusted to international units (IU) of citrate synthase activity. N = 15. (G) Citrate synthase activity of SH-SY5Y-APP₆₉₅ cells incubated with ID63 and (H) Citrate synthase activity incubated with ID64 compared to control. N = 12. Significance was determined by Student's unpaired *t*-test. Data are displayed as the mean ± SEM. ID63 = 200 μM MgOr and 10 μM Fol; ID64 = 200 μM MgOr, 10 μM Fol and 100 nM Vit B6. Leak I (G + M) = leak respiration with glutamate and malate; OXPHOS = oxidative phosphorylation system; ETC = electron transport chain; Leak II (Omy) = leak respiration with oligomycin.

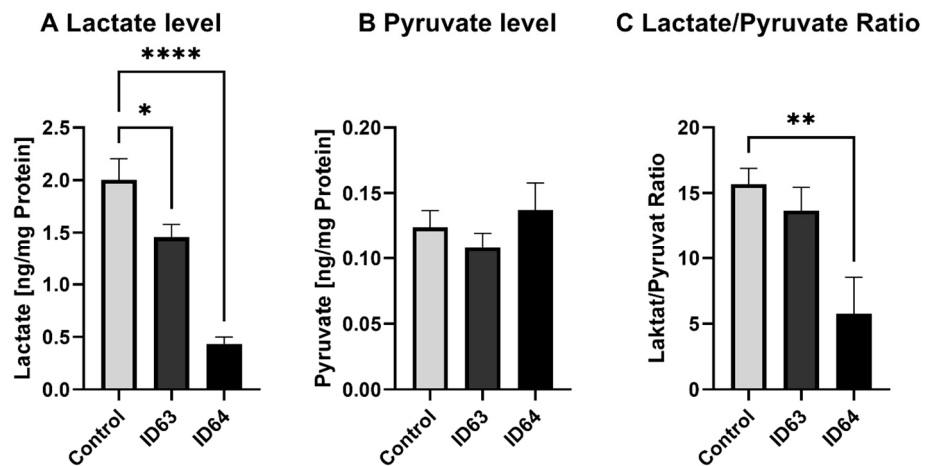


Figure 5. Effect of the incubation with ID63 or ID64 on lactate and pyruvate level after 24 h of incubation. (A) Lactate level of SH-SY5Y₆₉₅ cells after the incubation with ID63 or ID64 compared to the control. (B) Pyruvate level of SH-SY5Y₆₉₅ cells after the incubation with ID63 or ID64 compared to the control. (C) Lactate to pyruvate ratio. N = 6. Levels were adjusted to the protein content. Significance was determined by one-way ANOVA. * $p < 0.05$, ** $p < 0.01$ and **** $p < 0.0001$. Data are displayed as the mean \pm SEM. ID63 = 200 μ M MgOr and 10 μ M Fol; ID64 = 200 μ M MgOr, 10 μ M Fol and 100 nM Vit B6.

Gene expression

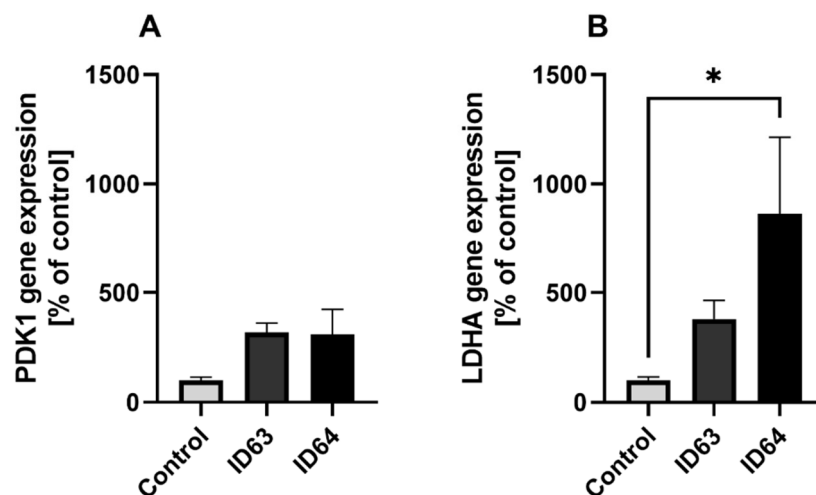


Figure 6. Effect of the incubation with ID63 or ID64 on the gene expression after 24 h of incubation. (A) Gene expression of pyruvate dehydrogenase kinase 1 (PDK1) of SH-SY5Y₆₉₅ cells after the incubation with ID63 or ID64 compared to the control. (B) Gene expression of lactate dehydrogenase A (LDHA) of SH-SY5Y₆₉₅ cells after the incubation with ID63 or ID64 compared to the control. N = 8. Significance was determined by one-way ANOVA. * $p < 0.05$. Data are displayed as the mean \pm SEM. ID63 = 200 μ M MgOr and 10 μ M Fol; ID64 = 200 μ M MgOr, 10 μ M Fol and 100 nM Vit B6.

2.7. qPCR

To investigate the molecular basis of altered lactate and pyruvate levels, the gene expression of pyruvate dehydrogenase kinase 1 (PDK1) and lactate dehydrogenase A (LDHA) were examined after 24 h incubation using qRT-PCR. ID63 and ID64 had no significant effect on PDK1 gene expression compared to the control (Figure 6). Both combinations increased LDHA mRNA levels (Figure 6B), with ID64 ($p = 0.0148$) showing a significant increase in gene expression.

2.8. Effect on the Lifespan of *C. elegans* in Heat Stress Survival Assay

To test the effect of the combinations in vivo two invertebrate AD-models were used. *C. elegans* CL2006 were incubated with the same compounds but in different concentrations. The single compounds Fol 50 μM ($p > 0.0001$), Vit B6 100 μM ($p = 0.0397$) and MgOr 100 μM ($p = 0.001$) had a significant life-extending effect compared to the control (see Figure 7A,B). As shown in Figure 7A, the ID63_{worm} extended lifespan of CL2006 compared to the control by trend ($p = 0.0502$), whereas in Figure 7B, it can be seen that ID64_{worm} had a significant life-prolonging effect ($p = 0.0002$) compared to the control. Subsequently, the mean survival of the nematodes after the incubations of the combinations and single substances was assessed. Thereby Figure 7C shows that ID63_{worm} ($p = 0.0196$), Fol 50 μM ($p < 0.0001$), MgOr 100 μM ($p = 0.0004$), ID64_{worm} ($p < 0.0001$) and Vit B6 ($p = 0.0107$) had a significant increasing effect on the mean survival of the nematodes compared to the control. Folic acid alone was numerically more effective than the combinations or the other single compounds.

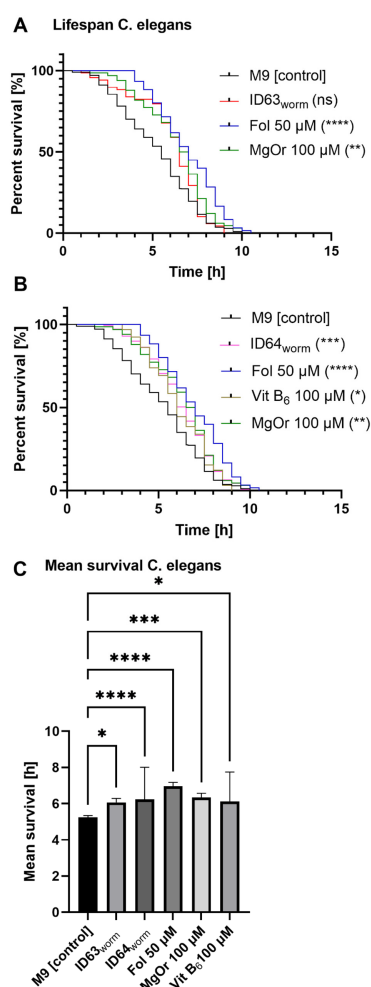


Figure 7. The lifespan under heat-stress of *C. elegans* after treatment with either ID63_{worm}, ID64_{worm} or their single substances in CL2006. (A) The single substances lead to an increase in heat-stress resistance of the nematodes although the combination of them does not have a significant impact. (B) After combination with vitamin B₆, the resulting treatment leads to a significant increase in heat-stress resistance. For heat-stress experiments, the survival was assessed according to the penetration of SYTOX Green nucleic acid stain into dead cells. $N > 60$. log-rank (MantelCox) test. (C) Mean Survival of *C. elegans* after treatment with either ID63_{worm}, ID64_{worm} or their single substances in CL2006. Significance was determined by one-way ANOVA. $p^* < 0.05$, $p^{**} < 0.01$, $p^{***} < 0.001$ and $p^{****} < 0.0001$. ID63_{worm} = Fol 50 μM and MgOr 100 μM ; ID64_{worm} Fol 50 μM , MgOr 100 μM and Vit B6 100 μM .

2.9. Effect on the Paralysis

To investigate the effect of combining biofactors on paralysis induced by A β , *C. elegans* GMC101 were incubated for 24 h at 25 °C with either ID63_{worm} or ID64_{worm}. Both combinations ($p = 0.0243$ for ID63_{worm} and $p = 0.0149$ for ID64_{worm}) were able to significantly decrease the paralysis induced by A β (Figure 8). Thus, the phenotype of this AD-worm was significantly enhanced by both biofactor combinations to a comparable extent.

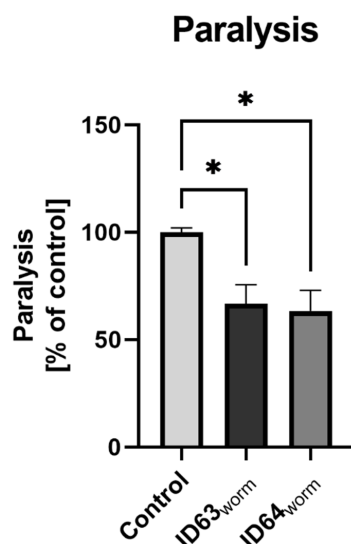


Figure 8. The A β induced paralysis after treatment with either ID63_{worm} or ID64_{worm} in GMC101. Both the feeding of both treatment combinations leads to significant reduction of A β induced paralysis after incubation of the nematodes for 24 h at 25 °C. N = 4. Mean \pm SEM. One-way ANOVA with Tukey's comparison post hoc test. $p^* < 0.05$ ID63_{worm} = Fol 50 μ M and MgOr 100 μ M; ID64_{worm} Fol 50 μ M, MgOr 100 μ M and Vit B6 100 μ M.

2.10. A β ₁₋₄₂ Production in GMC101

To study the production of A β ₁₋₄₂ in nematodes, GMC101 was incubated for 24 h at 25 °C with both combinations and then the A β level was examined. There was no effect on the A β ₁₋₄₂ levels compared to the control (Figure 9). Since A β ₁₋₄₀ is not produced in GMC101 this amyloid peptide was not investigated.

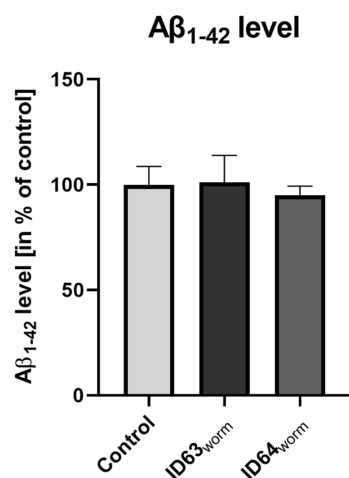


Figure 9. Effect of ID63 and ID64 in transgene nematodes GMC101 compared to the controls on the A β ₁₋₄₂ level after 48 h incubation. Neither ID63 nor ID64 did not lead to a significant alteration of A β ₁₋₄₂. N = 8. A β ₁₋₄₂ levels were adjusted to the protein content. N = 8. Mean \pm SEM. Student's *t*-test. ID63_{worm} = Fol 50 μ M and MgOr 100 μ M; ID64_{worm} Fol 50 μ M, MgOr 100 μ M and Vit B6 100 μ M.

2.11. A β_{1-42} Aggregation

To investigate the aggregation of A β in nematodes, GMC101 was incubated at 25 °C for 24 h and stained with thioflavine (ThT), which labels β -sheet structures of aggregated peptides. There was a significant reduction of A β -aggr by the combination ID63_{worm} ($p = 0.0324$) compared to the control, whereas ID64_{worm} was without an effect (Figure 10).

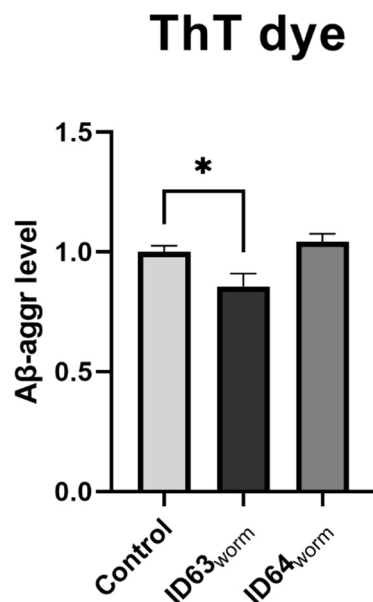


Figure 10. The effect of either ID63_{worm} or ID64_{worm} on the accumulation of aggregated A β (A β -aggr) in GMC101 after 48 h incubation. The treatment of the nematodes with ID63 leads to a significant reduction of accumulated A β . Whereby the treatment with ID64 combination did not lead to an alteration of A β levels; N = 8; mean \pm SEM; one-way ANOVA with Tukey's comparison post hoc test. * $p < 0.05$. ID63_{worm} = Fol 50 μ M and MgOr 100 μ M; ID64_{worm} = Fol 50 μ M, MgOr 100 μ M and Vit B6 100 μ M.

3. Discussion

In the present work, we examined the effect of MgOr, Fol and Vit B6 in different combinations on bioenergetic parameters including mitochondrial function and glycolysis, as well as A β production in cellular and invertebrate models of AD. We wanted to create a combination product that achieves an optimal result by combining several biofactors. The hit compounds used in the current study were selected after a screening of seven substances of interest whose concentrations used in the experiments were based on known literature values [37,43–47]. We selected the concentrations because they were mostly tested in our cell model, related to AD or cell survival and they were used in relative physiological concentrations. This resulted in the combinations ID63 and ID64. The combinations ID63 and ID64 significantly reduced A β_{1-40} levels in SH-SY5Y-APP₆₉₅ cells compared to the control (Figure 1). Even when compared to the individual components of the combinations, ID63 was able to show an over additively reducing effect on A β levels. Similar results were obtained by Li et al. who found a dose-dependent decrease of A β_{1-40} levels by incubation with Fol, modulating DNA methyltransferase activity [48]. In a study with AD patients, an intervention with Fol significantly reduced A β levels and increased the concentration of s-adenosyl methionine (SAM) [49]. Low SAM levels are a risk factor for AD, whereby incubation with SAM led to a decrease in A β levels in SK-N-SH cells [50]. Fol and Vit B6 are essential for the SAM cycle [51]. It seems that in our SH-SY5Y model the additional administration of Vit B6 shows no additional effect on A β_{1-40} levels. Furthermore, lower Mg²⁺ are associated with the occurrence of AD which negatively affects brain energy metabolism [52]. In addition, low Mg levels are also negatively correlated with the occurrence of A β_{1-40} and A β_{1-42} [53]. As a result, an administration of Mg²⁺

leads to a decrease in A β levels of N2a-APP cells [37]. After the incubation with ID63, SH-SY5Y-APP₆₉₅ cells showed reduced levels of A β ₁₋₄₂ compared to the control, although not significantly. In contrast, the incubation with ID64 showed a significant reduction of A β ₁₋₄₂ (Figure 2) compared to the control. Administration of a Fol-rich diet significantly reduced A β ₁₋₄₂ levels in APP/PS1 mice compared to the standard diet [54]. Similarly, the incubation with Mg significantly decreased A β ₁₋₄₂ production in Na2 neuroblastoma cells and transgenic mice [37,55,56]. In our work, we could not reproduce these described effects shown despite the administration of both substances in SH-SY5Y-APP₆₉₅ cells. However, it seems that Vit B6 has a crucial role in the reduction of A β ₁₋₄₂ levels (ID64) even if Vit B6 alone showed no effects. In contrast, it did not provide any benefit at the A β ₁₋₄₀ level (Figure 1). Next, we examined sAPP α and - β levels after incubation with ID63 and ID64. The reducing effects on A β ₁₋₄₂ levels by ID64 in SH-SY5Y-APP₆₉₅ cells could not be confirmed in transgene nematodes GMC101. On the one hand, incubation decreased the sAPP α levels significantly compared to the control (Figure 3A). On the other hand, both ID63 and ID64 had a reducing but not significant effect on the sAPP β levels compared to the control (Figure 3B). Whereas it should be noted that the concentration of sAPP β is 100 times lower than sAPP α . Studies showed that the concentration of sAPP α is generally higher than that of sAPP β [57,58]. An application of Mg²⁺ increased the amount of sAPP α in APP/PS1 transgenic mice and simultaneously decreased the concentration of sAPP β [37]. The results shown in this study [37], where both sAPP α and - β were decreased, could not be reproduced in our work. We assume that this was due to a general decrease in APP production, since the application of Fol can reduce APP processing [59,60], which in turn can affect sAPP α and - β , resulting in generally lowered levels.

Next, we tested ID63 and ID64 on MD in SH-SY5Y-APP₆₉₅ cells. SH-SY5Y-APP₆₉₅ cells show reduced ATP level, MMP and O₂ consumption compared to their non-transfected SH-SY5Y-MOCK cells [61]. Mg and Fol are vital compounds for enzymes and ATP production in cells [31,33,36]. A deficit leads to the reduced production of ATP [31,34]. In our case, which is not a deficit model, we did not observe any improvement in ATP level, MPP, O₂ consumption or citrate synthase activity in SH-SY5Y-APP₆₉₅ cells (Figure 4). In a study by Viel et al. using a similar cocktail consisting of some of our compounds, the mitochondrial complex activity in a transgenic rat model was increased to that of wild type rats [28]. This effect is not found in our case, possibly due to the additional substances contained in the cocktail, which had been the decisive factor here.

It has been shown that in the brains of AD patients there is a switch from aerobic respiration to glycolytic metabolism [62,63]. This is accompanied by an increase in lactate and pyruvate values [64,65], which results from insufficient utilization in oxidative phosphorylation [66]. Both biofactor combinations used in our investigations were able to significantly reduce the lactate values compared to the untreated control cells. The switch to increased oxidative phosphorylation could not be shown in our work, because there was no effect on ATP, MMP, OXPHOS or citrate synthase activity in the respiratory chain. To investigate the impact of the two combinations on the glycolytic genes, mRNA levels of PDK1 and LDHA were determined (Figure 6). There was an increase in the expression of PDK1 by both combinations compared to the control. PDK1 phosphorylates pyruvate dehydrogenase and inactivates it. This may lead to a reverse transport of pyruvate from the mitochondrion into the cytosol, where it is used for glycolytic energy production via lactate [67,68]. Both combinations were able to increase the expression of LDHA, while ID64 did so significantly. LDHA converts pyruvate to lactate and vice versa [23,69], although the expression was increased there were decreased lactate levels with both ID63 and ID64 (Figure 5). By upregulating PDK1 and LDHA expression, the cell may reduce the effects of A β toxicity and ROS production by shifting from mitochondrial to glycolytic energy production [23,69]. Both combinations appear to support this process, resulting in an upregulation of expression relative to control.

Based on the results obtained, especially those related to A β , we wanted to test our compounds in another model of AD. For this purpose, we adjusted the concentrations

and tested them in two invertebrate models of AD. CL2006 and GMC101 both express human A β in their muscle cells. While CL2006 produces A β continuously at 20 °C [70], GMC101 needs a temperature shift to 25 °C to initiate A β production [71]. Phenotypically, both are identified like wild-type N2 [72]. ID63_{worm} had no significant life-prolonging effect on CL2006 compared to the control. Whereas the single substances and ID64_{worm} had a significant effect on the lifespan (Figure 7A,B). In contrast, all tested combinations and single substances, have a significantly increased effect on mean survival (Figure 7C). CL2006 continuously produces A β , which has a toxic effect on the lifespan [70,73]. In particular, it has effects on DAF-16, which is expressed in the nucleus during stress and has an effect on life span extension [74]. DAF-16 is notable for being responsible for activating genes involved in longevity, lipogenesis, heat shock survival and oxidative stress responses [75,76], homologs being found in *C. elegans*, humans and mice [77]. It was demonstrated that incubation with Fol extended the lifespan by increasing the expression of DAF-16 [78]. It seems that the substances used have a similar effect on lifespan, whereas the single substance Fol seems to be superior. Furthermore, it was investigated whether the substances can reduce the toxic effect caused by A β . For this purpose, it was examined whether the paralysis in GMC101 changes because of the administration (Figure 8). Both combinations were able to reduce the paralyzes, indicating the significantly reduced toxicity of A β . Similar effects have been shown with other substances, which reduced the paralysis and thus the toxicity of A β [73,79–81]. The reduced toxicity could be a consequence of the reduction of A β levels in *C. elegans*. It could be shown that the administration of single substances leads to a reduction of the A β levels [37,48,50,54,55]. To verify this, the A β _{1–42} levels were determined in GMC101 (Figure 9). Here, we found no significant effect in reduced A β level after incubation with both combinations. It should be noted here that the effects on A β _{1–42} were also relatively moderate in the cells (see above). A β _{1–40} was not measured in GMC101 because the worm does not produce these peptides. To examine the aggregation of A β after incubation with both combinations, samples were stained with ThT, which labels A β -structures in proteins (Figure 10). A significant reduction in A β -aggr was observed after incubation with ID63_{worm}. The combination with additional vitamin B6 did not reduce aggregation further. A similar effect was found in a study by Yu et al., in which the aggregation of A β in mice was reduced by incubation with magnesium [82].

It remains to be considered that there are both advantages and disadvantages to administering the substances as a single or combination preparation. Combined administration could lead to synergistic effects that enhance the positive effects and thus lead to an additive effect, as shown in the A β _{1–40} values Figure 1. However, it cannot be ruled out that negative effects may occur when more than one substance is administered, as the substances may influence each other. Complex formation or competition for transport systems into the cell could occur. Especially when moving from an in vitro to an in vivo experiment. In order to exclude these effects, further experiments will have to be carried out in the future.

4. Materials and Methods

4.1. Cell Culture

The cultivation of SY5Y cells was performed in 75 cm² cell culture flasks under sterile conditions. To ensure optimal growth, cells were split several times per week once a cell density of approximately 70–80% was reached. All cells were cultured in an incubator at 37 °C and 5% CO₂ in DMEM supplemented with 10% (*v/v*) heat-inactivated fetal calf serum, 60 μ g/mL streptomycin, 0.3 mg/mL hygromycin, 1% MEM non-essential amino acids, and 1 mM sodium pyruvate 1%, 60 units/mL penicillin. SH-SY5Y cells were stably transfected with DNA constructs harboring human wild-type APP₆₉₅ (APP₆₉₅) and were kindly donated by A. Eckert (Basel, Switzerland) (for details; please refer to [83]).

4.2. Cell Treatment

First of all, cells were incubated for 24 h with different concentrations of zinc orotate (ZnOr), magnesium-orotate (MgOr), benfotiamine (vitamin B1), folic acid (Fol), cholecalciferol (Vit D3), cobalamin (Vit B12), and pyridoxine (Vit B6) for possible hit substances. After the hit substances were determined, cells were incubated with 200 μ M magnesium-orotate (MgOr) and folic acid 10 μ M (Fol) (combination ID63) or 200 μ M MgOr, 10 μ M folic acid and 100 nM Vit B6 (combination ID64). We have derived the concentrations used here from the literature [37,43–47]. The solution medium NaOH diluted 1:8 with water served as a control. The ratio had the best dissolving properties without being toxic to the cells.

4.3. ATP Measurement

A bioluminescence assay was used to determine the ATP levels, which is based on the production of light from ATP and luciferin in the presence of luciferase. The test was performed using the ATPlite Luminescence Assay System (PerkinElmer, Rodgau, Germany) according to the previously published protocol [84].

4.4. MMP Measurement

Mitochondrial membrane potential (MMP) was measured using the fluorescence dye rhodamine-123 (R123). Cells were incubated for 15 min with 0.4 μ M R123 and centrifuged at $750 \times g$ for 5 min before being washed with Hank's Balanced Salt Solution (HBSS) buffer supplemented with Mg^{2+} , Ca^{2+} , and HEPES. The cells were suspended with fresh HBSS before they were evaluated by measuring the R123 fluorescence. The excitation wavelength was set to 490 nm and the emission wavelength to 535 nm with CLARIOstar (BMG Labtech, Ortenberg, Germany).

4.5. Cellular Respiration

Respiration in SH-SY5Y₆₉₅ cells was assessed using an Oxygraph-2k (Oroboros, Innsbruck, Austria) and DatLab 7.0.0.2. The cells were treated according to a complex protocol developed by Dr. Erich Gnaiger [85]. They were incubated with different substrates, inhibitors and uncouplers. First, cells were washed with PBS (containing potassium chloride 26.6 mM, potassium phosphate monobasic 14.705 mM, sodium chloride 1379.31 mM and sodium phosphate dibasic 80.59 mM) and scraped into mitochondrial respiration medium (MiRO5) developed by Oroboros [85]. Afterwards, the cells were centrifuged, resuspended in MiRO5, and diluted to 10^6 cells/mL. After 2 mL of cell suspension was added to each chamber and endogenous respiration was stabilized, the cells were treated with digitonin (10 μ g/ 10^6 cells) to permeabilize the membrane, leaving the outer and inner mitochondrial membrane intact. OXPHOS was measured by adding the complex I and II substrates malate (2 mM), glutamate (10 mM) and ADP (2 mM), followed by succinate (10 mM). Gradual addition of carbonyl cyanide-4- before it is evaluated by measuring the R123 fluorescence (trifluoromethoxy) phenylhydrazon showed the maximum capacity of the electron transfer system. Rotenon (0.1 mM) was added to measure the activity of complex II. To investigate the leak respiration, oligomycin (2 μ L/mL) was injected. To inhibition of complex III to determined residual oxygen consumption, antimycin A (2.5 μ M) was added. This value was subtracted from all respiratory states. Adding N measured N, N', N'-tetramethyl-p-phenylenediamine (0.5 mM) and ascorbate (2 mM) the activity of complex IV. To measure the sodium autoxidation rate, azide (≥ 100 mM) was added. Afterwards, complex IV respiration was corrected by subtracting the autoxidation rate of azid. NaOH served as control.

4.6. Citrate Synthase Activity

Cell samples from respirometry measurements were frozen and stored at -80 °C for the determination of citrate synthase activity. Samples were thawed while the reaction mix (0.1 mM 5,5'-dithiol-bis-(2-nitrobenzoic acid) (DTNB), 0.5 mM oxaloacetate, 50 μ M EDTA, 0.31 mM acetyl coenzyme A, 5 mM triethanolamine hydrochloride, and 0.1 M Tris-HCl)

was mixed and heated at 30 °C for 5 min. Afterwards, 40 µL of samples were submitted in triplets and mixed with 110 µL of the reaction mix. The absorption was measured at 412 nm.

4.7. $A\beta_{1-40}$ Measurement

After 24 h incubation, the $A\beta_{1-40}$ levels were determined in SH-SY5Y-APP₆₉₅ cells using HTRF Amyloid-Beta 1–40 kit (Cisbio, Codolet, France). The protocol was the same as described earlier [61]. $A\beta$ concentrations were normalized against protein content.

4.8. $A\beta_{1-42}$ Measurement

After 24 h incubation, the $A\beta_{1-42}$ levels were determined in SH-SY5Y-APP₆₉₅ cells and nematodes GMC101 using the Human- $A\beta_{1-42}$ ELISA Kit (Invitrogen™, Waltham, MA, USA). The protocol was performed according to the manufacturer's instructions. $A\beta$ concentrations were normalized against protein content.

4.9. Protein Quantification

Protein content was determined using a Pierce™ Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Bovine serum albumin was used as a standard.

4.10. Quantification of Human Soluble Amyloid Precursor Protein α (sAPP α)

The sample preparation was the same according to the $A\beta_{1-40}$ quantification. The sAPP α levels were determined using the Human Soluble Amyloid Precursor Protein α (sAPP α) ELISA Kit (Cusabio, Wuhan, China). The process was carried out according to the manufacturer's instructions. Levels were normalized to the protein content.

4.11. Quantification of Human Soluble Amyloid Precursor Protein β (sAPP β)

The sample preparation was the same according to the $A\beta_{1-40}$ quantification. The sAPP β levels were determined using the Human Soluble Amyloid Precursor Protein β (sAPP β) ELISA Kit (BT LAB, Zhejiang, China). The process was carried out according to the manufacturer's instructions. Levels were normalized to the protein content.

4.12. Pyruvate and Lactate Content

Frozen cells, which were previously harvested and incubated for 24 h, were thawed at room temperature. Pyruvate and lactate concentrations were assessed using a pyruvate assay kit (MAK071, Sigma Aldrich, Darmstadt, Germany) and a lactate assay kit (MAK064, Sigma Aldrich, Darmstadt, Germany) according to the manufacturer's instructions.

4.13. Real-Time qRT-PCR

To isolate RNA, we used the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's guidelines. Nanodrop™ 2000c spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to quantify RNA. The TURBO DNA-free™ kit was used according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA) to remove residual genomic DNA. cDNA was synthesized from 1 µg total RNA using an iScript cDNA Synthesis Kit (Bio-Rad, Munich, Germany). qRT-PCR was conducted using a CFX 96 Connect™ system (Bio-Rad, Munich, Germany). All used primers are listed in Table 2. The cDNA aliquots were analyzed in triplicate and diluted 1:10 with RNase-free water (Qiagen, Hilden, Germany). PCR cycling conditions were as follows: initial denaturation for 3 min at 95 °C, followed by 45 cycles at 95 °C for 10 s, 58 °C for 30 s (or 56 °C for 45 s, depending on the primer), and 72 °C for 29 s. Expression was analyzed with $-(2\Delta\Delta Cq)$ using Bio-Rad CFX manager software. To normalize the values a factor was calculated based on the geometric mean of the levels of multiple control genes of β -actin (ACTB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and phosphoglycerate kinase 1 (PGK1) according to the MIQE guidelines [86]. RNA free water served as an assay control to exclude impurities.

Table 2. Oligonucleotide primer sequences, product sizes, and primer concentrations for quantitative real-time PCR.

Primer	Sequence	Manufacturer	Product Size	Concentration (nM)
β -Actin (ACTB) NM_001101.2	5'-ggacttcgagcaagagatgg-3' 5'-agcactgtgttgccgtacag-3'	Biomol, Hamburg, Germany	234	200
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) NM_002046.2	5'-gagtcaacggatttgctgt-3' 5'-ttgattttggaggatctcg-3'	Biomol, Hamburg, Germany	238	200
Phosphoglycerate kinase 1 (PGK1) NM_000291.2	5'-ctgtgggggtatttgaatgg-3' 5'-cttcaggagctccaaa-3'	Biomol, Hamburg, Germany	198	200
Pyruvate dehydrogenase kinase, isozyme 1 (PDK1) NM_002610	5'-atacggatcagaaaccgaca-3' 5'-cagacgcctagcatttcat-3'	Biomol, Hamburg, Germany	291	100
Human lactate dehydrogenase A like 6B (LDHA) NM_033195	5'-ggtgtcccttgaaggatct-3' 5'-tgcagtcacttcttgtgga-3'	Biomol, Hamburg, Germany	87	400

4.14. Nematode and Bacterial Strain

C. elegans strains were obtained from the Caenorhabditis Genetics Center (University of Minnesota, MN, USA) and included CL2006 [Punc-54::human A-beta 3–42; pRF4 (rol-6(su1006))] and GMC101 [Punc-54::A-beta::unc-54 3Prime UTR; Pmtl2::GFP]. The strain GMC101 expresses the full-length human A β _{1–42} peptide in body-wall muscle cells that aggregates in vivo. Shifting L4 or young adult animals from 20 °C to 25 °C could induce the expression of A β and cause paralysis. The strain CL2006 constitutively expressed A β when cultured at 20 °C.

Nematodes were maintained on nematode growth medium (NGM) agar plates seeded with the bacterial *E. coli* strain OP50. According to standard protocols, the seeded plates were stored at 20 °C [87]. Synchronous populations were generated for all experiments by using a standard bleaching protocol [88].

4.15. Cultivation and Treatment

Post-bleaching generated larvae were washed twice in M9-buffer and the number of larvae in 10 μ L were adjusted to 10 larvae. Afterward, the synchronized larvae were raised in cell culture flasks (Sarstedt, Nümbrecht, Germany) in either an amount of 1000 or 5000 nematodes, depending on the experiments. OP50-NGM was added to the flasks as a standardized source of food. The larvae were maintained under shaking at 20 °C until they reached young adulthood within 3 days. The micronutrients were dissolved in advance in M9 buffer. For each micronutrient observed in this study, we generated a series of concentrations as follows. Folic acid (Fol) 50 μ M, magnesium orotate (MgOr) 0.1 mM (together ID63_{worm}) and Fol 50 μ M, MgOr 0.1 mM and vitamin B6 (Vit B6) 100 μ M (together ID64_{worm}). After reaching adulthood (48 h before the experiment), the micronutrients were added to the flasks. Pure M9-buffer was used as a control. Then, 24 h before the experiment amyloid aggregation was proceeded by upshifting young adult GMC101 from 20 °C to 25 °C.

4.16. Paralyze Assay

Cell culture flasks containing approximately 1000 adult amyloid beta producing nematodes were incubated for several hours to achieve A β induced paralysis (GMC101 24 h at 25 °C). On a NGM Agar Plate, 25 nematodes were placed and by physically touching with a platinum tip the paralysis status was recorded. Nematodes which normally act

after being touched by the wire are recognized as “not paralyzed” whereas uncoordinated movements or just head movements were recorded as “paralyzed”.

4.17. Heat-Stress Survival Assay

After 48 h of incubation of CL2006 with the mentioned effectors, the time till death was determined using a microplate thermotolerance assay [89]. In preparation, the nematodes were washed out of the flasks with M9-buffer into 15 mL tubes followed by 3 additional washing steps. Each well of a black 384-well low-volume microtiter plate (Greiner Bio-One, Frickenhausen, Germany) was prefilled with 6.5 μ L M9-buffer/Tween[®]20 (1% *v/v*). In the following step, one nematode in 1 μ L M9-buffer was transferred and immersed in the well under a stereomicroscope (Breukhoven Microscope systems, Netherlands). SYTOX[™] Green (Life Technologies, Karlsruhe, Germany) in a final concentration of 1 μ M was added to reach a final volume of 15 μ L in the well. SYTOX[™] Green creates a fluorescent signal after binding to DNA. The plates were sealed with a Rotilab sealing film (Greiner Bio-One, Frickenhausen, Germany). The heat-shock was applied and the fluorescence was measured every 30 min for 17 h at 37 °C following the protocol previously described [90]. The excitation was set at 485 nm and the emission was detected at 538 nm.

4.18. ThT Dying of A β Aggregates

Detection and quantification of A β aggregates (A β -aggr) in GMC101 were performed using the fluorescent dye thioflavin T (ThT) according to a previously described method with minor modifications [91]. Synchronized and heat incubated nematodes were washed out of the cell culture flasks with M9-buffer/Tween[®]20 (1% *v/v*) and separated from larvae. After centrifugation, 200 μ L of a thick pellet of nematodes was transferred into a microcentrifuge tube and were frozen in liquid nitrogen. Samples were thawed with 500 μ L of PBS including proteinase inhibitor. Afterward, the samples were homogenized with a sonifier 3 \times 20 s on ice. Protein contents in the homogenate were assessed according to the Pierce[™] BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Bovine serum albumin was used as a standard. Finally, fluorescence was measured in a black 96-well plate by adding 1 mM ThT (final concentration 20 mM). The volume in each well was 100 μ L by adding M9. To determine the fluorescence of ThT, samples were measured by excitation at 440 nm and emission at 482 nm.

4.19. Statistics

Unless otherwise stated, values are presented as mean \pm standard error of the mean (SEM). Statistical analyses were performed by applying one-way ANOVA with Tukey’s multiple comparison post-hoc test, log-rank (Mantel-Cox) test and student’s unpaired *t*-test (Prism 9.1 GraphPad Software, San Diego, CA, USA). Statistical significance was defined for *p* values ns = not significant, * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001 and **** *p* < 0.0001.

5. Conclusions

In the present study, we reported that different combinations of folic acid, magnesium orotate, and vitamin B6 had significant effects on glycolysis, A β production, and A β aggregation in SH-SY5Y-APP₆₉₅ cells and *C. elegans*. The phenotype of the in vivo model was significantly improved, highlighting the potential of the tested biofactor combinations as candidate therapeutics in AD. However, since the data did not consistently show a benefit of either combination, this study does not allow a clear statement as to whether vitamin B6 is required in addition to the combination of folic acid and magnesium orotate.

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