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Beneficial effects of primidone in Niemann-Pick disease type C (NPC)-model cells and mice: Reduction of unesterified cholesterol levels in cells and extension of lifespan in mice

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ABSTRACT

Niemann-Pick disease type C (NPC) is caused by a loss of function of either NPC1 or NPC2 protein, resulting in the accumulation of unesterified, free-cholesterol (free-C) in cells/tissues and thus leading to cell/tissue damage. In the brain of patients/animals with NPC, as a consequence of the accumulation of free-C in late endosomes/lysosomes (LE/LY) in cells, multiple lipids including complex sphingolipids are accumulated, and almost all patients/animals ultimately develop progressive/fatal neurodegeneration. Several reagents that are considered to act in the brain show beneficial effects on NPC-model animals. In the present study, we investigated the effects of antiepileptic drugs, such as primidone and valproic acid, on the accumulation of free-C in NPC1-null CHO cells and NPC1* fibroblasts, human fibroblasts established from a patient with *NPC1* mutation. Like valproic acid, treatment with primidone reduced free-C levels in LE/LY in NPC1-null/mutant cells. Down-regulation of cholesterol ester levels in NPC1-null cells and up-regulation of HMG-CoA reductase and low-density lipoprotein receptor mRNA levels in NPC1* cells were partially recovered by primidone treatment. Thus, primidone was suggested to enhance free-C trafficking from LE/LY to endoplasmic reticulum in NPC1-null/mutant cells. In NPC1-null mice, oral application of primidone (100 mg/kg/day) extended lifespan by approximately 5 days, although the first days showing ataxia, a typical symptom of neuromotor dysfunction, were not affected. Our findings suggest the potential of primidone for the treatment of NPC.

1. Introduction

Cholesterol supply is essential for the homeostasis of various tissues including the viscera and the brain. Cholesterol is mainly synthesized in the liver and transported to other tissues as a component of low-density lipoprotein (LDL), in which cholesterol exists as an esterified form, cholesterol ester (CE). LDL is taken up via the LDL receptor, and then LDL containing CE is transported into late endosomes/lysosomes (LE/LY) in cells. CE is cleaved to unesterified free cholesterol (free-C) in the LE/LY, and free-C is transported out of LE/LY to other compartments/organelles including the plasma membrane (PM). Niemann-Pick disease type C (NPC) is a rare neurovisceral endosomal/lysosomal lipid storage disorder, and approximately 95% of NPC patients have mutations in the *NPC1* gene, while 5% of patients have a defect in the *NPC2* gene (Loftus et al., 1997; Vanier, 2010). A loss of function of either NPC1 and NPC2 proteins results in the progressive accumulation of free-C specifically in the LE/LY.

Many NPC patients will ultimately develop progressive and fatal neurological disease due to damages/dysfunctions of neurons in the brain (Vanier, 2010; Vance, 2012; Pineda et al., 2018). Actually, increases in total cholesterol (free-C and CE) levels occur to a lesser extent in the brain than that in peripheral tissues in NPC patients/animals (Dietschy and Turley, 2004; Rosenbaum and Maxfield, 2011; Vance, 2012). Thus, some of the neurological deficits in NPC might be due to a deficiency of cholesterol trafficking to axons, synaptic sites, and/or PM, rather than an excess of free-C in LE/LY in neurons. Another hypothesis was reported for neurodegeneration in NPC (Smith et al., 2009; Platt et al., 2016): multiple lipids including sphingosine and complex sphingolipids are accumulated in LE/LY as a consequence of abnormal trafficking of cholesterol in cells, and these lipids cause neuroinflammation and neuronal damage/dysfunction in the brain of NPC patients/animals. Miglustat (Zavesca^{TM}), which decreases the abnormally increased glycosphingolipids in neurons, is approved for NPC treatment in many countries (Zervas et al., 2001; Pineda et al., 2018), although miglustat

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showed almost no effect on cholesterol levels in the brain. Thus, additional development of the reagents that modulate cholesterol trafficking in the brain of NPC patients is required. Valproic acid, an antiepileptic drug, corrected the abnormal cholesterol trafficking and accumulation in cells with NPC mutations (Kim et al., 2007; Subramanian et al., 2020). In their studies, valproic acid is considered to modulate lipid metabolism via the inhibition of histone deacetylase. Treatment with carbamazepine, another antiepileptic drug, corrected cholesterol accumulation in neuronal cells derived from NPC patient-specific induced pluripotent stem cells by restoring impaired autophagy (Maetzel et al., 2014). Phenobarbital, which is an active metabolite of primidone, reduced neurotoxicity and showed behavioral neuroprotection against kainate-induced toxicity (Calabresi et al., 2003; Caccamo et al., 2016). These reports suggest that antiepileptic drugs, which are centrally acting reagents, prevent the progression of several neurological diseases including NPC. In order to find drugs modifying cholesterol trafficking/accumulation, firstly we have screened several antiepileptic drugs in cells, NPC1-null CHO cells and NPC1* cells (an established cell line from the skin of a patient with a mutation in the *NPC1* gene). We found the beneficial effects of primidone on cellular levels, thus, pharmacological effects of primidone on body weight, neuromotor dysfunction ataxia, and lifespan were examined in NPC1-null mice.

2. Materials and Methods

2.1. Reagents

The following antiepileptic drugs and reagents were used: primidone, valproic acid sodium, diazepam, carbamazepine, phenobarbital sodium, phenytoin, and mefenamic acid (Wako, Osaka, Japan); acetazolamide (Alfa Aesar-ThermoFisher, Waltham, MA, USA); phenylethylmalonamide (2-ethyl-2-phenylmalonamide, Sigma-Aldrich, St. Louis, MO); levetiracetam (Combi-Blocks, San Diego, CA); ononetin (Tocris, Minneapolis, MN). We used the antiepileptic drugs at 1 mM or lower concentrations, which are much greater than the concentrations causing pharmacological responses in their established target proteins/enzymes, to monitor their effects on cellular cholesterol levels. In several exploratory studies, the effects of antiepileptic drugs were examined using mM order concentrations in an enzyme in serum (Beydemir and Demir, 2017) and in cells (Kobayashi et al., 2009; Lee et al., 2016), and primidone at 0.2 mM did not show cytotoxicity in human glial cells (Lee et al., 2016). In the cases of primidone and valproic acid, details regarding their concentrations *in vitro* and therapeutic doses *in vivo* are described in the text. Fetal bovine serum (FBS, serum) was from Gibco-ThermoFisher. Lipoprotein-deficient serum (LPDS, fetal bovine) was from Sigma. LDL (human) was from Alfa Aesar-ThermoFisher.

2.2. Cells

Parental JP17 and NPC1-null A101 CHO cells were kind gifts from Dr. Haruaki Ninomiya (Tottori University, Japan, Higaki et al., 2001). The CHO cells were maintained in Ham's F-12 medium supplemented with 10% serum as described previously (Hashimoto et al., 2016; Wanikawa et al., 2020). Untransformed skin fibroblasts from a patient with NPC mutation (GM03123 cells, NPC1* cells) and fibroblasts from a healthy control (GM00038 cells, wild-type cells) were purchased from Coriell Institute (Camden, NJ, USA). The donor subject (9-year-old female) of GM03123 cells is a compound heterozygote; one allele carries a missense mutation resulting in a substitution of a serine for a proline at codon 237 (P237S) and the second allele carries a missense mutation resulting in a substitution of a threonine for an isoleucine at codon 1061 (I1061T). The donor subject of GM00038 is an apparently healthy 9-year-old female. The fibroblasts were maintained in MEM with 15% serum and antibiotics. Fibroblasts express a variety of signaling proteins including ion channels and receptors. Also, CHO cells endogenously express various ion channels including melastatin-related transient receptor potential (TRPM) channel (Yarishkin et al., 2008; Leitner et al., 2016) and cation channels (Lalik et al., 1993; Michel et al., 1998). Thus, we used the two cell lines, not neuronal cells, for screening the effects of antiepileptic dugs on NPC1-null/mutant cells.

2.3. Detection of accumulation of free-C and assays of free-C and CE levels in cells

Filipin III (from *Streptomyces filipinensis*, Sigma) is a fluorescent compound that specifically binds to free-C. Accumulation of free-C in cellular compartments was analyzed by filipin staining, as previously reported (Kim et al., 2007; Hashimoto et al., 2016; Wanikawa et al., 2020). Briefly, cells seeded on glass-bottomed dishes after fixation with paraformaldehyde were treated with 100 μg/ml filipin. Fluorescence images of free-C/filipin were taken with a LAM780 confocal laser scanning microscope (Zeiss, Jena, Germany). In an experiment, the views per well were selected blindly and the mean intensity of fluorescence (approximately 20 cells per a view, 2–3 different views) in cells was calculated. The mean intensity of fluorescence of free-C/filipin in cells fluctuated, the values were 3,000–9,000, with each experiment, thus the values with tested reagents were expressed as relative values with the control value in each time taken as 1. Levels of free-C and CE were examined using two assays. In the thin layer chromatography (TLC) assay, free-C and CE in the lipids extracted were analyzed on a TLC plate as described (Wanikawa et al., 2020). After air-drying of the plates, the samples were made visible by spraying the TLC plates with 47% aqueous sulfuric acid and charring on a hot plate. The intensity of staining was measured using LAS1000 (Fuji-Film, Tokyo). Because of fluctuated values by staining with sulfuric acid, the data were expressed as relative values with the control value in each time taken as 1. In order to measure absolute free-C levels, the lipids extracted were analyzed with the Amplex® Red cholesterol assay kit (Invitrogen-ThermoFisher) in some cases.

2.4. Acyl-CoA cholesterol acyltransferase (ACAT) activity

ACAT protein is located mainly in the endoplasmic reticulum (ER), and plays important roles in cellular cholesterol homeostasis. For the activity of the ACAT assay, cells cultured on 100-mm dishes were scraped and homogenized with a homogenizer in 300 μl of buffer A (20 mM Tris-HCl, pH 7.7, 1 mM EDTA, and proteases inhibitors). The lysates were centrifuged at 17,400 x g for 30 min, and equal amounts of protein from the soluble fractions were prepared. The samples (250 μl) were mixted with 250 μl of buffer B (buffer A supplemented with 250 μM [^{14}C]oleoyl CoA (PerkinElmer, Boston, MA), 4 mg/ml albumin, 1 mM dithiothreitol) for 1 h at 37 °C, as previously described (Wanikawa et al., 2020). The lipids extracted were analyzed by the TLC assay. The ACAT activity in the fractions of the control cells was 7.50 pmol/300 μg protein/1 h in a typical experiment and the radioactivity in CE formed was less than 0.01% of substrate [^{14}C]oleoyl CoA. The activity changed between 1-3 times dependent on each experiment, thus quantitative data from repeated experiments were expressed as relative values with the control in each time taken as 1.

2.5. Quantitative real-time PCR

Total RNAs in human NPC1* and wild-type cells were isolated with the ISOGEN-II (Nippon Gene, Tokyo, Japan). cDNAs were prepared using the ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Tokyo). We used the following primers for PCR: 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase), (sense) 5′-GGACCCCTTTGCTTAGATGAAA-3′ and (antisense) 5′-CCACCAAGACCTATTGCTCTG-3'; LDL receptor, (sense) 5′-AGTTGGCTGCGTTAATGTGAC-3′ and (antisense) 5′-TGATGGGTTCATCTGACCAGT-3'; liver X receptor α (LXRα), (sense) 5′-ACGGTGATGCTTCTGGAGAC-3′ and (antisense) 5′-AGCAATGAGCAAGGCAAACT-3'; and β-actin, (sense)

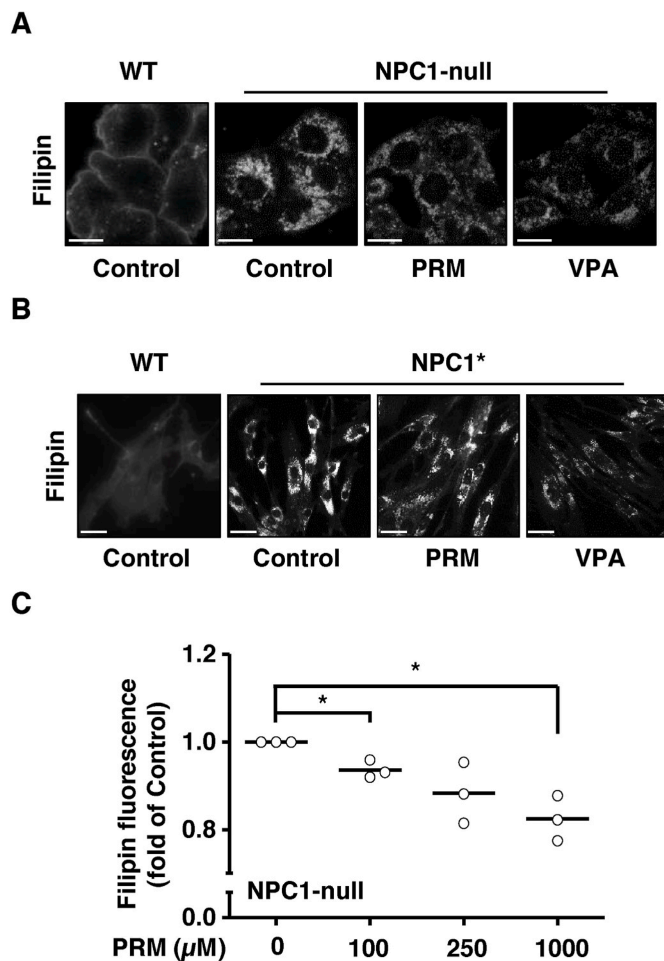

Fig. 1. Effects of primidone and valproic acid on filipin-sensitive free-C levels in NPC1-null and NPC1* cells. CHO cells lacking NPC1 (NPC1-null cells), fibroblasts established from a patient with NPC1 mutation (NPC1* cells), and their control wild-type cells (WT) were cultured with vehicle, 1 mM primidone (PRM), and 1 mM valproic acid (VPA) for 24 h in the presence of 10% serum. Then, the cells were subjected to second culture in the fresh medium containing the same indicated reagents with serum for 24 h. The accumulation of free-C was analyzed by filipin staining. Typical images of filipin fluorescence in NPC1-null and NPC1* cells are shown in A and B, respectively, and those in wild-type cells are shown in the left panels. The concentration-dependent effects of primidone in NPC1-null cells are shown in C. The intensity of filipin fluorescence in cells fluctuated with each experiment, thus the data were expressed as fold changes from the value in NPC1-null cells with vehicle in each time taken as 1. Individual data points (n = 3) and means were plotted. * $P < 0.05$, significantly different from the value without primidone. Scale bar, 5 μm in A and 20 μm in B.

5′-AGCGAGCATCCCCCAAAGTT-3′ and (antisense) 5′-GGGCACGAAGGCTCATCATT-3’. qPCR was performed using the PowerUp™ SYBR® Green Master Mix (Applied Biosystems, ThermoFisher). Reactions were run with the Eco-Real-Time PCR System (Illumina, San Diego, CA).

2.6. Assay of BODIPY-labeled LDL uptake in intact cells

Cells seeded on glass-bottomed dishes were incubated in the medium containing human plasma LDL-BODIPY™ FL complex (BODIPY-LDL, 5 μg/ml, #L3483, Invitrogen-ThermoFisher) and 0.1% albumin for 20 min at 37 °C. After washing, the cells were fixed with 4% paraformaldehyde and the uptake was analyzed using a LSM780 confocal microscope. For quantitative analyses, the mean intensity of fluorescence in cells was calculated. Data are expressed as fold changes from the control, the value in wild-type cells without reagents.

Table 1
Effects of antiepileptic drugs on filipin-sensitive free-C levels in NPC-model cells.

Drugs	NPC1-null cells	NPC1* cells
Free-C levels (fold of control)		
Vehicle	1	1
AZ (1 mM)	0.91 ± 0.04	1.02 ± 0.09
CBZ (30 μM)	0.92 ± 0.05	0.92 ± 0.07
DZP (100 μM)	1.07 ± 0.04	1.01 ± 0.08
LEV (1 mM)	0.95 ± 0.03	1.00 ± 0.03
PB (1 mM)	0.96 ± 0.03	0.96 ± 0.06
PHT (200 μM)	1.11 ± 0.09	0.98 ± 0.09
PRM (1 mM)	0.79 ± 0.03[a]	0.88 ± 0.04[a]
VPA (1 mM)	0.69 ± 0.06[a]	0.70 ± 0.12[a]
PEMA (1 mM)	1.01 ± 0.03	ND

NPC1-null and NPC1* cells were cultured with vehicle, acetazolamide (AZ), carbamazepine (CBZ), diazepam (DZP), levetiracetam (LEV), phenobarbital (PB), phenytoin (PHT), primidone (PRM), valproic acid (VPA), and phenylethylmalonamide (PEMA) at the indicated concentrations for 24 h in the presence of 10% serum. The cells were subjected to second culture in the fresh medium containing the same indicated reagents with serum for 24 h. The accumulation of free-C was analyzed by filipin staining, and the mean intensity of fluorescence in cells was measured. The quantitative data were expressed as relative values with the values in NPC1-null/mutant cells with vehicle in each time taken as 1. Data are the means ± S.D. of 3–4 independent experiments. [a]$P < 0.05$, significantly different from the value with vehicle. Typical images of filipin fluorescence in cells cultured with primidone and valproic acid are shown in Fig. 1.

2.7. Animals and oral administration of primidone

BALB/cNctr-Npc^{m1N}/J heterozygote mice, kindly gifted from Prof. Makoto Michikawa (Nagoya City University, School of Medicine, Nagoya, Japan), were bred to generate wild-type and homozygous affected NPC1-null mice, and genotyped. Mice were cared for according to the animal care guidelines of Chiba University. Experiments were performed according to an animal protocol approved by the Animal Welfare Committee of Chiba University (Number: 30–44). Primidone (fine grain, Nichi-Iko Pharmaceutical Co., Toyama, Japan) in a stock water solution was diluted with water containing 1% carboxymethyl cellulose sodium salt (CMC, Wako) to 20 mg/ml. Young mice wean at 3–4 weeks of age, thus, oral application of primidone to mice was started from 25 days after birth. The primidone/CMC solution was diluted two times with water, and mice were treated every day to the final stage; the dose of primidone was 100 mg/kg/day and the final concentration of CMC was 0.5%. The number of animals in each group is as follows: control, n = 13 (male 7, female 6); primidone-treated, n = 16 (male 8, female 8). We measured/observed body weight and motor activity every day, and the presence of 5-times or greater motor dysfunction of the legs, a wobbly and/or lurching gait, in a period of 30 s per trial/day was judged as an index of ataxia in NPC. The initial day when mice showed the phenomenon for 2 consecutive days was defined as the appearance of cerebellar ataxia.

2.8. Statistical analysis

Survival curves (Kaplan-Meier plots) were analyzed using the Log-rank test. Other results are expressed as dot-plot or the mean ± S.D. for the indicated number (n) of independent experiments. Each experiment was conducted using different cells/animals and at different times. An ANOVA with Bonferroni *post-hoc* test and Student’s two-tailed t-test were used for multiple and pair-wise comparisons, respectively. *$P < 0.05$ shows a significant difference compared with the control values without reagents, such as primidone and valproic acid, in wild-type or NPC1-null cells. In some cases, *$P < 0.05$ shows a significant difference induced by NPC1 knockout. #$P < 0.05$ shows a significant difference

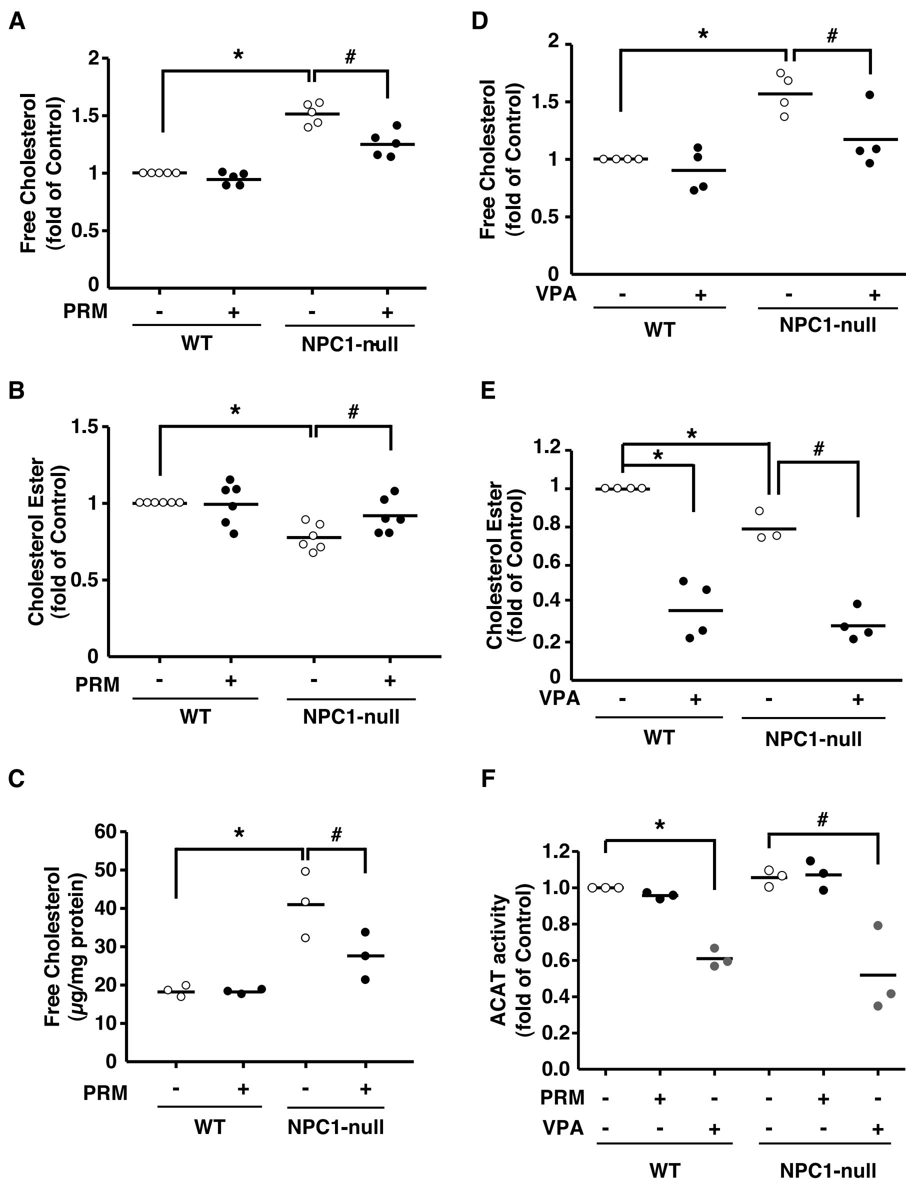

Fig. 2. Effects of primidone and valproic acid on levels of free-C and CE in NPC1-null cells. The wild-type CHO cells (WT) and NPC1-null cells were cultured with vehicle, 1 mM primidone (PRM, A-C and F), and 1 mM valproic acid (VPA, D-F) for 48 h (24-h cultivation, 2 times) in the presence of serum. Free-C (A and D) and CE (B and E) in extracted lipids were separated with the TLC method. The intensities of free-C and CE are expressed as fold changes from those in wild-type cells with vehicle. In C, absolute amounts of free-C (μg/mg protein) in cells treated with primidone were analyzed by the Amplex kit. In F, the activity of ACAT in the homogenate fractions prepared from cells treated with primidone and valproic acid was measured. Individual data points (n = 3–6) and means were plotted. The dyeing intensity of free-C/CE in a TLC plate (A, B, D, and E) and the ACAT activity (F) fluctuated with each experiment, as described in Methods, thus, the data were expressed as relative values with the value in wild-type cells with vehicle in each time taken as 1. * $P < 0.05$, significantly different from the control, wild-type cells with vehicle. # $P < 0.05$, significantly different from the values in NPC1-null cells with vehicle.

compared with the values without reagents in NPC1-null cells.

3. Results

3.1. Effects of various antiepileptic drugs on accumulation of free-C in NPC-model cells

First, accumulation of free-C in cells was detected by filipin staining. Fluorescence derived from filipin was markedly detected as vesicular specks in CHO cells lacking NPC1 protein (NPC1-null cells, Fig. 1A) and in an established human cell line, skin fibroblasts from an NPC patient with *NPC1* mutation (NPC1* cells, Fig. 1B). Treatment with 1 mM primidone (two-times every 24 h, total treatment for 48 h) markedly decreased accumulation of free-C in both NPC1-null and NPC1* cells. Quantitative data for 1 mM primidone in the two cell types and those for its concentration-dependency in NPC1-null cells are shown in Table 1 and Fig. 1C, respectively. Primidone is metabolized in cells, specifically in hepatocytes, to phenobarbital and phenylethylmalonamide. Neither treatment with phenobarbital nor phenylethylmalonamide at 1 mM (Table 1) or 200 μM (data not shown) changed free-C levels in NPC1-null cells. It was reported that primidone acted as an inhibitor of the TRPM3 channel and resulting attenuated nociception (Krügel et al., 2017). Treatment of NPC1-null cells with 50 μM mefenamic acid and 5 μM ononetin, which are inhibitors of TRPM3 (Straub et al., 2013; Thiel et al., 2017), did not affect free-C levels: the values were 1.08 ± 0.03 and 1.04 ± 0.07 (fold of control, n = 3), respectively. These results showed that treatment with primidone reduced free-C levels in not only NPC1* cells but also NPC1-null cells, although concentrations over 100 μM were required for the cellular effects.

Primidone has been established as an inhibitor of $GABA_A$ receptors/Cl^- channels, and also inhibits several Na^+ and Ca^{2+} channels. As described in Section 2.2., CHO cells, in addition to fibroblasts, have been shown to endogenously express several types of ion channels. Treatments with phenobarbital and carbamazepine, modulators of $GABA_A$ receptors, did not reduce free-C levels in NPC1-null/mutant cells. Treatments with carbamazepine and phenytoin, direct inhibitors of Na^+ channels, and acetazolamide, which causes acidosis and inhibits seizure via acid-sensing Na^+ channel, were also ineffective. These results suggest that primidone modulates cholesterol trafficking in cells via as yet unidentified mechanisms. Kim et al. (2007) reported that treatment with valproic acid at 0.5 and 1 mM decreased cholesterol accumulation in neural stem cells from NPC1-null mice. In the present study, treatment

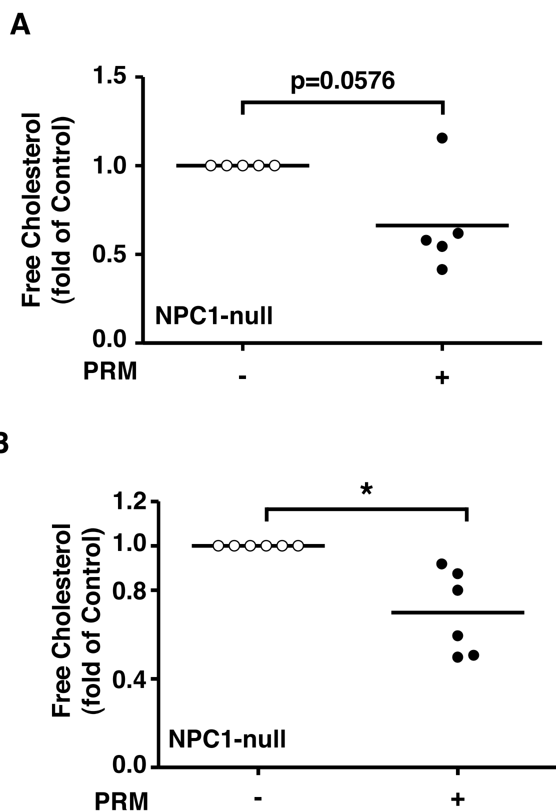

Fig. 3. Primidone-induced decrease in free-C levels in NPC1-null cells in the absence of LDL. NPC1-null cells were cultured with 1 mM primidone (PRM) for 48 h (24-h cultivation, 2 times) in medium containing 10% LPDS. Free-C levels were measured by the TLC method (A) and the Amplex kit (B). In A and B, the data were expressed as fold changes from the value in NPC1-null cells with vehicle. The absolute values of free-C levels examined with the Amplex kit (B) were shown in Supplementary Fig. 1. Individual data points (n = 5–6) and means were plotted. Student's two-tailed *t*-test was used for pair-wise comparison. * $P < 0.05$, significantly different from the control without primidone.

with 1 mM valproic acid reduced accumulation of free-C in NPC1-null and NPC1* cells.

3.2. Effects of primidone and valproic acid on levels of free-C and CE in NPC1-null cells

Levels of free-C and CE in wild-type and NPC1-null cells were analyzed by a TLC method. The levels of free-C (Fig. 2A) and CE (Fig. 2B) in NPC1-null cells increased and decreased, respectively, compared with those in wild-type cells. Treatment with 1 mM primidone significantly reversed the responses in NPC1-null cells. The primidone-induced reduction of free-C levels in NPC1-null cells was confirmed by measurement using a quantitative assay kit (Fig. 2C). Treatment with primidone did not affect the levels of free-C or CE in wild-type cells. Treatment with 1 mM valproic acid also reduced free-C levels in NPC1-null cells, but not in wild-type cells (Fig. 2D). Interestingly, valproic acid markedly reduced CE levels in both wild-type and NPC1-null cells (Fig. 2E). Free-C in LE/LY is transported to ER, and this is converted to CE by ACAT within it. Thus, we examined the effects of primidone and valproic acid on ACAT activity in cell homogenates prepared from wild-type and NPC1-null cells (Fig. 2F). ACAT activity in NPC1-null cells was almost the same as that in wild-type cells, and treatment with primidone did not affect the activity. The results suggest that primidone treatment up-regulated CE formation without changing ACAT activity in NPC1-null cells. Treatment with valproic acid reduced the ACAT activity to half in both cell types.

3.3. Effects of primidone on free-C levels in NPC1-null cells without LDL and on cellular uptake of BODIPY-LDL

LDL containing CE in serum was taken up by LDL-receptor-mediated endocytosis into cells. Next, we investigated the effect of primidone on free-C levels in NPC1-null cells in the medium containing LPDS, not normal serum (Fig. 3). Treatment with 1 mM primidone for 48 h reduced free-C levels, estimated in the TLC analysis (Fig. 3A) and the quantitative assay kit (Fig. 3B), in NPC1-null cells, although the values varied depending on experiments (Suppl. Fig. 1). In NPC1-null cells, uptake of BODIPY-LDL measured for 20 min was significantly greater than that in wild-type cells (Fig. 4). Treatment with 1 mM primidone slightly, not significantly, reduced uptake of BODIPY-LDL in NPC1-null cells, but did not change it in wild-type cells. Thus, primidone treatment is considered to reduce free-C levels in an extracellular LDL-independent manner. In contrast, treatment with 1 mM valproic acid markedly and significantly reduced BODIPY-LDL uptake in both wild-type and NPC1-null cells.

3.4. Regulation of cholesterol-related genes in NPC1* cells by primidone treatment

The decrease and/or depletion of cholesterol in ER has been shown to cause the trafficking of sterol regulatory element binding proteins (SREBPs) from ER to the Golgi complex, and mature SREBPs formed after proteolysis of the proteins regulate gene expression in the nucleus (Goldstein et al., 2006). Nuclear accumulation of SREBP-1 and up-regulations of cellular cholesterol synthesis and LDL uptake were reported in CHO cells lacking NPC1 (Higaki et al., 2001). In order to monitor the free-C trafficking from LE/LY to ER in cells, levels of HMG-CoA reductase and LDL receptor mRNAs, which are regulated by SREBPs, were measured in wild-type fibroblasts (GM00038) and NPC1* cells (GM03123). In NPC1* cells cultured with serum, the mRNA levels of HMG-CoA reductase and LDL receptor were significantly up-regulated compared with those in wild-type cells (Table 2). Interestingly, treatment with 1 mM primidone significantly reduced mRNA levels of HMG-CoA reductase and LDL receptor in NPC1* cells, but not in wild-type cells. The feedback-regulation by SREBPs in response to LDL was reported in wild-type cells (Higaki et al., 2001; Goldstein et al., 2006): cultivation with LPDS activated many pathways to increase cholesterol levels and the responses were canceled by re-addition of medium containing LDL. As shown in CHO cells with and without NPC1 mutation (Higaki et al., 2001), the feedback-regulation in response to LDL was active in wild-type fibroblasts, but not NPC1* cells (data not shown). These results suggest that free-C was not trafficked to ER in NPC1* cells and that primidone treatment improved the free-C trafficking from LE/LY to ER.

LXRs are sterol-activated transcription factors that play major roles in cellular homeostasis of lipids including cholesterol (Zhao and Dahlman-Wright, 2010). The levels of LXRα mRNA in NPC1* cells were lower compared with those in wild-type cells. Treatment with primidone, which did not change the levels in wild-type cells, up-regulated this gene in NPC1* cells. On the other hand, treatment with valproic acid showed a complicated response: the reduction of LXRα mRNA, but not HMG-CoA reductase and LDL receptor mRNAs, in wild-type cells, and reduction of HMG-CoA reductase and LDL receptor mRNAs, but not LXRα mRNA, in NPC1* cells (Table 2).

3.5. Effects of oral administration of primidone on body weight, onset of ataxia, and lifespan in NPC1-null mice

As shown in Fig. 1C, treatment with 0.1 mM primidone slightly but significantly reduced free-C levels in NPC1-null cells. The concentration of primidone was 2-fold greater than the maximal therapeutic plasma concentration, 12 μg/ml (0.05 mM, Himmerich et al., 2013). As a pilot study *in vivo*, we examined the effects of primidone (100 mg/kg/day), that was approximately 2-fold greater than the recommended maximal

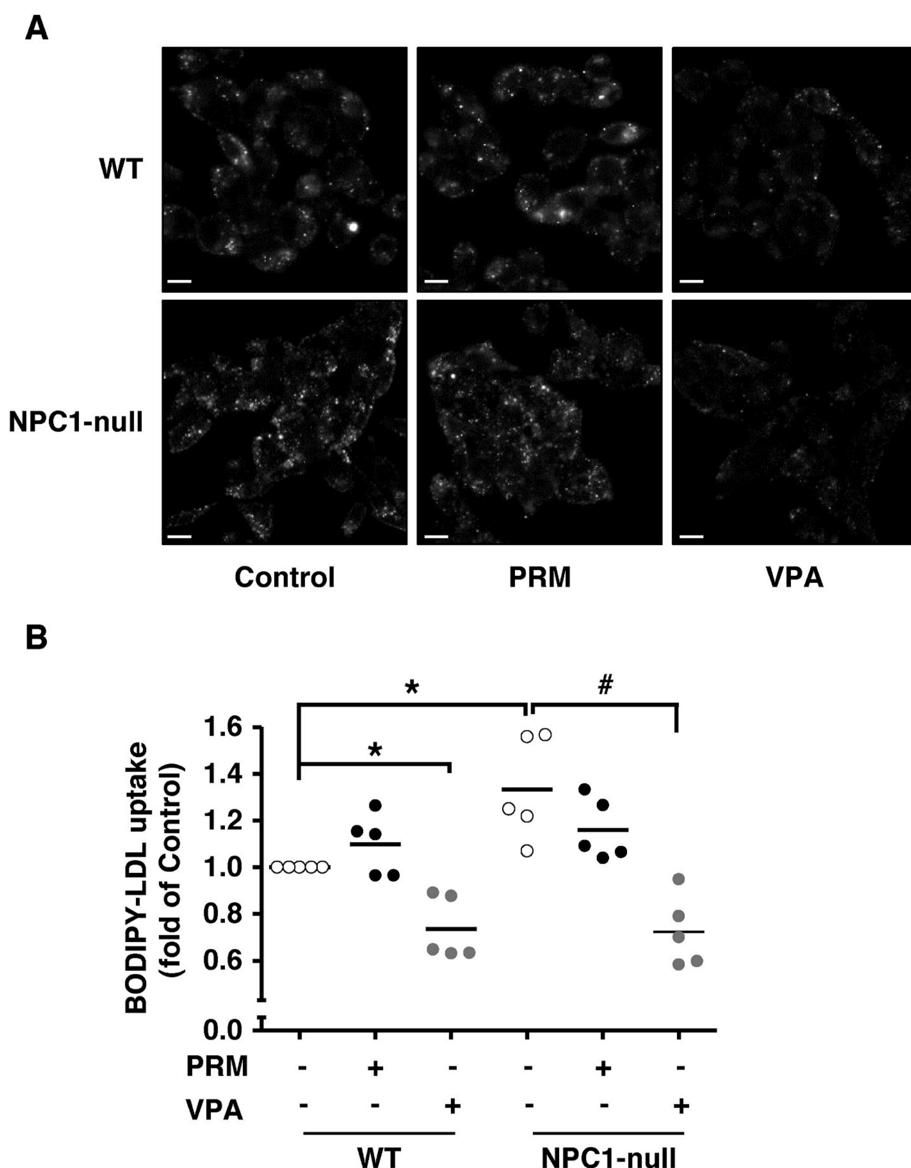

Fig. 4. Effects of primidone and valproic acid on cellular uptake of BODIPY-LDL. The wild-type CHO cells (WT) and NPC1-null cells were cultured with vehicle, 1 mM primidone (PRM), and 1 mM valproic acid (VPA) for 48 h (24-h cultivation, 2 times) in the presence of serum. Then, the cells were incubated with 5 μg/ml of BODIPY-LDL for 20 min. After washing and fixation of cells, the fluorescence of BODIPY-LDL taken up by each cell was measured as described in Materials and Methods. Typical images and quantitative data are shown in A and B, respectively. The quantitative data of the intensity of BODIPY-LDL fluorescence were expressed as relative values with the value in wild-type cells with vehicle. Quantitative data are expressed as the relative values of wild-type cells with vehicle. Individual data points (n = 5) and means were plotted. * $P < 0.05$, significantly different from the control, wild-type cells with vehicle. # $P < 0.05$, significantly different from the value in the NPC1-null cells with vehicle. Scale bar, 5 μm.

therapeutic dose in human adults, 2.0 g/day (40–50 mg/kg/day), on NPC1-null mice. Solutions of primidone and vehicle were orally administered every day to NPC1-null mice from 25 days old. Treatment with primidone at the dose for 70 days did not show toxic/adverse effects such as poor weight gain and abnormal behaviors in wild-type mice (data not shown). Treatment with primidone slightly but significantly extended the lifespan of NPC1-null mice, by approximately 5 days (Fig. 5A and B). The same tendency was observed in both male and female mice (Suppl. Fig. 2A and 2B), although there was no significant difference due to the small number of mice (P values were 0.075 and 0.055 in male and female mice, respectively). In NPC1-null mice, the rapid loss of body weight at 7–8 weeks old is concurrent with the visible onset of ataxia and/or intentional tremor (Loftus et al., 2002; Nietupski et al., 2012; Williams et al., 2014). Under our conditions, the first day showing ataxia was approximately 60 days old in both NPC1-null mice with and without primidone treatment (Fig. 5C). The maintenance of body weight was another indicator of therapeutic efficacy in NPC1-null mice (Smith et al., 2009; Davidson et al., 2009; Williams et al., 2014). In male NPC1-null mice, the body weight increased up until approximately 50 days old and then rapidly decreased, and treatment with primidone slowed both the increase and decrease of body weight (Suppl. Fig. 2C). In female NPC1-null mice, treatment with primidone slowed the decrease of body weight from approximately 48 days old without changing the increase of body weight (Suppl. Fig. 2D). In NPC1-null mice (male and female), the peak day reaching the maximum body weight was significantly delayed by primidone treatment (Fig. 5D). At 63 days old (9 weeks old), during the period of body-weight reduction, the mean body weight of male NPC1-null mice with primidone was significantly greater than that without the reagent (Fig. 5E and Suppl. Fig. 2C). A similar maintenance of body weight was observed in females with primidone (Suppl. Fig. 2D). These results showed that oral administration of primidone from 25 days old delayed the loss of body weight observed from approximately 50 days old and extended the lifespan, although the onset of ataxia was not affected.

4. Discussion

In order to find drugs modifying cholesterol trafficking/accumulation in the brain, we have screened several antiepileptic drugs in NPC-model cells. In this study, we showed that: i) primidone treatment reduced intracellular free-C levels in both NPC1-null and NPC1* cells, and ii) primidone treatment appeared to enhance the free-C trafficking from LE/LY to ER in NPC cells. In addition, we revealed that oral administration of primidone extended the lifespan and delayed the loss

Table 2

Effects of primidone and valproic acid on mRNA levels of HMG-CoA reductase, LDL receptor, and LXRα in NPC1* cells.

Drugs	Wild-type cells	NPC1* cells
Relative mRNA levels (fold of control)		
HMG-CoA reductase		
Vehicle (4)	1	1.69 ± 0.09[a]
PRM (4)	1.09 ± 0.11	1.24 ± 0.10[b]
VPA (3 or 4)	0.95 ± 0.11	0.83 ± 0.12[b]
LDL receptor		
Vehicle (4)	1	2.81 ± 0.11[a]
PRM (3 or 4)	1.27 ± 0.20	1.75 ± 0.22[a,b]
VPA (4)	0.98 ± 0.04	1.03 ± 0.26[b]
LXRα		
Vehicle (4 or 5)	1	0.63 ± 0.10[a]
PRM (5)	0.91 ± 0.09	1.21 ± 0.12[b]
VPA (5)	0.50 ± 0.09[a]	0.64 ± 0.18

NPC1* and wild-type cells were cultured in 6-well plates with vehicle, 1 mM primidone (PRM), and 1 mM valproic acid (VPA) for 24 h in the presence of 10% serum. The cells were subjected to second culture in the fresh medium containing the same indicated reagents with serum for 24 h. Total RNA was isolated by Isogen-II, and quantitative real-time PCR was performed as described in Materials and Methods. The numbers of independent samples are shown in parentheses. The quantitative data were expressed as relative values with the values in wild-type cells with vehicle. Data are expressed as fold changes from the control, wild-type cells with vehicle. [a] $P < 0.05$, significantly different from the control. [b] $P < 0.05$, significantly different from the respective values in NPC1* cells with vehicle.

of body weight in NPC1-null mice.

4.1. Cellular mechanisms for primidone-induced down-regulation of free-C: Comparisons with those for valproic acid

In the present study, we showed that the increased levels of free-C in NPC1-null cells were decreased by primidone treatment. The decreased levels in CE in NPC1-null cells were up-regulated by primidone without changing ACAT activity, which converts free-C to CE in ER in cells. Primidone-induced decrease of free-C levels was significantly observed in NPC1-null cells cultured in medium containing LPDS, and the uptake of BODIPY-LDL was almost not inhibited by primidone. These results suggest that primidone treatment enhances the free-C trafficking from LE/LY to ER, and so reduces free-C levels in NPC cells. This is also supported by the following results. Cholesterol depletion in ER activates the SREBP pathway from ER to Golgi complex and up-regulates expression of HMG-CoA reductase and LDL receptor genes (Higaki et al., 2001; Goldstein et al., 2006). The up-regulated expression of the two genes in NPC1* cells was decreased by primidone treatment. The lack of a change in expression of the genes in primidone-treated wild-type cells might exclude a possibility: the primidone-induced direct inhibition of the SREBP pathway. From these results, we propose that primidone treatment reduced free-C levels by enhancing the free-C trafficking from LE/LY to ER.

Valproic acid acted as an inhibitor of histone deacetylase and modified expression of several genes including HMG-CoA reductase in neuronal cells from NPC1-null mice (Kim et al., 2007). Also, we showed that treatment with valproic acid affected mRNA levels involved in cholesterol metabolism in not only NPC1* cells but also wild-type cells. Treatment with valproic acid showed many cellular effects, such as inhibition of ACAT activity and inhibition of BODIPY-LDL uptake, in wild-type cells. Recently, it is reported that treatment with valproic acid corrected the folding and trafficking defect associated with NPC1* (I1061T-NPC1) via an increase in the acetylation of NPC1* and restored cholesterol homeostasis in cells (Subramanian et al., 2020). The effects of valproic acid on cholesterol metabolism appeared to be complicated, and additional studies are required in the future.

4.2. Possible targets of primidone for reducing free-C levels in NPC cells

The antiepileptic drugs such as phenobarbital and carbamazepine, except for primidone and valproic acid, did not affect cholesterol trafficking in cells (Table 1). Kralic et al. (2005) reported that treatment with primidone, in a Ca^{2+} channel-independent manner, effectively reduced tremor severity in $GABA_A$ receptor-null mice. The precise mechanisms for primidone to enhance free-C trafficking from LE/LY to ER are still unclear. Recent studies showed that LE/LY apposes various cellular organelles, and ER apposes PM and organelles (Luo et al., 2017; Höglinger et al., 2019). Numerous sterol-transfer proteins including NPC1/2 mediate cholesterol transfer at membrane contact sites between closely apposed compartments, suggesting free-C trafficking from LE/LY to the Golgi complex and mitochondria, in addition to ER and PM. Endocytic and/or recycling vesicles with sterol-transfer-related proteins also carry cholesterol to far sites including PM (Luo et al., 2017; Höglinger et al., 2019). In lysosomal storage disorders including NPC, expression of sterol-transfer proteins in cells was changed (Torres et al., 2017). Thus, primidone might affect activities of sterol-transfer proteins. TRP channels may be another target of primidone. TRP channels exist and function in various intracellular membranes/organelles including LE/LY, and cholesterol can directly regulate TRP channels (Zhang et al., 2018; Lee, 2019). Mucolipidosis type IV (ML-IV) is a neurodegenerative lysosomal storage disorder caused by mutations in the gene *MCOLN1* that encodes mucolipin-1, a member of the TRP channel family (Soyombo et al., 2006). In ML-IV* cells, cholesterol levels were 3-fold higher than in wild-type cells (Soyombo et al., 2006). Thus, primidone might regulate cholesterol trafficking via modification of TRP channels except for TPRM3 in NPC1-null cells.

Cholesterol has both hydrophilic and hydrophobic regions, and is thus referred to as an amphipathic molecule. Membrane-interacting amphiphiles have been shown to displace sterols from their complexes with phospholipids/sphingolipids. For example, chlorpromazine, an antipsychotic drug with an amphipathic character, transferred the cholesterol pool in LE/LY to extracellular acceptors including serum; thus, it might be useful in treating NPC (Lange et al., 2012). However, a probable amphipathic character of primidone did not appear to contribute to cholesterol trafficking, since phenobarbital, having a primidone-like chemical structure, had no effect on free-C levels in NPC cells.

4.3. Extended lifespan in primidone-treated NPC1-null mice: Comparisons with other therapeutics/reagents

We showed that a daily oral administration of primidone (100 mg/kg/day) from 25 days old delayed the loss of body weight in the late phase (over 50 days old) and slightly but significantly extended lifespan (by approximately 5 days) in NPC1-null mice. As the treatments for NPC, a therapeutic, miglustat, and several candidates have been reported (Rosenbaum and Maxfield, 2011; Pipalia et al., 2011; Platt et al., 2016; Pineda et al., 2018). Miglustat (Zavesca™, Actelion Pharm. Ltd), an inhibitor of glucosylceramide synthase, is approved in countries including the EU and Japan (Zervas et al., 2001; Pineda et al., 2018). Miglustat taken orally decreases the abnormally increased glycosphingolipids in neurons, maintains the number/functions of neurons including cerebellar Purkinje cells, and markedly extended lifespan (over 30 days) in model animals with NPC (Zervas et al., 2001; Smith et al., 2009; Davidson et al., 2009; Nietupski et al., 2012; Williams et al., 2014). Treatment with miglustat showed almost no effect on cholesterol levels in the brain, but reduced inflammatory responses in the brain of NPC-model animals depending on studies. Several unclear and/or non-beneficial effects of miglustat were reported: alteration in the microglial phenotype(s) in CNS (Stein et al., 2012) and accumulation of macrophages in the liver (Nietupski et al., 2012) in an NPC-model animal, and miglustat treatment up-regulated, not reduced, cholesterol levels in NPC1-null cells (Sillence et al., 2002; Hashimoto et al., 2016).

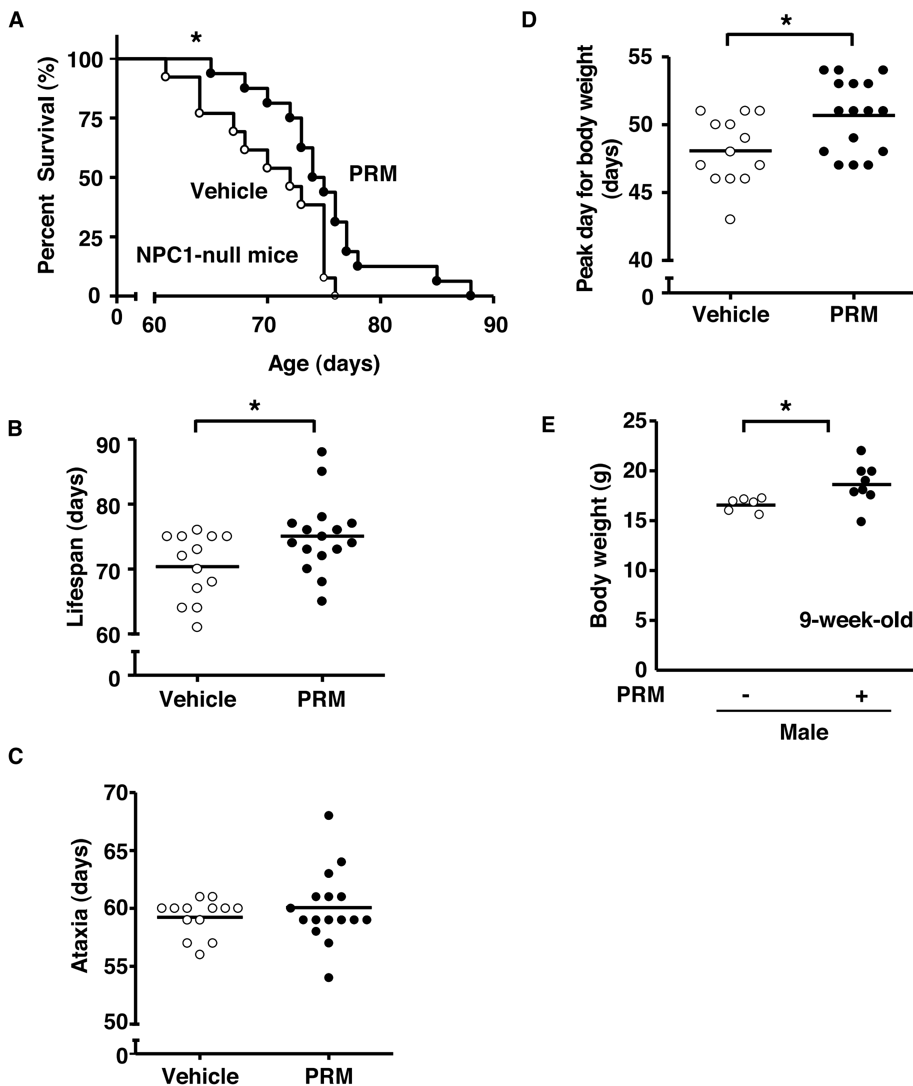

Fig. 5. Effects of oral application of primidone on NPC1-null mice. Primidone (100 mg/kg/day, PRM) in 0.5% CMC solution was orally administrated every day from 25 days old. As the control, 0.5% CMC solution was administrated (Vehicle). Survival curves (males and females) and lifespans of mice are shown in A and B, respectively. In C, the first day that ataxia was observed was examined. In D, the day of reaching maximum body weight was plotted. In E, body weights of 9-week-old male mice with and without primidone application were measured. Individual data and means were plotted. Survival curves (Kaplan-Meier plots) in A were analyzed using the Log-rank test. In B-E, Student's two-tailed *t*-test was used. * $P < 0.05$, significantly different from the control without primidone.

As a fundamental treatment for NPC, administration of cyclodextrins (CDs), sterol-binding agents including 2-hydroxypropyl-β-cyclodextrin, has been proposed. Treatment with CDs corrected cholesterol levels preferentially in peripheral tissues, slowed and/or reduced the magnitude of neurodegeneration in the brain, and extended lifespan of NPC1-null mice (Davidson et al., 2009; Liu et al., 2009; Ramirez et al., 2010; Lopez et al., 2014). Recent studies showed that subcutaneous administration of CDs reduced free-C storage levels in the cerebral cortex in NPC-model mice (Davidson et al., 2016) and cats (Vite et al., 2015). Intracisternal, in addition to intravenous, administration of CD showed neurological benefits in NPC patients (Ory et al., 2017; Hastings et al., 2019). Changes in application methods to other less-invasive ways and in structures of CDs will overcome the low blood-brain barrier permeability of CDs in future. We showed a reduction in free-C levels in primidone-treated NPC1-null and NPC1* cells; thus, the oral administration of primidone may be useful for treatment of NPC. Interestingly, supplementation with aspirin (Smith et al., 2009) or ibuprofen (Williams et al., 2014) has been reported to show beneficial effects on the immunological and neurological changes in the brain of NPC1-null mice. Anti-inflammatory drug treatment by itself showed a marginal effect, but the combination with miglustat further extended lifespan compared with that in the miglustat-treated group. Interestingly, incubation of whole-blood preparations from healthy subjects with antiepileptic drugs including primidone (12 μg/ml) has been shown to modulate productions of cytokines including interleukin-1β and tumor necrosis factor-α (Himmerich et al., 2013). In NPC patients, defective myelination, in addition to neuron loss, occurred in the brain. Statins, including lovastatin, inhibitors of HMG-CoA reductase, increased proliferation and differentiation of oligodendrocyte progenitor cells and resulting remyelination (Paintlia et al., 2010). Yang et al. (2018) reported that treatment with lovastatin decreased free-C accumulation in the LE/LY of NPC1-null oligodendrocytes and accelerated their maturation. The pharmacological effects of primidone on immune-related cells and oligodendrocytes should be studied in NPC-model cells/mice.

4.4. Future perspectives

We showed that primidone treatment reduced free-C levels in NPC cells and propose that primidone improves free-C trafficking from LE/LY to ER. Oral administration of primidone extended the lifespan of NPC-null mice. Recent studies that administration of CDs improved both accumulation of cholesterol/sphingolipids and loss of Purkinje cells in the brain of NPC-model animals (Vite et al., 2015; Davidson et al., 2016). The effects of primidone on these changes in neuronal cells including Purkinje cells of NPC-model mice remained to be solved. Almost 95% of NPC patients carry mutations in the *NPC1* gene, not lacking the gene; thus, a chaperone therapy for and/or an inhibition of degradation of NPC1* protein was reported to potentially ameliorate NPC (Pipalia et al., 2011; Nakasone et al., 2014). The combination of treatments with primidone and other reagents/therapeutics and/or

chaperone therapy may be worthwhile for NPC treatment. Accumulation and/or abnormal trafficking of cholesterol has been reported in the brains of patients with Alzheimer's and Parkinson's diseases (Vance, 2012; Arenas et al., 2017). Trials of primidone for treatment of these neurodegenerative diseases may be of interest.

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CRediT authorship contribution statement

Hitomi Ashikawa: Investigation. **Hinako Mogi:** Investigation. **Takuya Honda:** Visualization. **Hiroyuki Nakamura:** Conceptualization, Methodology, Writing - original draft, Funding acquisition. **Toshihiko Murayama:** Conceptualization, Methodology.

Declaration of competing interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2021.173907.

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