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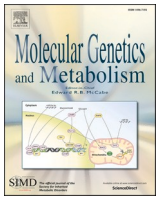

Research Paper

Mechanistic insights into arimoclomol mediated effects on lysosomal function in Niemann-pick type C disease

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ABSTRACT

Niemann-Pick disease type C (NPC) is an ultra-rare, fatal neurodegenerative disease. It is characterized by lysosomal dysfunction with cytotoxic accumulation of unesterified cholesterol and glycosphingolipids in lysosomes, which causes neurodegeneration and peripheral organ dysfunction. Arimoclomol, an orally available small molecule, is the first FDA-approved treatment for NPC when used in combination with miglustat. Here, we present the results of a series of in vitro studies performed to explore the pathways by which arimoclomol targets the fundamentals of NPC etiology. While the precise cellular interactions of arimoclomol remain unclear, the increased translocation of the transcription factors EB and E3 (TFEB and TFE3) from the cytosol to the nucleus is a key initial step for triggering a cascade of downstream events that can rescue cellular functions. Activation of TFEB and TFE3 raises the expression rates of coordinated lysosomal expression and regulation (CLEAR) genes including *NPC1* that are essential for the regulation of lysosomal function. The subsequent upregulation of CLEAR network proteins combined with increased unfolded protein response activation was shown to enlarge the pool of matured NPC1 capable of reaching the lysosome to reduce cholesterol accumulation. By also amplifying expression of CLEAR genes associated with autophagy, arimoclomol has the potential to act on different pathways and improve cell viability independent of NPC1 protein levels and functionality.

In summary, the findings presented illustrate how arimoclomol improves lysosomal function and potentially autophagy flux to decrease lipid burden in NPC patient fibroblasts.

1. Introduction

Lysosomal Storage Diseases (LSDs) are a class of metabolic disorders characterized by a progressive accumulation of undigested macromolecules in the lysosomes, e.g., lipids, glycoproteins, and oligosaccharides. LSDs are caused by genetic mutations in lysosomal enzymes or transport proteins [1]. As a key player in autophagic processes, lysosomes are responsible for the removal of cellular waste products including cytoplasmic organelles, proteins, and other macromolecules. Since efficient autolysosomal function is critical for cellular survival, accumulation of waste materials in the lysosomes impairs important degradation and recycling processes leading to disruption of cellular homeostasis.

Niemann-Pick disease type C (NPC) is an ultra-rare and fatal neurodegenerative LSD in which normal lysosomal function is disrupted by mutations in the *NPC1* (95 % of cases) or *NPC2* genes [2,3]. Both genes encode lysosomal proteins that are essential for intracellular transport and metabolism of lipids. Mutations in either of these genes can result in a reduced amount of properly folded and mature NPC protein, or even a complete lack of NPC protein. The consequence is lysosomal dysfunction leading to accumulation of unesterified cholesterol and glycosphingolipids (GSLs) in lysosomes and late endosomes which is cytotoxic and causes neurodegeneration and peripheral organ impairment [4,5].

The most common mutations (70–80 %) found in the NPC1 protein of NPC patients are missense mutations, which typically result in misfolded protein and premature degradation [6–8]. One example is endoplasmic reticulum (ER) missense mutations (I1061T mutation is the most common) that result in mutant NPC1 proteins which are blocked in the ER

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due to misfolding and early degradation [9]. Functional null mutations caused by frameshift, splicing, or premature stop mutations in the *NPC1* gene are less frequent (20–30 %) [10,11]. Arimoclomol is the first FDA-approved treatment for NPC when used in combination with miglustat [12]. Arimoclomol is an orally available small molecule that has been shown to cross the blood-brain-barrier [13]. Arimoclomol was initially identified as a stress response amplifier that increases the production of heat shock proteins (HSPs) [14,15]. New data have now expanded the understanding of how arimoclomol targets the primary biochemical mechanisms implicated in NPC etiology. While the precise target interactions of arimoclomol remain unknown, the earliest effect observed so far is the increased translocation of the transcription factors EB (TFEB) and E3 (TFE3). This triggers a cascade of downstream events that depending on the genotype can rescue cellular functions via two pathways.

In most genotypes, arimoclomol can increase the pool of functional and properly trafficked NPC1 protein. In all genotypes, the expression of coordinated lysosomal expression and regulation (CLEAR) genes is broadly upregulated to promote the production of lysosomal and autophagy-related proteins. Activation of CLEAR network genes has been shown to rescue impaired autophagy in the NPC phenotype to mitigate the deleterious effects of faulty cholesterol trafficking and to improve overall cell health in cells and animal models of LSDs, including NPC [16,17].

Here we present the results of in vitro studies performed to explore the pathways by which arimoclomol targets the fundamentals of NPC etiology.

2. Materials and methods

Fibroblast cell lines derived from skin biopsies of clinically confirmed NPC patients and from healthy donors (wild type controls) were purchased from Coriell Biorepositories and maintained in culture under standard conditions (37 °C and 5 % CO_2, High glucose Dulbecco's Modified Eagle Medium supplemented with non-essential amino acids, 1 % Penicillin-Streptomycin and 12 % Fetal Bovine Serum). HeLa cells clone CCL-2 were acquired from American Type Culture Collection and cultured in the same medium and conditions as the fibroblast cell lines.

The following cell lines were obtained from the NIGMS Human Genetic Cell Repository at the Coriell Institute for Medical Research: NPC patient cell lines: GM17911 (I1061T/T1036M), GM17912 (P1007A/T1036M), GM17918 (T137M/null), GM17919 (I1061T/R404W), GM18390 (D242H/S940L), GM18393 (G248V/M1142T), GM18420 (P1007A/null), and GM18453 (I1061T/I1061T). Healthy control (wildtype) cell lines: GM00409, GM00498, and GM00969.

As applicable, test solutions of arimoclomol, miglustat, or U-18666 A in PBS, or just PBS (vehicle control) were added to the cell medium. The effect of arimoclomol on the nuclear vs cytosolic distribution of the TFEB and TFE3 was assessed by immunostaining, high-content imaging and automated image analysis, measuring the ratio of nuclear to cytosolic staining intensity. Binding of TFE3/TFEB to the promoter regions of CLEAR genes was assessed with chromatin immunoprecipitation and qPCR in wild type (WT) fibroblasts treated with arimoclomol. The effects of arimoclomol on the expression levels of CLEAR genes were examined by qPCR in WT and NPC1 fibroblasts. Endo H assays and Western blotting were conducted to assess NPC1 protein levels and maturation. Filipin staining was used to evaluate clearance of unesterified cholesterol from lysosomes following treatment with arimoclomol alone and with arimoclomol and miglustat combined. Filipin is a polyene antibiotic that binds 3-beta-hydroxysterols including unesterified cholesterol. Filipin is brightly fluorescent in the UV spectrum and is commonly used as a histochemical stain of cholesterol in NPC cells and tissues for clinical diagnosis and research purposes. Following formaldehyde fixation, cells were stained with 50 μg/mL filipin-III (cat. no. F4767-1MG, Sigma-Aldrich) diluted in PBS containing 10 % FBS for two hours, followed by washing with PBS. Nuclei were stained with 5 μM DRAQ5 (cat. no. 62251, ThermoFisher Scientific). Fractions of cells from each treatment condition were transferred to separate culture vessels for analyses and imaging was performed using a high-content imaging system, together with an unbiased automated image analysis method. 9 to 36 image fields per sample were acquired with a ImageXpress Pico system using a 20×/numerical aperture 0.4 objective. Images were analyzed using CellProfiler v. 4.2.1. Briefly, Nuclei objects were segmented from the DRAQ5 signal and used as seeds for segmentation of cell objects from the dim filipin signal of the cell membrane, using the Watershed Image/Otsu method predefined in the software. Lysosome objects were segmented with a global manual threshold in the filipin image, fused per encompassing parent cell object, resulting in one lysosomal compartment object per cell. The filipin integrated intensity in the lysosomal compartment per cell was normalized by dividing with the area of the parent cell object.

All experiments were performed in the former Orphazyme laboratory in Copenhagen, Denmark.

Effects of treatment relative to control were assessed by a paired 2-tailed *t*-test or by repeated-measures two-way analysis of covariance (ANCOVA). Multiplicity was adjusted using Šídák's or Dunnett's multiple comparisons test.

For the analyses that included both arimoclomol and miglustat, the means of replicate experiment medians of drug-treated samples were compared with the respective vehicle control sample per cell line in two separate multiple comparison tests, comparing either the miglustat concentrations within each arimoclomol concentration group, or the arimoclomol concentrations within each miglustat concentration group.

Expression levels of target genes relative to reference genes were calculated using the ΔΔCT-method.

Statistical significance was considered at a threshold value of 0.05. Analyses were performed using GraphPad Prism (versions 9.1.0, 9.1.1or 9.3.1).

3. Results

3.1. Effect of Arimoclomol on the Localization of Transcription Factors TFE3 and TFEB

A critical step in the mechanism of action (MOA) of arimoclomol is its involvement in the trafficking of TFEB and TFE3 between the cytosol and the nucleus.

After 1 day of treatment with arimoclomol (400 μM), translocation of TFE3 from the cytosol to the nucleus was significantly increased compared to vehicle in both healthy human and NPC patient fibroblasts across three genotypes containing either the I1061T or P1007A mutation (I1061T/I1061T, P1007/null, P1007/T1036M); see Fig. 1.

In addition, a dose-dependent (0–400 μM) arimoclomol-enhanced translocation of TFE3 and TFEB from the cytosol to the nucleus was demonstrated in HeLa cells in which NPC1 protein was disabled by U-18666 A; see Fig. 2. U-18666 A is an NPC1 protein inhibitor that has been shown to induce an NPC-like phenotype [18]. The nucleus-to-cytosol ratio was less pronounced in WT cells vs cells with non-functional NPC1 indicating that the NPC phenotype is more sensitive to the effects of arimoclomol on the nuclear translocation of TFEB and TFE3.

3.2. Effects of Arimoclomol on the Expression of CLEAR Network Genes

Treatment with arimoclomol (400 μM) was shown to significantly enhance the binding of TFE3 to the CLEAR promoter elements of *NPC1, NPC2, GBA, MCOLN1,* and *GLA* in healthy human fibroblasts, whereas binding of TFE3 to the negative control *ACTB* promoter was not affected; see Fig. 3. This indicates that arimoclomol raises TFE3 recruitment to promoter regions of CLEAR genes resulting in increased expression of lysosomal genes including *NPC1*.

Subsequently, seven relevant CLEAR genes related to lysosomal

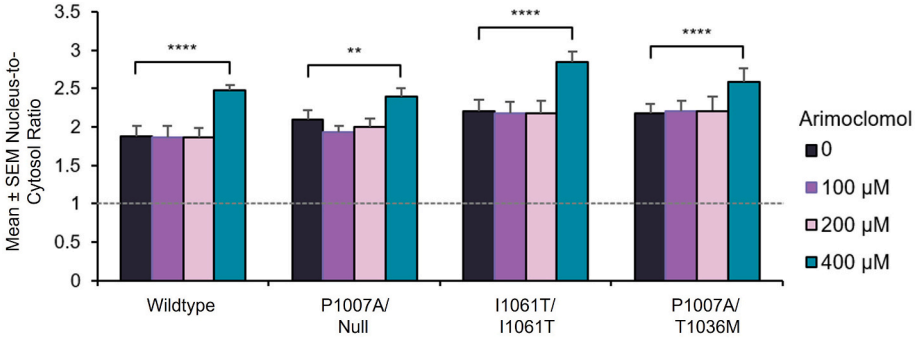

Fig. 1. TFE3 nucleus-cytosol intensity ratios in healthy human (wild type) and human NPC fibroblasts.
Immunofluorescence staining of TFE3, in GM00498 (wild type), GM18420 (NPC), GM18453 (NPC), and GM17912 (NPC) fibroblasts treated with PBS (control) or 100–400 μM arimoclomol for 1 day. The experiment was repeated 3 times, each column representing more than 2300 cells in total. The mean intensity ratio of the TFE3 staining in the nuclear to cytosolic compartments per cell was quantified and displayed as a bar graph with mean + SEM ($n = 3$). $^{**}p < 0.01$; $^{****}p < 0.0001$. NPC = Niemann-Pick disease type C; PBS = phosphate buffered saline; SEM = standard error of the mean; TFE3 = transcription factor E3.

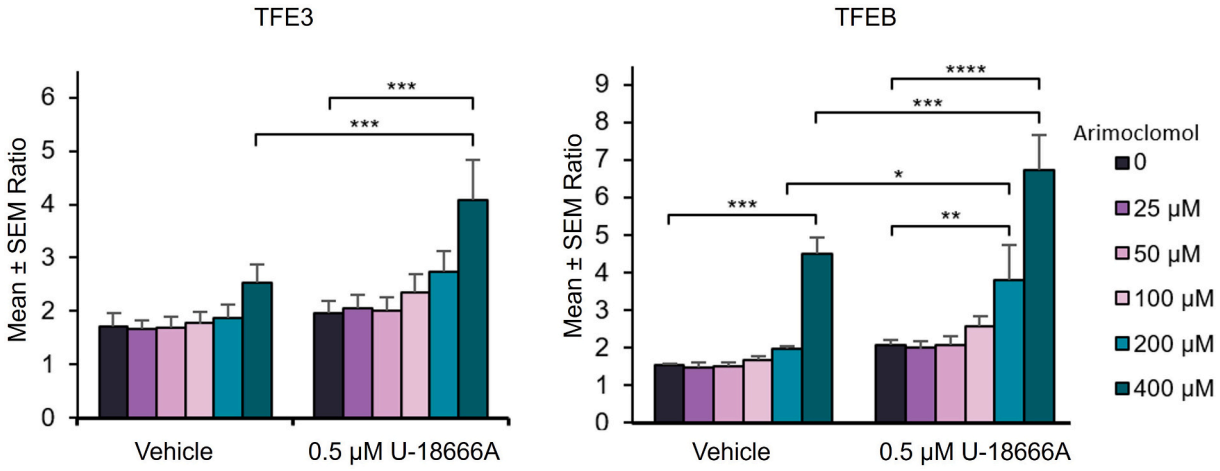

Fig. 2. TFE3 and TFEB nucleus-cytosol intensity ratios in HeLa cells treated with arimoclomol in combination with the NPC1 inhibitor U-18666 A.
Quantifications of the nucleus-to-cytosol mean fluorescence intensity ratios of immunostainings of TFE3 and TFEB in HeLa cells. Cells were treated with 0–400 μM arimoclomol in combination with either vehicle or 0.5 μM NPC1 inhibitor U-18666 A for 3 days. Bars depict the means of median ratios of independent experiments ($n = 4$), each with >160 cells per sample, error bars show SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. TFE3 = transcription factor E3; TFEB = transcription factor EB.

function (*NPC1*, *NPC2*, *GBA*, *GLA*, *MCOLN1*, *RRAGD*, *SQSTM1*) were investigated to determine if their expression was upregulated by arimoclomol in healthy human fibroblasts. Transcriptional upregulation with arimoclomol (400 μM) was observed for all seven tested CLEAR genes; see Fig. 4. The mean expression levels ranged from approximately 1.5-fold to 11-fold higher vs control with most genes being expressed at approximately 2–3-fold higher rates.

The finding of transcriptional upregulation of the seven selected CLEAR genes, was confirmed in NPC patient fibroblasts with the ER missense mutation I1061T in a dose-dependent manner following treatment with 0–400 μM arimoclomol. Similar upregulation was found for *HSPA1A* (encoding HSP70, a protein of the heat shock family) which was evaluated in the same experiment. Moreover, an additive effect on the upregulation of the selected CLEAR genes and *HSPA1A* was demonstrated following combination treatment with miglustat (0–100 μM); see Supporting Information Figure S1.

3.3. Concentrations, maturation, and trafficking of NPC1 protein

The effect of arimoclomol on NPC1 biosynthesis and maturation was explored in fibroblasts from NPC patients and healthy donors. The NPC fibroblasts included three common types of mutations that can produce partially functional NPC1 protein if folded properly and transported to the late endosomes and lysosomes. The first type comprised ER missense mutations. The second type consisted of mutants that can reach the target late endosomes, but at lower rates compared to the wildtype protein. The third type exhibited trafficking patterns like the wildtype protein and typically results in milder phenotypes with adult-onset of disease symptoms (e.g., P1007A mutation). At baseline, all genotypes, except for one (GM18390: D242H/S940L) resulted in 21–73 % lower NPC1 protein concentrations compared to the wildtype. Arimoclomol (0–400 μM) increased NPC1 protein concentrations in all NPC fibroblast cell lines in a dose-dependent manner with the most pronounced effects observed in the I1061T/I1061T (ER/ER) genotype; see Fig. 5.

To explore the potential clinical relevance of increased cellular NPC1 protein concentrations following arimoclomol treatment, the mobility and localization of the mutant NPC1 protein complex was evaluated. Nascent membrane proteins like NPC1 are glycosylated with mannose-rich N-glycans as they enter the lumen of the ER [19]. These immature glycans can be cleaved by endoglycosidase H (Endo H). However, as the proteins mature during migration to the Golgi, the glycan chains are heavily processed and become resistant to Endo H hydrolysis [20]. Thus, arimoclomol (400 μM) treatment for 5 days was evaluated in Endo H assays with fibroblasts of two different genotypes (ER missense I1061T and wildtype-like P1007A). Increased NPC1 resistance to Endo H degradation was demonstrated in cells treated with arimoclomol

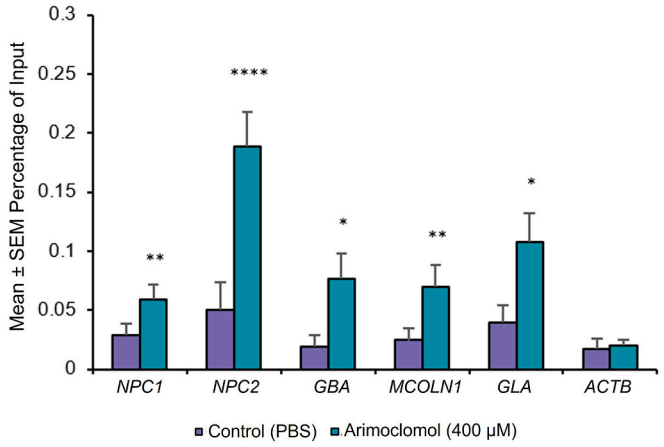

Fig. 3. Enhanced Binding of TFE3 to CLEAR Elements in Target Gene Promoters after Arimoclomol Treatment.
For each gene promoter, ChIP data are presented as TFE3 immunoprecipitated DNA as % of input in control (PBS) or arimoclomol (400 μM, 84–86 h) treated healthy human fibroblasts ($n = 3$, mean is shown as a bar. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. ACTB = actin beta; ChIP = chromatin immunoprecipitation; CLEAR = coordinated lysosomal expression and regulation; DNA = deoxyribonucleic acid; GBA = β-glucosylceramidase; GLA = α-galactosidase; IgG = immunoglobulin G; MCOLN1 = mucolipin TRP cation channel 1; NPC1/2 = NPC intracellular cholesterol transporter 1/2; PBS = phosphate buffered saline; qPCR = quantitative polymerase chain reaction; SEM = standard error of the mean; TFE3 = transcription factor E3.

compared to untreated control; see Fig. 6. This indicates that the mutant NPC1 proteins in arimoclomol-treated cells could mature sufficiently to escape from the ER and migrate through the cis-Golgi. As expected, the arimoclomol-induced increase in mature protein was more pronounced in I1061T/I1061T fibroblasts with impaired ER transport that have a higher potential for improvement when compared to the P1007A genotype that exhibits trafficking patterns already close to WT cells.

Overall, the data supports that arimoclomol does not only upregulate expression of certain CLEAR genes including *NPC1* at the transcriptional level, but also that this overexpression results in the amplification of protein levels which improves the probability of mutant NPC1 protein to pass through the Golgi and reach the target late endosomes. It is likely that the arimoclomol-induced upregulation of CLEAR genes other than *NPC1* also leads to increased biosynthesis of the respective lysosomal proteins.

In summary, activation of TFE3/TFEB by arimoclomol was shown to upregulate expression of CLEAR genes including *NPC1* as well as *HSPA1A*. On a cellular level, this finding supports that arimoclomol has the potential to improve lysosomal function and possibly autophagy flux to clear toxic components from the cell. More specifically, the amplified expression response was shown to increase cellular concentrations of mutant NPC1 protein including a mature, glycosylated complex with resistance to Endo H hydrolysis.

3.4. Clearance of unesterified cholesterol in human NPC fibroblasts

While protein maturation is a critical step in the ER-Golgi-lysosomal pathway of NPC1, it is not clear if successful glycosylation of the mutant protein is sufficient to allow for proper lysosomal targeting. To assess whether functional mutant NPC1 can reach the late endosome, the effects of arimoclomol on the clearance of cholesterol from the lysosomal compartment were explored in I1061T/I1061T mutant and WT fibroblasts. Unesterified cholesterol levels in the fibroblasts were assessed through high-content imaging followed by automated image analysis of filipin staining intensity (filipin is an unesterified cholesterol binder). Reduced staining intensities following arimoclomol treatment (0–200 μM) indicated improved cholesterol clearance from the lysosome compared to vehicle control at the prespecified timepoints (7, 14, 21, 28 days); see Fig. 7. The reduction in cholesterol levels plateaued after 14–21 days and was more pronounced at the highest arimoclomol concentration. These results support that the arimoclomol-enhanced transcription processes can lead to increased cholesterol transport out of the lysosomes and reduce the lipid burden in NPC cells.

Subsequently, the effects of combined treatment with arimoclomol (0–200 μM) and the glycosylceramide synthase inhibitor miglustat (0–100 μM) on the cholesterol clearance in NPC fibroblasts was explored; see Supporting Information Figs. S2 (heatmap) and S3 (microscopic pictures). Combining arimoclomol and miglustat resulted in an additive decrease in lysosomal cholesterol content that was dose-dependent for both compounds. After 14 days of treatment at the lowest test concentrations of 50 μM arimoclomol and 10 μM miglustat,

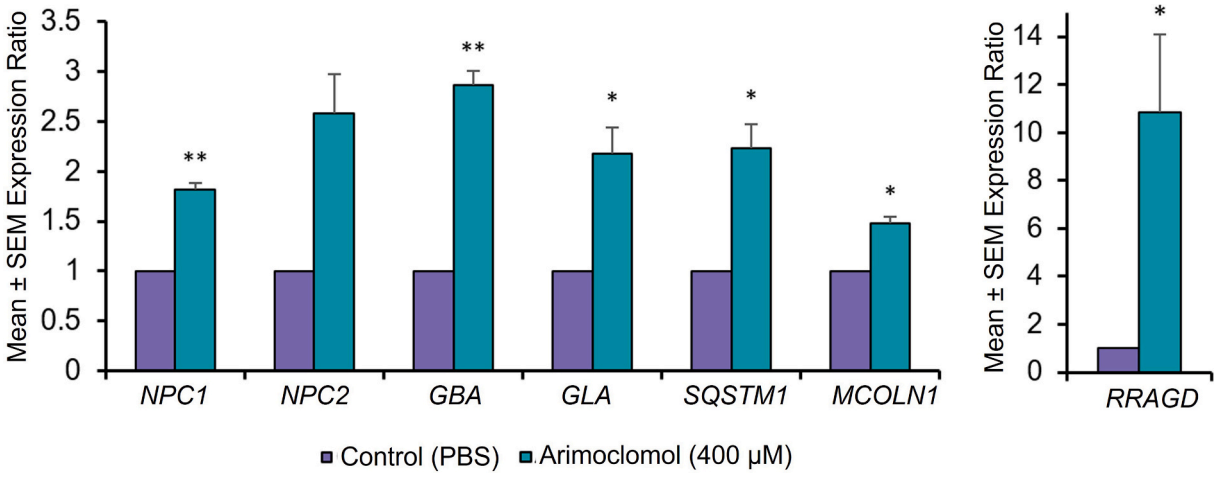

Fig. 4. Expression levels of selected CLEAR network genes after treatment with 400 μM arimoclomol in wild type fibroblasts.
Relative gene expression following 5-day treatment with 400 μM arimoclomol vs control (PBS). Left: *NPC1*, *NPC2*, *GBA*, *GLA*, *SQSTM1*, and *MCOLN1* (values on left y-axis). Right: *RRAGD* (shown separately as the expression rate was markedly higher compared to the other genes). Gene quantification was performed by quantitative RT-PCR. Treatment effects were evaluated by a paired 2-tailed *t*-test: *$p < 0.05$, **$p < 0.01$.
CLEAR = coordinated lysosomal expression and regulation; *GBA* = β-glucosylceramidase; *GLA* = α-galactosidase; *MCOLN1* = mucolipin TRP cation channel 1; *NPC1/2* = NPC intracellular cholesterol transporter 1/2; PBS = phosphate buffered saline; *RRAGD* = Ras related GTP binding D; RT-PCR = reverse transcription quantitative polymerase chain reaction; SEM = standard error of the mean; *SQSTM1* = sequestosom.

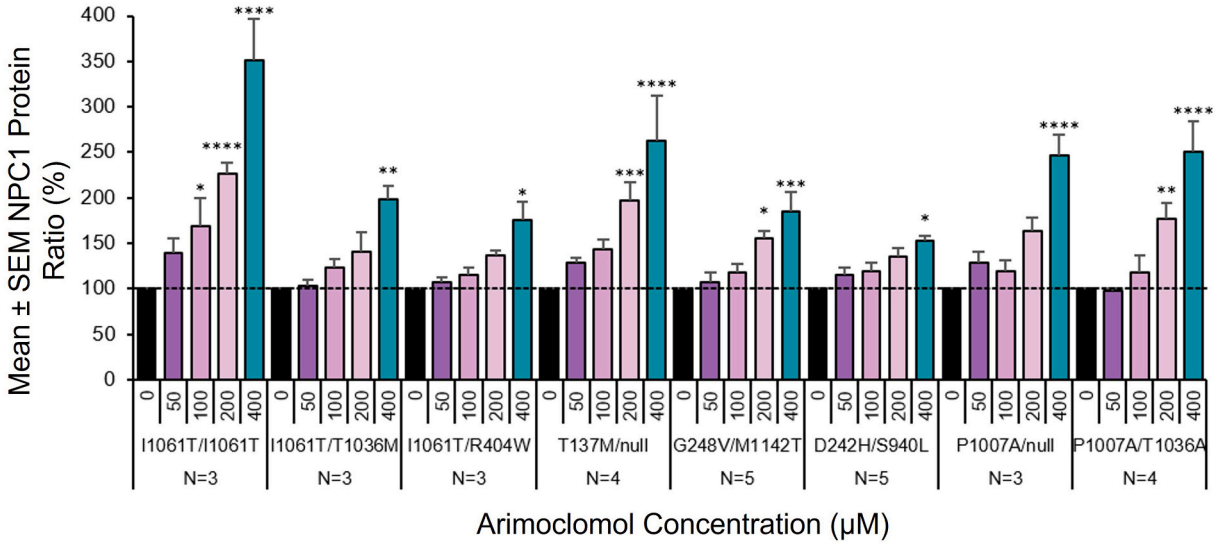

Fig. 5. Effects of arimoclomol treatment on NPC1 protein concentrations in NPC fibroblasts with different mutations.
Western blot data presented as the mean change in % + SEM of NPC1 protein expression after arimoclomol treatment at concentrations of 50, 100, 200 and 400 μM for 5 days, relative to PBS-treated control cells from a total of 3 to 5 independent experiments, as indicated. NPC1 levels have been normalized to tubulin for GM18453 (I1061T/I1061T) and GM18420 (P1007A/null), and to ponceau staining of total protein for the other cell lines. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.
NPC = Niemann-Pick disease type C; NPC1 = NPC intracellular cholesterol transporter 1; PBS = phosphate buffered saline; SEM = standard error of the mean.

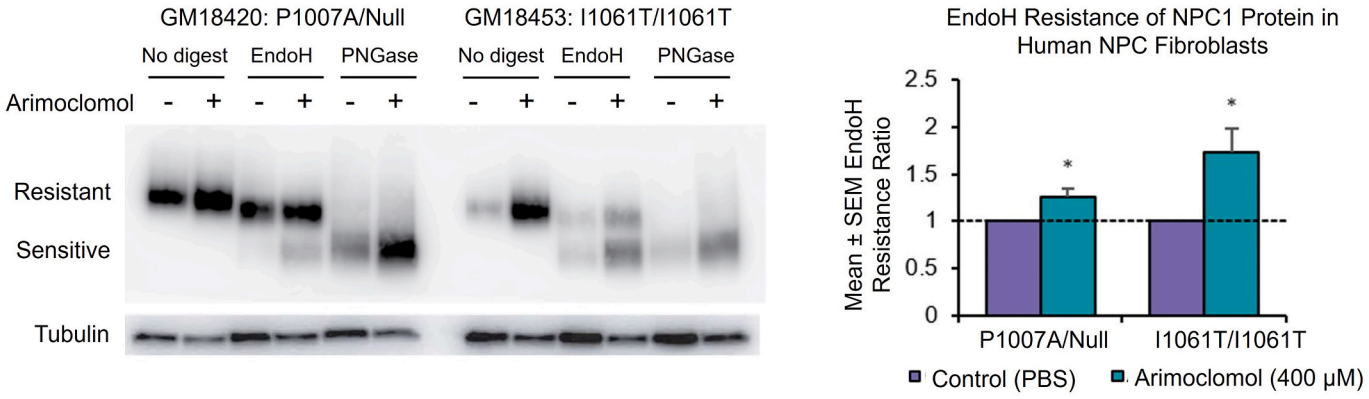

Fig. 6. Results of Endo H assays with arimoclomol to assess NPC1 protein maturation and localization.
A: Western blotting of extracts from NPC fibroblast cell lines harboring P1007A and a functional null allele (GM18420), or homozygous I1061T NPC1 (GM18453). PNGase cleaves all glycans from the NPC1 protein regardless of maturation status and is included as a control. Endo H sensitive, immature NPC1 is seen in PBS-treated GM18453 extracts. B: Quantification of the Endo H resistant NPC1, shown as arimoclomol-treated relative to untreated cells. Cells were treated for 5 days with 400 μM arimoclomol. Average of three independent experiments, mean ± SEM. *$p < 0.05$.Endo H = endoglycosidase H; NPC = Niemann-Pick disease type C; NPC1 = NPC intracellular cholesterol transporter 1; PBS = phosphate buffered saline; PNGase = peptide:N-glycosidase; SEM = standard error of the mean.

the unesterified cholesterol level was 35 % lower compared to vehicle control. Combination therapy for 14 days at the highest test concentrations of 200 μM arimoclomol and 100 μM miglustat led to a reduction in filipin staining intensity of nearly 80 % compared to vehicle control.

These data demonstrate that arimoclomol can decrease unesterified cholesterol in the lysosomal compartments of human NPC fibroblasts and that the cholesterol clearance is further enhanced in the presence of miglustat.

A proposed MOA of arimoclomol, based on the data presented herein, is shown in Fig. 8 and illustrates how arimoclomol can mitigate the deleterious effects of impaired cholesterol trafficking and potentially improve overall cell health by increasing lysosomal biogenesis and autophagic flux.

The additive effect observed when miglustat was combined with arimoclomol treatment indicates that arimoclomol and miglustat act via distinct yet complementary MOAs.

4. Discussion

Lysosomal proteins play an important role in autophagy, lipid and glycan catabolism and export, and have shown to reduce lipid accumulation in several cells and animal models of lysosomal storage disorders including NPC [16,17,21]. TFEB/TFE3 is a chief regulator of lysosomal function and binds to the CLEAR motif in the promoter region of *NPC1* and other lysosomal genes that encode a host of proteins including lysosomal hydrolases, lysosomal membrane proteins, and other proteins involved in autophagy [22]. The binding of TFEB/TFE3 to CLEAR motifs activates gene transcription thereby increasing expression rates and subsequent protein biogenesis to enhance lysosomal functions such as clearance of substrates, cell membrane repair, cell metabolism and autophagy [23].

Arimoclomol was found to activate TFE3 and TFEB by promoting their transport from the cytosol to the nucleus in healthy cells, NPC

A

Day	Arimoclomol Concentration (μM)					
	0	12	25	50	100	200
7	0%	-11%	-14%	-21%	-21%	-34%
14	0%	-17%	-27%	-25%	-32%	-55%
21	0%	-12%	3%	-15%	-29%	-55%
28	0%	-8%	-1%	-11%	-39%	-38%

B

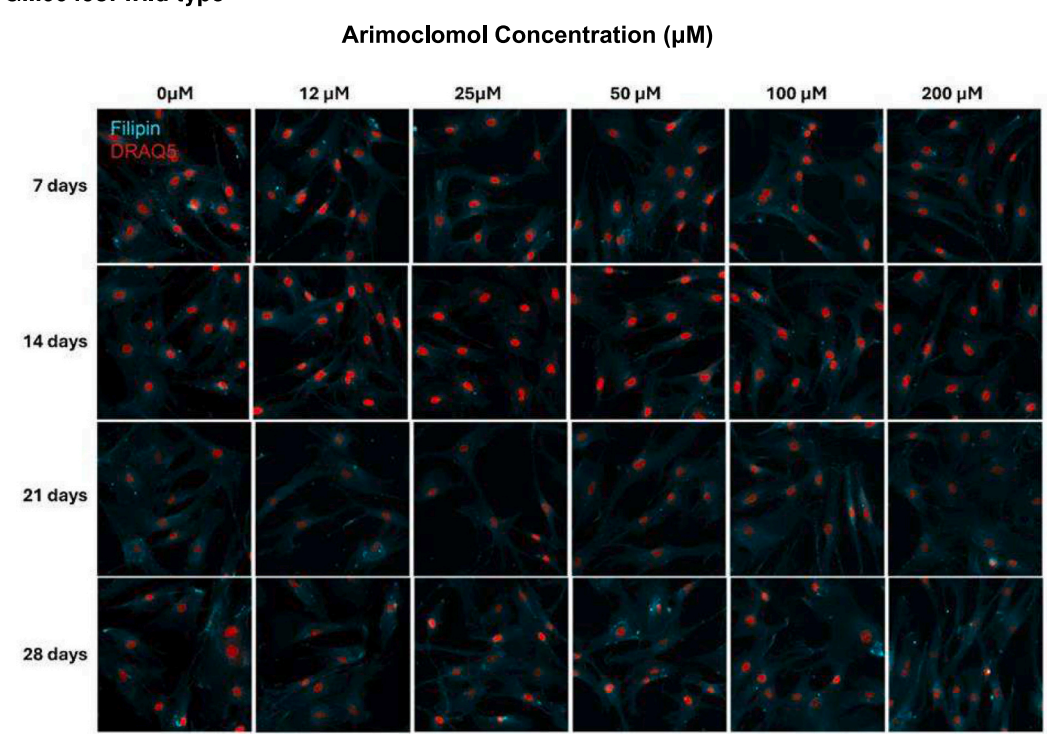

Fig. 7. Heatmap (A), microscopic pictures (B) and quantification (C) of filipin staining intensity in lysosomal compartment following arimoclomol treatment. Unesterified cholesterol levels were measured via high-content imaging of filipin staining in lysosomal compartments of NPC (GM18453, I1061T/I1061T) and wild-type (GM00498) fibroblasts. Cells were treated with 0, 12, 25, 50, 100, or 200 μM arimoclomol for 7, 14, 21, or 28 days. Data represent means of medians from three replicate experiments (>1400 cells/condition) and are expressed as % reduction in staining intensity relative to vehicle control (0 μM). (A) Heatmap: Green (100 % reduction), white (control level), red (100 % increase) across concentrations and time points. (B) Representative filipin-stained images (cyan, cholesterol) and DRAQ5-stained nuclei (red). (C) Corresponding plots. Statistics: Two-way repeated measures ANOVA with Dunnett's test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$). SEM = standard error of the mean.

patient fibroblast, and NPC-like cells where the NPC1 protein was disabled by U-18666 A. In addition, arimoclomol improved the binding of TFE3 and TFEB to the CLEAR promoter elements of *NPC1*, *NPC2*, *GBA*, *GLA, and MCOLN1*. The proteins encoded by these genes are responsible for important lysosomal functions including the intracellular/lysosomal transport of cholesterol, lysosomal breakdown of glucosylceramide and globotriaosylceramide (Gb3), and lysosomal ion transport and cellular waste processing.

There are phosphorylated and de-phosphorylated pools of TFEB both in the cytosol and the nucleus. The phosphorylation state of TFEB is mediated by mechanistic target of rapamycin complex 1 (mTORC1). In the cytosol, mTORC1 can activate TFEB by initiating its translocation to the nucleus via dephosphorylation. *Re*-phosphorylation and export from the nucleus may also be modulated by mTORC1 although other kinases may be involved [24].

While a specific molecular target of arimoclomol that would lead to enhanced TFEB activation has not yet been identified, one potential mechanism may include the blockage or delay of nuclear TFEB phosphorylation which could prolong its activated state. However, the extensive feedback system that controls transcription processes would likely offset that shift by increasing cytosolic phosphorylation of TFEB unless this response could be counteracted by lysosomal stress signals that reduce mTORC1 activity. Indeed, misfolded protein aggregation, lipid accumulation, impairment in lysosomal biogenesis, and autophagy failing in the NPC disease state may trigger lysosomal stress signals that downregulate mTORC1 activity to promote the synthesis of proteins that can mitigate lysosomal and autophagic deficiencies [25].

Our data supports a potential NPC-induced stress response by indicating a slight increase in TFE3/TFEB nuclear translocation in NPC-like cells (acute inhibition of NPC1 by U-18666) in the absence of arimoclomol when compared to WT cells. A modest, endogenously driven reduction in basal mTORC1 activity in response to NPC1 dysfunction combined with the presumed arimoclomol-induced retention of dephosphorylated TFE3/TFEB in the nucleus could explain how arimoclomol is able to meaningfully shift the cytosol-to-nucleus ratio of these transcription factors. While mTOR signaling is involved in various

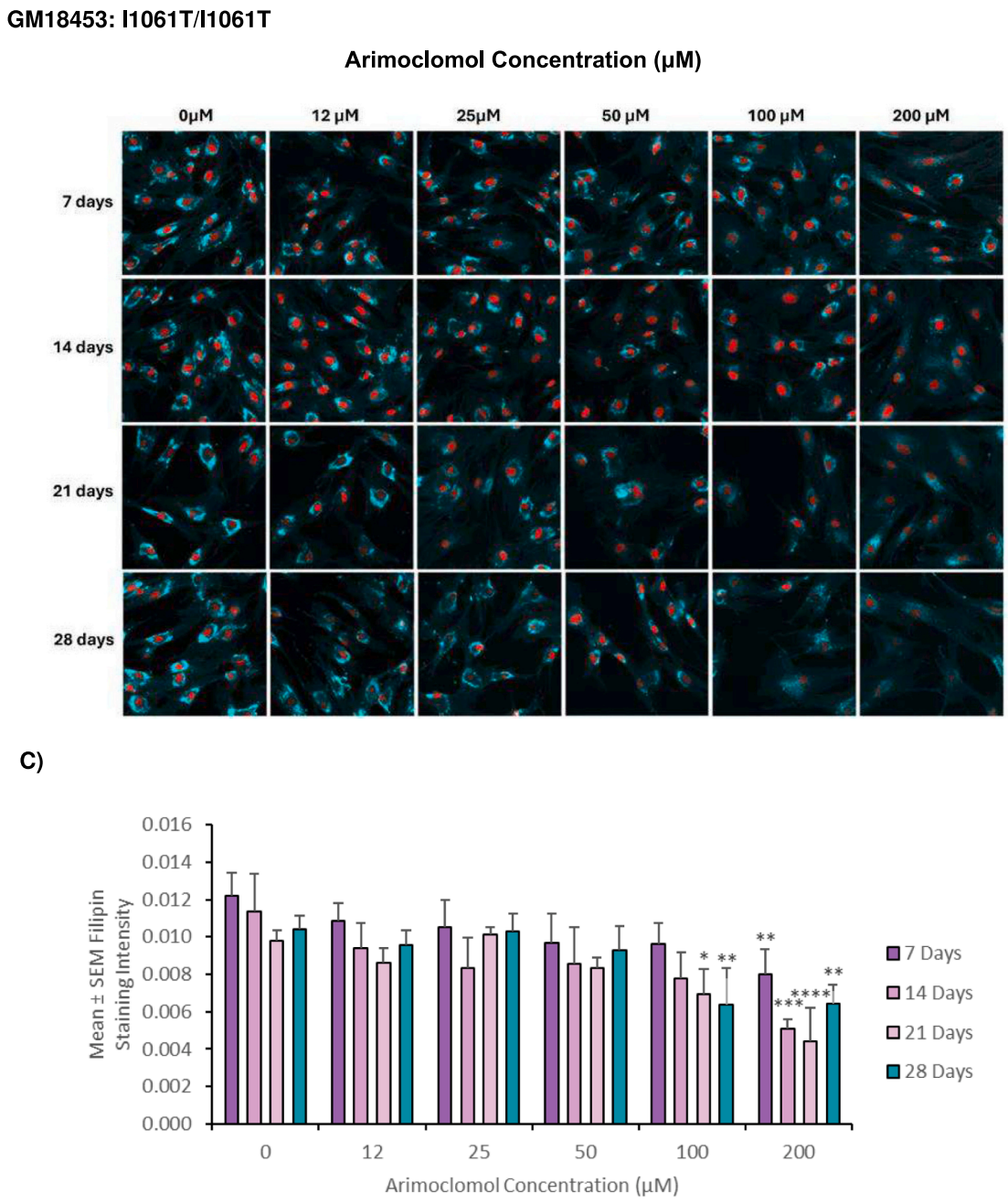

Fig. 7. (*continued*).

processes such as growth and metabolism, there is no evidence to suggest that arimoclomol's effects in NPC are due to direct mTOR inhibition or activation. Should future research reveal additional interactions between arimoclomol and mTOR beyond its influence on TFEB/TFE3 regulation, further studies will be warranted.

In our evaluation of potential benefits of arimoclomol on the pathophysiology of NPC, its effects on the gene expression and trafficking of NPC1 protein were of particular interest. In healthy cells, nascent NPC1 is synthesized, processed and folded in the ER before being further modified (e.g., by glycosylation and phosphorylation), sorted, and prepared for trafficking in the Golgi apparatus. NPC1 is then transported to the lysosome where it is embedded into the lysosomal membrane to facilitate removal of cholesterol and other lipids [23,26,27].

NPC1 phenotypes can be classified into three categories based on the ability of the mutant protein to be trafficked out of the ER, which is a marker for successful folding and for a certain level of protein maturation [9]. The first group of mutations results in proteins that fail proper folding and are unable reach the lysosome as they fall victim to the ER quality control checks. The second group of mutations gives rise to proteins that are trafficked to the lysosomal membrane but with a certain delay and with potentially limited functionality. The third group leads to proteins that exhibit wildtype-like trafficking and proper localization to the lysosome [9]. Out of these three groups, the *NPC1* variants that produce misfolded proteins with impaired ER trafficking are the most common. Few of these mutant proteins make it to the Golgi apparatus, and even fewer become mature and reach the lysosome. This can lead to aggregation of misfolded protein in the ER that triggers the endoplasmic reticulum-associated degradation (ERAD) response mechanisms [20,28]. If protein aggregates exceed cell capacity, cell death occurs [29].

One example of a variant that belongs in this 'common' category is the I1067T mutation in the *NPC1* gene. The high Endo H sensitivity of

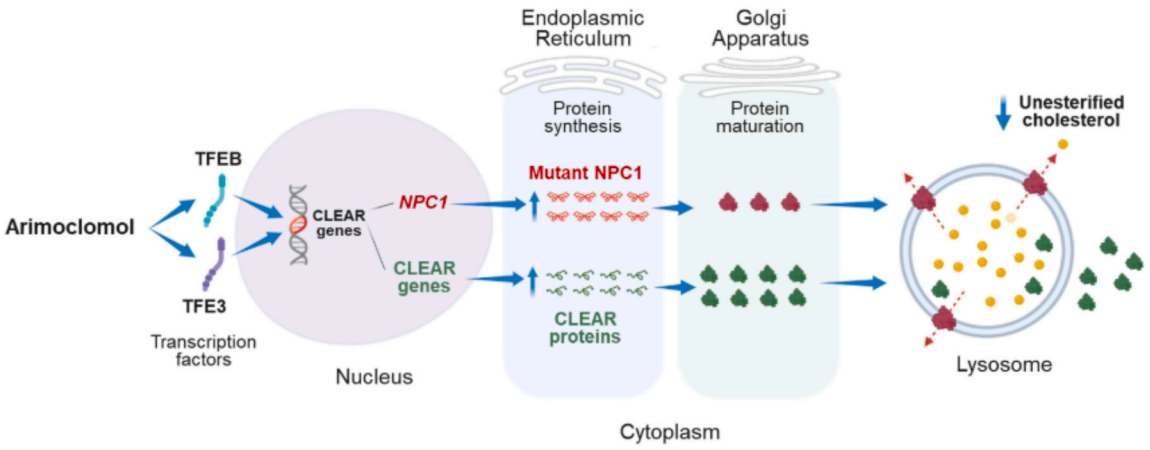

Fig. 8. Arimoclomol targets the pathophysiology of Niemann-Pick disease type C by two pathways.
The figure illustrates the key steps in the proposed MOA of arimoclomol, based on the presented in vitro findings. 1) Arimoclomol initiates translocation to and/or prolongs the localization of the transcription factors TFE3 and TFEB in the cell nucleus. 2) In the nucleus, TFE3 binding to CLEAR motifs is enhanced in the promoter regions of lysosomal genes (CLEAR network genes). Prolonged activation and improved binding of TFE3 to CLEAR gene promoters increases CLEAR gene expression. 3) Upregulation of CLEAR gene expression raises CLEAR network protein levels including NPC1 and HSP70 (heat shock protein). Higher concentrations of post-translational NPC1 mutant protein increases the odds for proper folding (possibly amplified by more available HSP70) and maturation to escape the ER quality control and migrate through the cis-Golgi to ultimately reach the late endosomes. 4) Greater availability of at least partially functioning NPC1 protein (depending on genotype) in the late endosomal/lysosomal membranes results in improved clearance of unesterified cholesterol. In an NPC1-independent pathway, upregulation of key CLEAR genes likely improves general cell health through heightened lysosomal efficiency and autophagy flux independent of NPC1 protein availability and functional capacity. Hereby, arimoclomol works through two pathways to improve autophagy, reduce cholesterol accumulations and prevent cell death.

the NPC1^{I1061T} protein indicates that it is heavily targeted for ER degradation and fails to mature [9,28,30]. Although biochemical studies have shown that this mutant protein can retain some functionality if folded properly, its inability to localize to the lysosomal membrane means that genotypes with the I1067T mutation are typically associated with moderate to severe forms of NPC.

Other types of mutations may allow proper trafficking of mutated NPC1 protein but due to structural defects may lack functionality leading to accumulation of cholesterol, dysfunctional autophagy, and neuronal death. The archetype of mutant proteins with efficient trafficking patterns is NPC1^{P1007A}, a mostly dysfunctional mutant protein characterized by its ability to localize properly at the lysosome [9].

Arimoclomol was assessed in a panel of human fibroblasts harboring different mutations in the *NPC1* gene. In all cell lines NPC1 was expressed at lower levels compared to the WT (except D242H/S940L that showed similar protein concentration to WT). The ER missense mutation I1061T resulted in low levels of NPC1, which is consistent with previous observations [20,28,30]. Arimoclomol was demonstrated to induce a significant increase in NPC1 concentration in these cell lines.

Evaluation of NPC1 maturation through Endo H assay in two cell lines (I1061T/I1061T and P1007A/null) showed that arimoclomol treatment increases the amount of NPC1 that is properly processed in the ER and shuttled to the Golgi. These data support that arimoclomol not only upregulates the expression of *NPC1* at the transcriptional level, but that this overexpression results in amplification of NPC1 protein levels and more successful NPC1 processing. A potential explanation for the enhanced trafficking of NPC1 protein after arimoclomol treatment could be that the increased protein production improves the odds of proper folding allowing more protein to undergo post-translational glycosylation, pass through Golgi apparatus, and reach the target lysosome. In many NPC1 genotypes that produce trafficking impaired but at least partially functional protein (e.g., NPC1^{I1061T}), overexpression of *NPC1* may therefore improve cholesterol clearance and lysosomal function [20].

Evidence of NPC rescue because of *NPC1* overexpression has been reported with histone deacetylase inhibitors (HDACis). HDACis was shown to increase protein expression of homozygous and heterozygous NPC1^{I1061T} thereby correcting cholesterol accumulation in human fibroblasts [31]. Conversely, rapid NPC1^{I1061T} protein degradation was observed in untreated cells indicating that increased NPC1 biosynthesis can improve the survival rate of mutant protein and enable its transport to the lysosomal membrane.

In another study, the effects of HDACis (vorinostat or panobinostat) on NPC1 protein expression and cholesterol clearance were evaluated in engineered U2OS cell lines with silenced *NPC1* expression and transiently transfected *NPC1* mutations [32]. Out of 60 tested mutants, HDACi treatment rescued 52 phenotypes as demonstrated by a statistically significant reduction in cholesterol accumulation. Non-significant outcomes were typically found in mild phenotypes and in mutants with structural defects near the sterol sensing or cholesterol binding regions that are essential for function.

In addition to simple upregulation of NPC1 protein levels, arimoclomol may enhance other pathways that may aid in the maturation of mutant protein with high misfolding and ER degradation rates. It has previously been reported that arimoclomol amplifies the gene expression of *HSPA1A* and *HSPA5* in human fibroblasts of patients with Gaucher disease [33]. HSPA5 encodes BiP/Grp78, another protein of the HSP70 heat shock family.

Our data confirm the upregulation of *HSPA1A* expression in NPC fibroblasts which would be expected to increase the availability of HSP70 protein. While one of the primary functions of HSP70 is to assist in the folding of cytosolic proteins, this heat shock protein can also bind to the ER stress sensor, IRE1α, located at the ER membrane to trigger the unfolded protein response (UPR) and prolong apoptosis [34–36]. UPR activates a number of pathways including the recruitment of ER-resident chaperones such as BiP/Grp78 to assist the folding of secretory proteins [37]. We therefore speculate that upregulation of HSP70 by arimoclomol may activate UPR to improve NPC1 folding in a process that is further enhanced by the additional expression of BiP/Grp78. This combined effort may provide greater processing capacity to the already stressed ER folding machinery to deal with the increased demand created by the arimoclomol amplified synthesis of NPC1 protein.

It is of interest to note that others have reported endogenous activation of UPR in a subset of NPC phenotypes [38]. The UPR activity appears to depend on the mutation that determines the level of NPC1 protein retention in the ER. Highly ER degraded NPC1^{V562V} protein

stimulated a significant upregulation of *HSP1A1* and *HSPA5* while delayed ER trafficking showed minimal effect on UPR genes. In NPC phenotypes with low UPR activity, treatment with arimoclomol may therefore provide additional benefits by improving the ER folding efficiency complementary to the upregulation of NPC1 expression.

Overall, multiple lines of evidence suggest that arimoclomol can boost the de novo synthesis of NPC1 protein and mobilize HSP70 heat shock proteins to potentially induce a coordinated response via ERAD and UPR. While initial data indicate that these effects result in a larger pool of more Endo H resistant and thus, more mature NPC1 in the Golgi, the ultimate fate of these mutant proteins remains a matter of future investigation. However, indirect evidence suggests that once mature, functional NPC1 mutant protein is shuttled out of the Golgi and embedded into the lysosomal membrane as demonstrated by a decrease in unesterified cholesterol in the lysosomal compartments of human NPC fibroblasts treated with arimoclomol. Notably, cholesterol clearance was further enhanced in the presence of miglustat. A likely explanation of this finding is based on reports that miglustat can lower glycosphingolipid accumulation with downstream reduction of cholesterol in NPC fibroblasts [12,39,40]. Therefore, it appears that arimoclomol and miglustat can act on independent pathways to provide additive effects with respect to reducing cholesterol burden in the lysosomes of NPC patients. This complementary effect was also observed in a clinical trial where a subset of pediatric and adult NPC patients co-administered with arimoclomol and miglustat demonstrated a robust treatment effect when compared to placebo [41].

One further aspect of the mechanistic actions of arimoclomol relates to its potential benefits on autophagic processes in NPC cells. While any specific effects described herein remain mostly speculative, the arimoclomol-induced overexpression of autophagy associated genes is another area that warrants further research.

Previous studies have demonstrated that lysosomal dysfunction in NPC patients causes a disruption of autophagosome-lysosome fusion which leads to a defective autophagic pathway and ultimately to cell death [42,43]. Autophagy is a lysosome-mediated catabolic process with a key role in maintaining cellular lipid homeostasis [44]. Conversely, lysosomal cholesterol accumulation impairs the autophagy pathway in NPC1 mutant cells [25].

Arimoclomol increased the expression of the autophagy mediator gene *SQSTM1* in human NPC fibroblasts. This upregulation may promote selective autophagy by enhanced targeting of ubiquitinated proteins for degradation in the autophagosome [45,46].

Arimoclomol also raised expression levels of *MCOLN1* which encodes transient receptor potential mucolipin 1 (TRPML1), an ion channel responsible for releasing Ca^{2+} from the lysosome. Calcium signaling regulates important cellular processes including autophagy, lysosomal trafficking, and lysosomal membrane fusion events [47,48]. Due to lipid accumulation in the lysosome, TRPLM1 function is compromised in NPC cells. Increasing TRPML1 activity or expression was shown to mitigate cholesterol accumulation and impaired lysosomal trafficking in NPC cells [49]. It is therefore conceivable that overexpression of *MCOLN1* by arimoclomol can improve NPC-induced defects in autophagosome-lysosome fusion as well as lysosomal biogenesis and reformation to boost overall lysosomal function and autophagy flux [49,50]. With its potential to directly enhance autophagic processes, arimoclomol may provide benefits in the NPC phenotype that are mechanistically independent of NPC1 protein levels, trafficking competency, or functionality.

There are numerous other CLEAR genes governed by TFEB that encode lysosomal proteins with critical roles in autophagy processes including autophagosome membrane elongation, and autophagosomes trafficking and fusion with lysosomes [23,26,27,51]. This provides an opportunity for future investigations of arimoclomol to assess its effects on various aspects of autophagy in NPC and other LSDs.

5. Conclusion

The presented in vitro data demonstrate that arimoclomol has significant effects along a mechanistic pathway that appears clinically relevant for NPC therapy. The increased translocation of the transcription factors TFEB and TEF3 from the cytosol to the nucleus is a crucial step that results in the upregulation of a series of CLEAR genes that improve lysosomal function. Arimoclomol-induced overexpression of the *NPC1* gene resulted in higher concentrations of post-translational NPC1 mutant protein due to increased odds for proper folding in the ER. The folding efficiency may be further enhanced by the upregulation of *HSPA1A* and *HSPA5* genes that play pivotal roles in UPR activity. In phenotypes with at least partially functional NPC1 protein, overexpression was shown to reduce accumulation of unesterified cholesterol in the lysosome. Combining arimoclomol treatment with miglustat demonstrated even higher upregulation of CLEAR genes and further reduction in lysosomal cholesterol compared to each compound alone. In NPC1-independent pathways, arimoclomol amplified the expression of key CLEAR genes associated with autophagic processes. While further investigation is required to identify specific downstream effects on autophagy in NPC cells, arimoclomol appears to have the potential for improving lysosomal efficiency and cell viability regardless of NPC1 protein availability and functional capacity.

CRediT authorship contribution statement

Hadeel Shammas: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration. **Cathrine Kloster Fog:** Writing – review & editing, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **Pontus Klein:** Writing – review & editing, Methodology, Formal analysis, Data curation, Conceptualization. **Anja Koustrup:** Writing – review & editing, Data curation. **Marianne Terndrup Pedersen:** Writing – review & editing, Methodology, Data curation, Conceptualization. **Anne Sigaard Bie:** Writing – review & editing, Methodology, Data curation, Conceptualization. **Travis Mickle:** Writing – review & editing, Formal analysis. **Nikolaj Havnsøe Torp Petersen:** Writing – review & editing, Methodology, Data curation, Conceptualization. **Thomas Kirkegaard Jensen:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Sven Guenther:** Writing – review & editing, Writing – original draft, Formal analysis.

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Declaration of competing interest

Hadeel Shammas: Employee at Zevra and owner of stock options in the company. Cathrine Kolster Fog: Employee at Orphazyme and received consulting fees from Zevra. Pontus Klein: Employee at Orphazyme. Anja Koustrup: Employee at Orphazyme. Marianne Terndrup Pedersen: Employee at Orphazyme. Anne Sigaard Bie: Employee at Orphazyme. Travis Mickle: Stock options, chairs at Zevra, pending patent. Nikolaj Havnsøe Torp Petersen: Employee at Orphazyme. Thomas Kirkegaard Jensen: Employee at Orphazyme, co-inventor of issued patents related to arimoclomol, and received reimbursement as a consultant to Zevra within the last 5 years. Sven Guenther: Employee at Zevra and stock owner in the company, co-inventor of pending patents

related to arimoclomol.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymgme.2025.109103.

Data availability

Data will be made available on request.

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