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COMMUNICATION

Mitochondrial dysfunction in NPC1-deficiency is not rescued by drugs targeting the glucosylceramidase GBA2 and the cholesterol-binding proteins TSPO and StARD1

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Niemann–Pick type C disease (NPCD) is a rare neurodegenerative disorder most commonly caused by mutations in the lysosomal protein Niemann–Pick C1 (NPC1), which is implicated in cholesterol export. Mitochondrial insufficiency forms a significant feature of the pathology of this disease, yet studies attempting to address this are rare. The working hypothesis is that mitochondria become overloaded with cholesterol which renders them dysfunctional. We examined two potential protein targets—translocator protein (TSPO) and steroidogenic acute regulatory protein D1 (StARD1)—which are implicated in cholesterol transport to mitochondria, in addition to glucocerbrosidase 2 (GBA2), the target of miglustat, which is currently the only approved treatment for NPCD. However, inhibiting these proteins did not correct the mitochondrial defect in NPC1-deficient cells.

Keywords: GBA2; mitochondria; Niemann–Pick C; NPC1; StARD1; TSPO

Although Niemann–Pick type C disease (NPCD) originates with dysfunction of the lysosomal protein Niemann–Pick C1 (NPC1), it does not end there but encompasses numerous aspects of cellular physiology (reviewed in Ref. [1]) including mitochondria; in this, it is similar to other lysosomal storage disorders [2]. Mitochondrial dysfunction is well-attested in NPCD where reduced mitochondrial potential [3,4], reduced oxygen consumption [5,6], reduced ATP production [3] and increased levels of lactate [5] have all been reported alongside increased reactive oxygen species (ROS) [5,7,8]. Most researchers attribute this respiratory impairment to excess cholesterol in the mitochondria poisoning the organelle; this excess presumably results from over-active cholesterol transport to mitochondria. All cells need to import cholesterol to mitochondria so that Cyp27A1 and related proteins can use it to synthesise liver X receptor (LXR) ligands as part of the cell's cholesterol homeostatic processes (recent study [9]). The idea that this process turns pathological in NPCD is supported by the observations that (a) mitochondrial dysfunction can be induced by cholesterol over-feeding in hepatic [10] and pancreatic [11] cells, (b) there is direct transport of cholesterol from lysosomes to mitochondria which involves NPC1 [12], (c) membrane contact sites (MCSs) between lysosomes and mitochondria increase in NPCD [13] and (d) cholesterol transport has been

Abbreviations

21-AcP, 21-acetoxypregnenalone; GBA2, glucocerbrosidase 2; LXR, liver X receptor; MCS, membrane contact sites; NPC1, Niemann–Pick C1; NPCD, Niemann–Pick type C disease; PBR, peripheral benzodiazepine receptor; ROS, reactive oxygen species; StARD1, steroidogenic acute regulatory protein D1; TSPO, translocator protein; VHTS, virtual high-throughput screening.

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observed at these sites [14]. The cholesterol overload hypothesis is not without its difficulties (see our discussion in [1]), nor is it obvious why cells with lysosomes that are normally considered as having a cholesterol export defect should also feature mitochondria with a cholesterol excess.

Perhaps reflecting the general confusion over the mechanism underlying this dysfunction, attempts to correct mitochondrial insufficiency have been few (exceptions [3,4,15,16]). This paper reports our work in the area focussing on the sphingolipid hydrolase glucocerbrosidase 2 (GBA2) and the cholesterol-binding proteins translocator protein (TSPO) and steroidogenic acute regulatory protein D1 (StARD1).

Materials and methods

Chemicals were purchased from Sigma, Cayman and Matreya and used without further purification. Healthy (GM00380, RRID: CVCL_V768) and diseased (GM03123, RRID: CVCL_7374) cells were obtained from the Coriell Institute.

Cell culture

Fibroblasts were incubated in cell culture flasks using high glucose Dulbecco's modified eagle medium supplemented with 10 mM glutamine, 10% foetal bovine serum and 50 $U \cdot mL^{-1}$ penicillin–streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were passaged or media refreshed every 2–5 days.

ATP production assay

Cells were placed in a white cell-culture treated 96-well plates (6000 cells/well) and incubated overnight. Medium was removed, wells washed with 100 µL sterile PBS, 100 µL fresh media (control or containing drug) added and the plate incubated for 24 h. One hundred microlitre of Promega Cell-Titer Glo reagent, which had been thawed in the dark for at least 2 h, was added. The plate was gently shaken for 2 min, equilibrated at room temperature for 10 min and the luminscence at 560 nm measured. In each experiment, measurements were taken in triplicate for each treatment.

O_2 consumption assay

Approximately 10^6 cells were suspended in MiR05 buffer [17] (2 mL) and placed in an Oroboros OxyGraph. After equilibration, cells were treated sequentially with digitonin (4 µM, to permeabilise the cells), (L)-malic acid (2 mM) and (L)-glutamic acid (10 mM, to feed complex I), ADP (1.2 mM, to provide an oxidative phosphorylation substrate), succinic acid (10 mM, to feed complex II) and rotenone (0.05 µM, to inhibit complex I).

Lipid quantitation

Prior to extraction, 25 µL of the internal standard $^{13}C_5$-Sphingosine (0.1 µM) was added to 80 µL of cell homogenate. Sphingosine was extracted as previously described [18]. Briefly, lipids were extracted by adding methanol, chloroform and water (1 : 1 : 0.9; v/v/v), and the upper phase was transferred to a new Eppendorf tube and further extracted with water/butanol extraction (1 : 1; v/v). Measurements were taken by reverse-phase liquid chromatography using a Waters UPLC-Xevo-TQS micro and a BEH C18 column, 2.1 × 50 mm with 1.7 µm particle size (Waters) at 23 °C.

Total cholesterol levels in cell lysates were determined after lipid extraction [19] using a colorimetric enzymatic kit (Biolabo) and measured in an EL-808 Ultra Microplate Reader (BIO-TEK Instruments Inc.).

Results and Discussion

NB-DNJ (miglustat, Zavesca®) is approved in Europe for the treatment of NPCD and is an inhibitor of GBA2, the enzyme that breaks down glucosylceramide on the cytosolic leaflet of membranes ('cytosolic GlcCer') [20]. Miglustat features a 4-carbon chain attached to nitrogen atom in a six-membered ring. Reasoning that GBA2 acts on a membrane bound lipid led to the idea that increasing the length of this chain would incorporate the inhibitor into membranes and so increase its effective concentration at the site of action. This tactic led to AMP-DNJ [21], a compound with greater GBA2 potency than miglustat though with some loss of selectivity over GlcCer synthase and lysosomal hydrolase GBA1 [20]. AMP-DNJ rather than miglustat was selected for use in this study as its greater potency is likely to reduce off-target effects and its efficacy in a mouse model of NPCD has been demonstrated [22]. We have previously demonstrated the correction of endocytic defects in NPCD cells using this compound at a concentration of 5–20 nM for 24 h [23]. In this case, treatment of NPC1-deficient fibroblasts with a concentration of 20 nM for the same length of time failed to correct reduced basal oxygen consumption or when specifically feeding respiratory complexes (Fig. 1A). Consistently, ATP production remained deficient on treatment (Fig. 1B) and was similarly unaffected in healthy cells. Reducing the incubation time to 4 h in search of a more transient effect was unsuccessful (Fig. 1B). This strengthens the idea that the clinical usefulness of GBA2 inhibitors in NPCD arises from their ability to correct endocytic errors and adds the contribution that such drugs'

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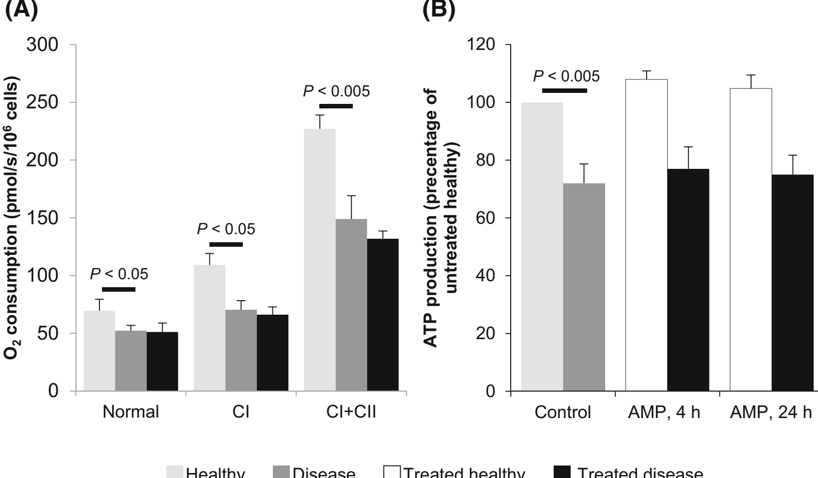

Fig. 1. AMP-DNJ does not correct respiratory insufficiency in NPC1-deficient cells. Neither reduced oxygen consumption (A, mean ± SEM, n = 4–8) nor reduced ATP production (B, mean ± SEM of four independent experiments conducted in triplicate) in NPC1-deficient fibroblasts is corrected by AMP-DNJ.

failure to halt disease progression completely may stem from their ineffectiveness at correcting mitochondrial defects. Niemann–Pick type C disease features an accumulation of sphingolipids and thus the clinical [24] and laboratory [22,23] observations that inhibition of GBA2, a sphingolipid hydrolase, is disease modifying is counter-intuitive. We wished to understand this further so we also tested a battery of drugs that target sphingolipid pathways and incubated both healthy and diseased cells with natural sphingolipids and synthetic analogues—none of these manipulations produced unambiguous evidence of an effect on mitochondrial function (Fig. S1).

Among the proteins implicated in transporting cholesterol into mitochondria is steroidogenic acute regulatory protein (StARD1), thought to transport cholesterol from the outer to the inner membrane [25]. Reduced expression of this protein led to improved mitochondrial function in NPC diseased cells [16]. Continuing with our pharmacological approach we investigated 21-acetoxy pregnenolone (21-AcP), which has been identified as an inhibitor of StARD1 [26]. Incubation with this agent at its IC_{50} of 10 μm did not alter ATP levels in either healthy or diseased cells (Fig. 2A). Thus, the concentration was increased to 40 μm and cells treated for either 4 or 24 h. At the longer time point, this yielded a small increase in ATP levels in diseased cells (79 ± 4% of untreated healthy to 86 ± 1%, Fig. 2A) but a similar change in healthy cells (100–110 ± 2%) questions whether this is through an impact on pathology or is a more general phenomenon.

To investigate this further, we identified small molecule binders of StARD1 through Virtual High-Throughput Screening (VHTS) using Atomwise's proprietary AI-based AtomNet® screening platform, as previously described [27,28]. Briefly, the sterol-binding domain of human apo-STARD1 (PDB: 3P0L [29]) was defined as shown in Fig. S2 and used to score and rank a curated library of small molecule binders (Mcule_v20201015 containing 4 251 237 compounds). The top ranked 200 molecules were reduced to a list of 86 compounds with drug-like properties calculated with ICM (Molsoft). These 86 compounds were purchased for testing at stock concentrations of 10 mm in DMSO and ≥ 85% purity by LC–MS. Identity of the compounds was blinded during experimental screening and compound identity and structures were revealed after data were returned to Atomwise (SMILES strings given in Table S1).

None of these compounds gave unambiguous evidence of correcting the mitochondrial defect as measured by cellular ATP levels (Fig. 2B). Although a 10 μm dose of few compounds increased ATP in diseased cells from 79 ± 4% of untreated healthy to ca. 90%, increasing the dose to 50 μm did not deliver further increases in ATP production. In fact the higher dose of these promising compounds gave ATP levels indistinguishable from untreated diseased cells. We suggest, therefore, that either the apparent improvements at 10 μm reflect noise in the assay rather than real changes or that the compounds were toxic at 50 μm mitigating any beneficial effects. We thus turned our attention elsewhere in our search for a drug that would correct the mitochondrial defect in NPC1-deficiency.

Translocator protein (TSPO) is a protein of the outer mitochondrial membrane, which has been implicated in numerous central nervous system pathologies [30–33]. It was initially named the peripheral benzodiazepine receptor (PBR) with the updated nomenclature being adopted to reflect the evolving view that this protein translocates substrates across the outer mitochondrial, a view that nonetheless remains controversial [34].

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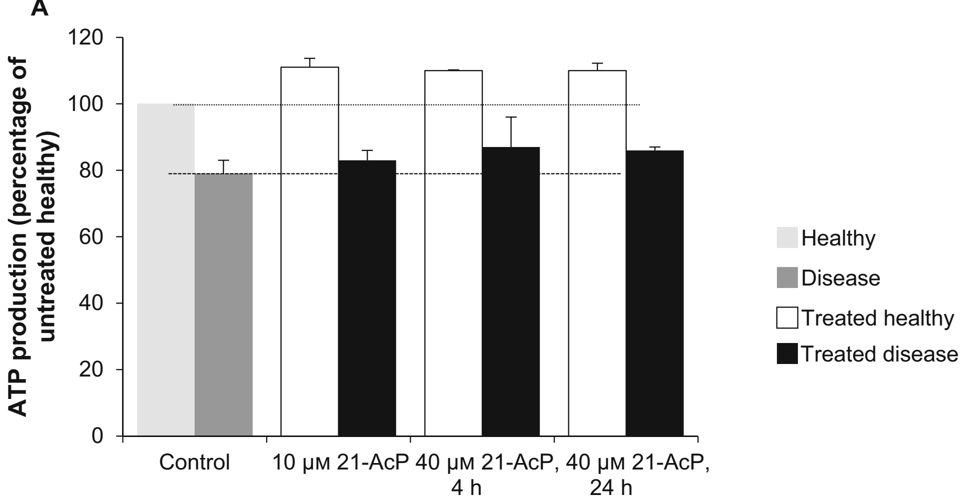

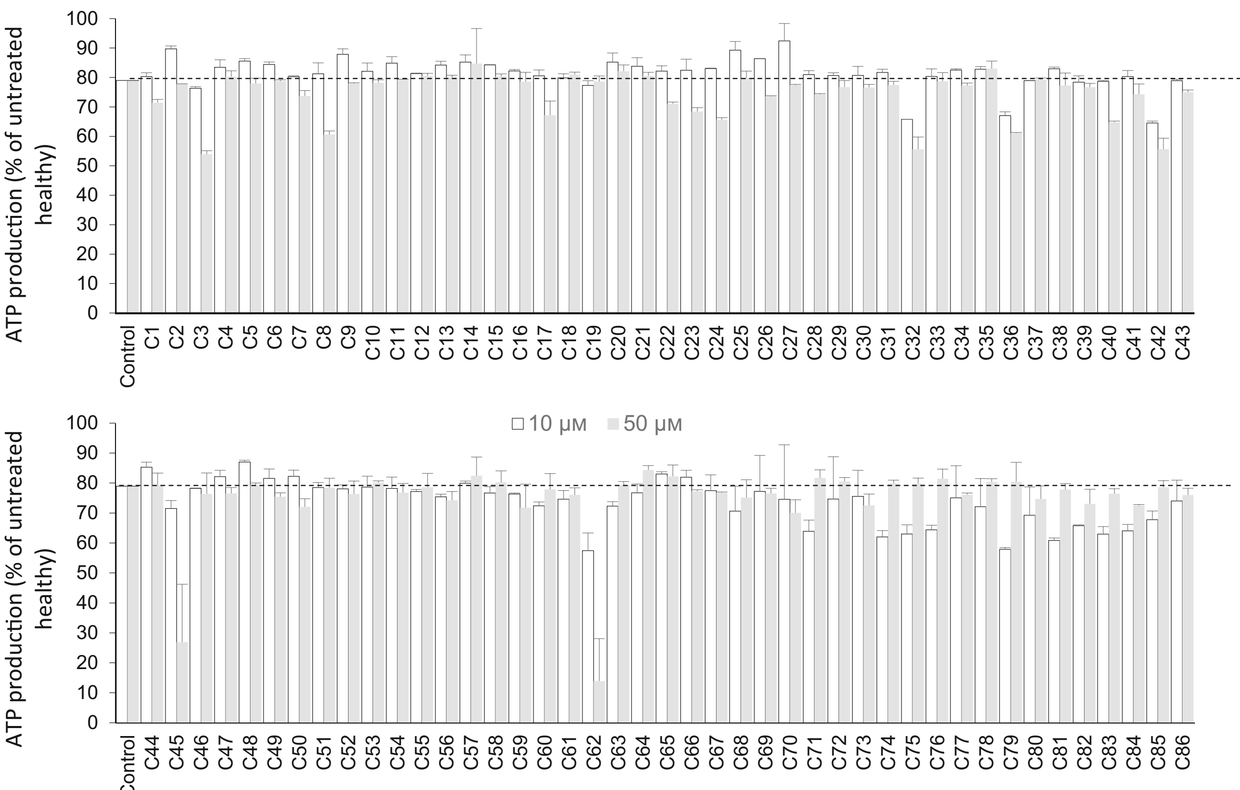

Fig. 2. StARD1 blockade does not correct respiratory insufficiency in NPC1-deficient cells. (A) Known inhibitor 21-AcP slightly increased ATP levels in disease and healthy cells. (B) A small library of putataive StARD1 blockers show only negligible effects on ATP levels in diseased cells. All data are mean ± SD of two independent experiments conducted in triplicate.

However, there is a growing body of work that finds this protein involved in mitochondrial cholesterol import [25,35]. Translocator protein was predicted to be a cholesterol-binding protein [36], and binding was later unambiguously demonstrated at the predicted region [37,38]. Since then, various TSPO ligands have been

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shown to provoke cholesterol efflux in eye epithelial cells [39,40], macrophages [41] and astrocytes and fibroblasts [42]. Thus, given the cholesterol overload hypothesis in NPCD, we considered TSPO to be a target worth investigating.

Previous work has identified two ligand binding sites on TSPO, both of which overlap with the cholesterol binding site (Fig. S3). As expected, incubation with drugs that target each of these binding sites (etifoxine, PK11195 and Ro5-4864) at the recommended concentrations [43] for 24 h produced decreases in cellular cholesterol in diseased cells that fell just outside the margin of statistical significance, probably due to the relatively large error in measurements of untreated cells. No change in cholesterol levels in healthy cells was observed (Fig. 3A). Effects on ATP production were mixed: PK11195 did not enhance it while Ro5-4864 increased it slightly (79 ± 4% of untreated healthy to 86 ± 3%, Fig. 3B) but, similar to 21-AcP (Fig. 2A), produced a similar increase in healthy cells (100–109 ± 6%) suggesting this treatment may not genuinely be disease modifying. In contrast, use of 20 µM etifoxine for 24 h increased ATP production in NPC1-deficient cells from 79 ± 4% of untreated healthy to 93 ± 3% but produced only a negligible increase in healthy cells (100–105 ± 5%, Fig. 3B). This treatment was then examined in more detail in the oxygen consumption assay but failed to correct the defect in oxygen consumption (Fig. 3C) either at basal levels or when specifically feeding complexes in the

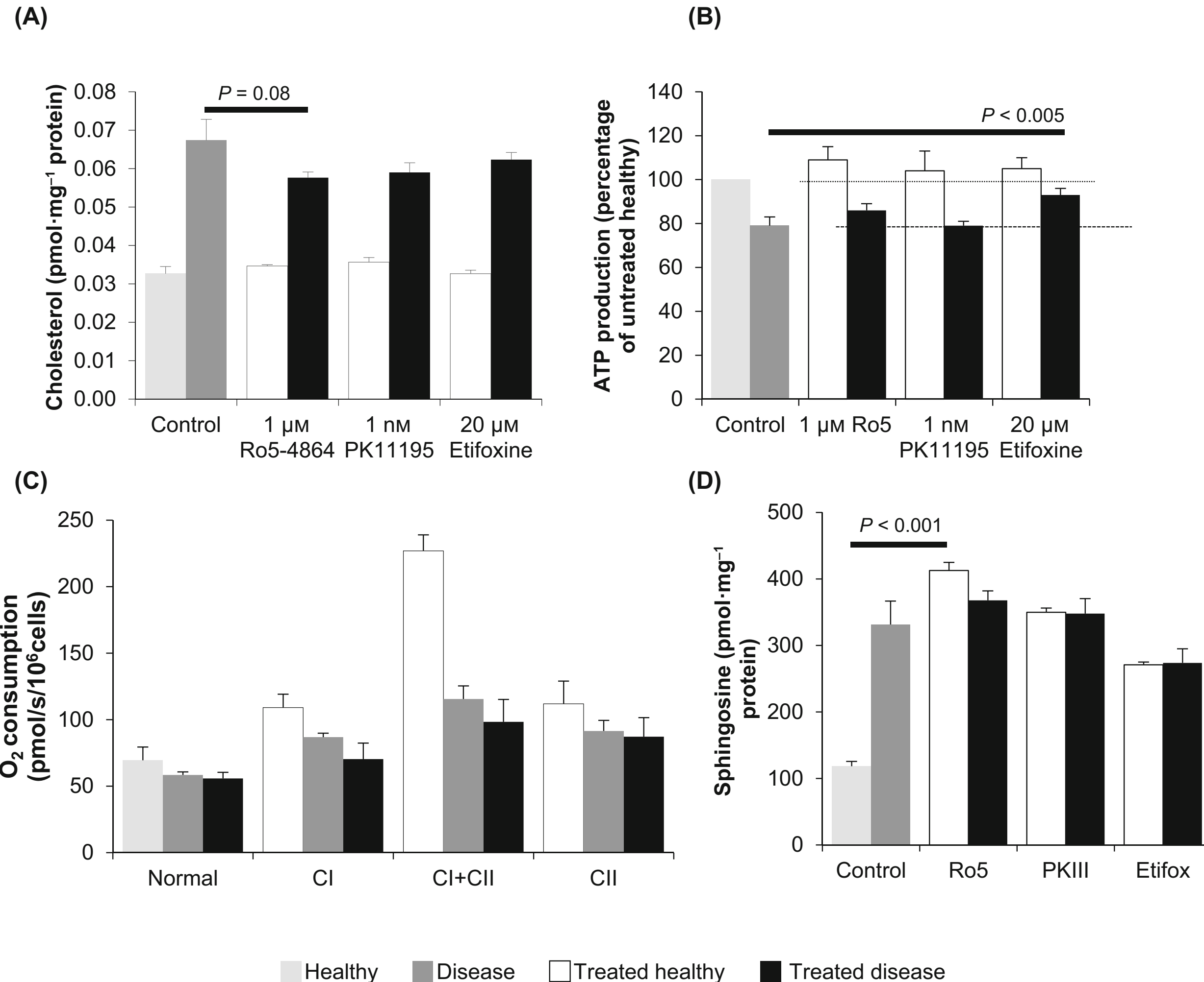

Fig. 3. Effects of TSPO ligands. (A) TSPO ligands produce only small reductions in whole cell cholesterol in diseased cells (mean ± SEM of three independent measurements). (B) Effects of TSPO ligands on ATP production in diseased cells (mean ± SEM of three independent experiments conducted in triplicate). (C) Etifoxine does not correct reduced O_2 consumption in diseased cells (mean ± SEM, n = 3–4). (D) Whole cell sphingosine is increased by TSPO ligands in healthy cells but not in diseased cells (mean ± SEM of three independent measurements).

electron transport chain. This may imply that the enhancements in ATP production arose from etifoxine acting through a nonmitochondrial pathway. This is consistent with recent work suggesting that off-target effects of TSPO ligands are not uncommon [44].

NPC1-deficient cells thus show a mitochondrial defect that appears immune to drugs targeting its hypothesised origin; we therefore asked whether a lipid other than cholesterol could be responsible for this aspect of NPCD pathology. Sphingosine is known to accumulate in NPC1-deficiency, has previously been proposed as a substrate for NPC1 [45], (an idea supported by recent evidence [46]) and has been shown to induce mitochondrial dysfunction at high doses [47]. To our surprise, treatment of healthy cells with all three TSPO ligands increased whole cell sphingosine to levels comparable to those seen in diseased cells (Fig. 3D). While the mechanism behind this increase is unknown, it was not accompanied by any reduction in ATP production (Fig. 3B) suggesting that sphingosine accumulation in diseased cells is unlikely to be responsible for the mitochondrial defect. (For sphingosine to be the culprit there would also have to be a plausible transport for this lipid from lysosomes to mitochondria). Computational work suggests that cholesterol-transporting StAR proteins cannot be used for this purpose (Fig. S4) though see also [48].

In summary, our attempt to correct the mitochondrial defect in NPCD with an inhibitor of GBA2, which is known to be disease-modifying in mice [22], was unsuccessful. Ligands of mitochondrial cholesterol-binding protein TSPO, which appeared to reduce mitochondrial cholesterol, also had no clear effect on mitochondrial function. Similarly, blockers of StARD1, a protein involved in transporting cholesterol to mitochondria, did not correct defective ATP production. This is particularly surprising as this parameter has been corrected in diseased cells by reduced expression of this protein [16]. This disconnect between genetic and pharmacological approaches to the same target underlines how incomplete our understanding is of the mitochondrial defect in NPCD.

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Author contributions

SW designed study, analysed data, performed experiments and drafted paper; MB performed experiments; VK designed StARD1 blockers; MJF lipid quantitation; JMFGA supervision; DJS designed study, analysed data, supervision and edited manuscript.

Peer review

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Data accessibility

Excel files containing the original data recorded and corresponding statistical analysis for all experiments are available upon request.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Sphingolipid manipulations do not correct impaired ATP production in NPC1-deficient cells.

Fig. S2. Residues used to define the cholesterol binding pocket of StARD1.

Fig. S3. Structure of the TSPO protein (PDB: 2no2, Jaremko M *et al* (2015) *ChemBioChem* **16**, 1483–1489) showing the overlap of the cholesterol binding site with those for known ligands used in this study; translucent discs represent the approximate positions of the membrane.

Fig. S4. Sphingosine is unlikely to be able to use the StAR family of cholesterol transport proteins.

Table S1. SMILES strings for putative StARD1 blockers used in Fig. 2.

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