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Ursocholanic acid rescues mitochondrial function in common forms of familial Parkinson's disease

Heather Mortiboys,[1] Jan Aasly[2] and Oliver Bandmann[1]

1 Sheffield Institute for Translational Neuroscience (SITraN), University of Sheffield, Sheffield, UK
2 Department of Neurology, St Olav's Hospital, Trondheim, Norway

Correspondence to: Oliver Bandmann, MD PhD
Sheffield Institute for Translational Neuroscience (SITraN),
Department of Neuroscience,
University of Sheffield,
385a Glossop Road,
Sheffield S10 2HQ, UK
E-mail: o.bandmann@sheffield.ac.uk

Previous drug screens aiming to identify disease-modifying compounds for Parkinson's disease have typically been based on toxin-induced *in vitro* and *in vivo* models of this neurodegenerative condition. All these compounds have failed to have a reliable disease-modifying effect in subsequent clinical trials. We have now established a novel approach, namely to screen an entire compound library directly in patient tissue to identify compounds with a rescue effect on mitochondrial dysfunction as a crucial pathogenic mechanism in Parkinson's disease. The chosen Microsource Compound library contains 2000 compounds, including 1040 licensed drugs and 580 naturally occurring compounds. All 2000 compounds were tested in a step-wise approach for their rescue effect on mitochondrial dysfunction in *parkin* (*PARK2*) mutant fibroblasts. Of 2000 compounds, 60 improved the mitochondrial membrane potential by at least two standard deviations. Subsequently, these 60 compounds were assessed for their toxicity and drug-like dose-response. The remaining 49 compounds were tested in a secondary screen for their rescue effect on intracellular ATP levels. Of 49 compounds, 29 normalized ATP levels and displayed drug-like dose response curves. The mitochondrial rescue effect was confirmed for 15 of these 29 compounds in *parkin*-mutant fibroblasts from additional patients not included in the initial screen. Of 15 compounds, two were chosen for subsequent functional studies, namely ursocholanic acid and the related compound dehydro(11,12)ursolic acid lactone. Both compounds markedly increased the activity of all four complexes of the mitochondrial respiratory chain. The naturally occurring compound ursolic acid and the licensed drug ursodeoxycholic acid are chemically closely related to ursocholanic acid and dehydro(11,12)ursolic acid lactone. All four substances rescue mitochondrial function to a similar extent in *parkin*-mutant fibroblasts, suggesting a class effect. The mitochondrial rescue effect depends on activation of the glucocorticoid receptor with increased phosphorylation of Akt and was confirmed for both ursocholanic acid and ursodeoxycholic acid in a Parkin-deficient neuronal model system. Of note, both ursocholanic acid and ursodeoxycholic acid also rescued mitochondrial function in *LRRK2*^{G2019S} mutant fibroblasts. Our study demonstrates the feasibility of undertaking drug screens in Parkinson's disease patients' tissue and has identified a group of chemically-related compounds with marked mitochondrial rescue effect. Drug repositioning is considered to be a time- and cost-saving strategy to assess drugs already licensed for a different condition for their neuroprotective effect. We therefore propose both ursolic acid as a naturally occurring compound, and ursodeoxycholic acid as an already licensed drug as promising compounds for future neuroprotective trials in Parkinson's disease.

Keywords: Parkinson's disease; parkin; LRRK2; mitochondria; disease-modifying therapy

Abbreviations: DUA = dehydro(11,12)ursolic acid lactone; MPTP = 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

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Introduction

Parkinson's disease is a common and relentlessly progressive, incurable neurodegenerative condition. Its world-wide prevalence is expected to double by 2030 (Dorsey *et al*., 2007). Currently available drugs only result in symptomatic improvement with limited efficacy. In the past, compounds were typically tested for their putative neuroprotective effect in toxin-induced, *in vitro* and *in vivo* models of Parkinson's disease. However, exposure to toxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) only partially resembles the mechanisms leading to Parkinson's disease, if at all. Subsequently undertaken clinical trials failed to confirm a beneficial, disease-modifying effect for any compound with a promising initial effect in these traditional MPTP models (Lang, 2006). Mitochondrial dysfunction is a key mechanism in the pathogenesis of both sporadic and familial Parkinson's disease (Exner *et al*., 2012). Mutations in the autosomal recessively inherited *parkin* (also known as PARK2) gene are the most common identifiable cause of early-onset Parkinson's disease. The *LRRK2*^{G2019S} mutation is the most common identifiable cause of monogenically inherited late-onset Parkinson's disease (Hardy, 2010). We have previously demonstrated abnormal mitochondrial function with specific lowering of complex I activity of the mitochondrial respiratory chain in skin fibroblasts of *parkin*-mutant patients with Parkinson's disease (Mortiboys *et al*., 2008). We and others subsequently also reported mitochondrial dysfunction in fibroblasts from patients with the *LRRK2*^{G2019S} mutation (Mortiboys *et al*., 2010; Papkovskaia *et al*., 2012).

The aim of this study was to undertake an *in vitro* compound screen in Parkinson's disease mutant patient tissue to identify mitochondrial rescue compounds. Our project is based on the hypothesis that any compound with a robust mitochondrial rescue effect in Parkinson's disease patient tissue is more likely to exert a subsequent beneficial effect in clinical trials than those compounds that have only been tested in toxin-induced model systems. Two thousand compounds from the Microsource Spectrum Collection (www.msdiscovery.com) were assessed for their rescue effect on mitochondrial function in several stages. This compound library consists of 1040 licensed drugs, 580 natural compounds and 420 other bioactive compounds. The large proportion of licensed drugs and natural compounds made it plausible to assume that any positive hits in our compound screen could rapidly be taken into clinical trials.

Materials and methods

Patients

The project was reviewed by the local ethics committee. Informed consent was taken from all research participants (see Supplementary Table 1 for further information on all patients included in this study). There was no significant difference in age between the four *parkin*-mutant patients and their four matched controls (age in years ± SD *parkin*-mutant patients, 40.5 ± 6.5; controls, 38.5 ± 5.5). Similarly, there was no significant difference in age between the three *LRRK2*^{G2019S} mutant patients and their three matched controls (*LRRK2*^{G2019S} mutant patients, age 59 ± 5.5; controls, age 61 ± 4.5). Groups were also sex matched.

Methods

Fibroblast cell culture conditions as well as measurement of mitochondrial membrane potential, respiratory chain function and cellular ATP production were carried out as previously described (Mortiboys *et al*., 2008).

Z-scores

In order to assess the robustness and reproducibility of the assays used as primary and secondary screens we undertook rigorous testing using Z′ and SW score calculations as described (http://www.ncats.nih.gov/). See Supplementary material for further information.

Primary drug screen

Stage 1

Parkin-mutant fibroblasts from two *parkin*-mutant patients were incubated with all 2000 Microsource Spectrum Collection compounds for 24 h at a concentration of 10 μM. Each drug treatment was carried out in duplicate, thus, a total of four drug exposure experiments were carried out at the first stage for each compound. A positive hit was defined *a priori* as a compound that would improve the mitochondrial membrane potential by more than 3 standard deviations (SD) in at least three of the experiments and by at least 2 SD in the fourth experiment. Positive hits were then tested further in cell-free assays to exclude a possible false-positive effect due to autofluorescence of the drug or a drug interaction with tetramethylrhodamine methyl ester (TMRM). In addition, compounds were tested for any cellular toxicity effects using the lactate dehyodrogenase (LDH) assay as described previously (Mortiboys *et al*., 2008). Furthermore, dose-response assessments (0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30 and 100 μM) were undertaken to determine the shape of the dose response curves.

Stage 2

Positive hits from the Stage 1 experiments were assessed for their rescue effect on intracellular ATP levels. As before, *parkin*-mutant fibroblasts from the same two patients and matched controls were treated twice at a concentration of 10 μM for 24 h. A positive hit at Stage 2 was again defined as a compound that improved intracellular ATP levels by at least 3 SD in at least three experiments and by at least 2 SD in the fourth. Positive hits were tested for dose response curves (0.01–100 μM) again. Positive hits with a sigmoidal dose-response curve were tested for their recovery effects on ATP levels in an additional two *parkin*-mutant patient and matched control fibroblast lines.

Stage 3

Selected top hits from Stage 2 were then assessed further for their effect on the four individual mitochondrial respiratory chain complexes in fibroblasts from four *parkin*-mutant patients and matched control subjects. Fibroblasts (~1.4×10^7 cells) were treated for 24 h with 100 nM of each compound before being harvested by trypsinization and used for all further analyses. Mitochondrially enriched fractions and individual mitochondrial respiratory chain assays were all done as described previously (Mortiboys *et al*., 2008). All data are expressed to mg protein. Protein was measured using the Bradford assay (Peirce) as per the manufacturers' instructions.

Functional studies

Pharmacological inhibition of glucocorticoid receptor

Fibroblasts were plated (5000 cells per well) into 96 well plates. After 24 h, cells were treated with 1 μM RU486 for 4 h before adding either 100 nM of selected compounds (see 'Results' section). Cellular ATP levels were measured 24 h later as described above.

Small interfering RNA glucocorticoid receptor knockdown

Small interfering RNA oligonucleotides were targeted to the glucocorticoid receptor gene (*NR3C1*), target sequence AAGTG CAAACCTGCTGTGTTT or scramble control small interfering RNA (both Qiagen). Small interfering RNAs (10 nM) (*NC3C1*-targeted or scramble negative) were transfected into fibroblasts using 0.5 mM Lipofectamine® 2000 according to the manufacturers' instructions. Knockdown efficiency of the glucocorticoid receptor protein was assessed using the glucocorticoid receptor ELISA (Abnova) at 48 h post-transfection as per the manufacturer's instructions. Twenty-four hours post-transfection cells were treated with 100 nM of selected compounds; cellular ATP levels were measured 24 h later as described above.

Quantification of total Akt and phosphorylated Akt at Ser^{473}

Akt and phosphorylated (p)Akt Ser^{473} ELISAs (Invitrogen) were performed on fibroblast cell lysates as per the manufacturer's instructions using the provided standards to calculate the amount of protein present. All data are presented as a ratio of pAkt (Ser^{473}): total Akt.

Pharmacological inhibition of the Akt pathway

Fibroblasts were plated (5000 cells per well) into 96 well plates. After 24 h, cells were treated with 1 μM LY294002 or 50 nM triciribine for 15 min before adding 100 nM of selected compounds. Cellular ATP levels were measured 24 h later as described above.

Confirmatory experiments

Mouse cortical neurons were prepared from embryonic Day 15 mouse embryos as previously described (Kasher *et al*., 2009). Approximately 6×10^4 neurons were plated into each well of a 96-well plate (previously coated with poly-L-lysine) or 2×10^5 neurons were plated into each well of a 24-well plate for either ATP assays or harvesting for western blot analysis or fixed for imaging. After 5 days in culture neurons we transfected using the Accell siRNA and Accell siRNA media (as per the manufacturer's instructions, Dharmacon) with either scramble-negative control small interfering RNA (Accell mouse control siRNA kit, Dharmacon) or *parkin* small interfering RNA (sequence GUUUCCACUUGUAUUGUGU). Forty-eight hours post-transfection neurons were dosed with various concentrations of compounds and 24 h later the cellular ATP assay was performed as described above, or neurons were harvested for western blotting. Western blotting was performed as described previously (Mortiboys *et al*., 2008). Coverslips were fixed with 4% paraformaldehyde for 30 min with subsequent PBS washes. Cells were permeabilized with 0.1% Triton™ X-100 for 10 min at room temperature and blocked with 1% goat serum for 1 h. Cells were incubated with primary antibodies (rabbit anti-Parkin; Abcam and mouse anti-TOM-20; BD Biosciences) at 1:500 overnight at 4°C with subsequent PBS washes and incubation with rabbit anti-mouse and goat anti-rabbit secondary antibodies for 1 h at room temperature. Cells were stained with Hoescht and then mounted into glass slides using ProLong® Gold (Invitrogen).

Cellular ATP levels were measured as described previously (Mortiboys *et al*., 2008) in fibroblasts from three $LRRK2^{G2019S}$ mutant patients with Parkinson's disease and three age and sex-matched controls. The cells were treated with 100 nM of the selected compounds for 24 h before measurement.

Statistical analysis

Values from multiple experiments were expressed as means ± SE (standard error). Statistical significance (Bonferroni corrected) was assessed using Student's *t*-test for data with a normal distribution, a non-parametric *t*-test was used for data with a skewed distribution. The effect of multiple factors was assessed using a two-way ANOVA test.

Results

A summary of our screening strategy is given in Fig. 1. Of 2000 compounds, 60 improved the mitochondrial membrane potential in *parkin*-mutant fibroblasts by >3 SD in three of the four experiments and by >2 SD in the fourth. Two compounds elicited an increase in the TMRM fluorescence signal in subsequent cell-free assays and were thus excluded as false-positive. A further nine compounds had to be excluded due to their toxicity (Table 1). Full dose-response curves were established for all 49 remaining compounds, which were then also further assessed for their effect on total intracellular ATP levels. Of 49 compounds, 35 increased the ATP levels in the *parkin*-mutant fibroblasts by >3 SD in at least three experiments and by >2 SD in the fourth (Table 1). Full dose-response curves were carried out using these top 35 compounds. Six compounds did not display a drug-like, sigmoidal dose response curve and were therefore excluded, leaving 29 compounds.

Each of these 29 compounds was then tested for their rescue effect on cellular ATP levels in a further two patient fibroblast lines and two control fibroblast lines. Of 29 compounds, 15 rescued cellular ATP levels by >3 SD in all four *parkin*-mutant fibroblast lines tested (Table 2).

Of 15 compounds, two, namely ursocholanic acid and dehydro (11,12) ursolic acid lactone (DUA), were selected for further assessment. Reasons for not investigating the remaining 13 compounds forward at this stage are listed in Table 2 and, in greater detail, in the Supplementary material. Ursocholanic acid and DUA were then further assessed for their effect on the activity of complexes I–IV of the respiratory chain. Ursocholanic acid significantly rescued and increased the activity of complexes I–IV by 200–500% (Fig. 2). Treatment with DUA achieved very similar results (Supplementary Fig. 1).

Interestingly, 7 of the 15 Stage 2 positive hits were steroids or related compounds with four carbon rings forming the (steroid) backbone of each particular compound, including ursocholanic acid and DUA (Table 2). We therefore hypothesized that their observed rescue effect was mediated through activation of the glucocorticoid receptor. To further test this hypothesis, *parkin*-mutant cells were pretreated with the glucocorticoid receptor antagonist RU486 to determine whether glucocorticoid receptor

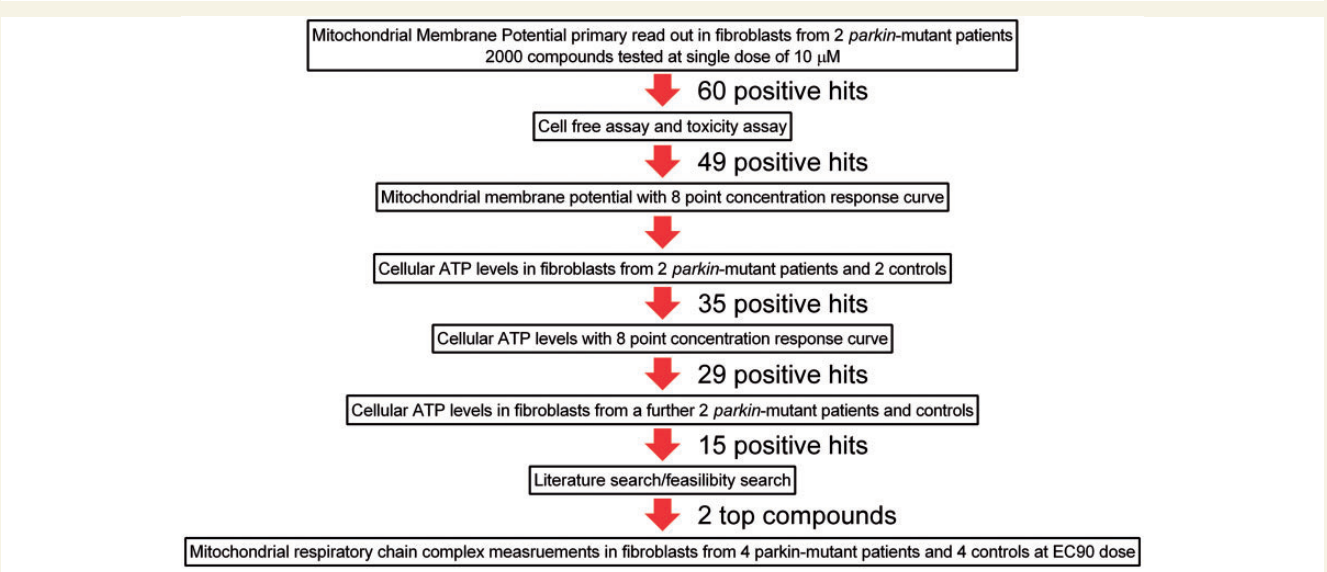

Figure 1 This flowchart shows an overview of the screening strategy used. Each part of the screen is depicted as is the number of positive hit compounds that were taken to the next stage of the screen.

inhibition may abolish the observed rescue effect of DUA and ursocholanic acid on ATP levels. As predicted, RU486 completely eliminated the rescue effect of all tested compounds on cellular ATP levels (Fig. 3A). To further validate these results, we used a different method, namely small interfering RNA-mediated glucocorticoid receptor knockdown before treatment with ursocholanic acid or DUA. Glucocorticoid receptor protein knockdown was confirmed to be 75% ± 3.8% (mean ± SD) using ELISA at 48 h post transfection (data not shown). As predicted, small interfering RNA-mediated glucocorticoid receptor knockdown abolished the rescue effect of 100 nM ursocholanic acid or DUA on intracellular ATP levels (Fig. 3B).

Ursolic acid and ursodeoxycholic acid were not part of the initially screened Microsource Compound Library, but are chemically related to ursocholanic acid and DUA (Fig. 4). Ursolic acid exerts its beneficial effect on muscle atrophy through Akt activation, namely by increased phosphorylation of Akt at Ser^{473} (Kunkel *et al.*, 2011). Similarly, ursodeoxycholic acid exerts its protective effect against mitochondria-dependent programmed cell death in SH-SY5Y cells through Akt activation (Chun and Low, 2012).

We therefore assessed the effect of DUA and ursocholanic acid in *parkin*-mutant fibroblasts on Akt phosphorylation at Ser^{473}. There was a marked increase in the pAktSer^{473}:Akt protein ratio by 400% after treatment with DUA and 305% after treatment with ursocholanic acid ($P < 0.05$) in *parkin*-mutant fibroblasts compared with the ratio in untreated *parkin*-mutant fibroblasts (Fig. 5A). Interestingly, this change was only evident in *parkin*-mutant fibroblasts, the pAktSer^{473}:Akt ratio in control fibroblasts remained constant after drug treatment. We next aimed to confirm that both ursocholanic acid and DUA are exerting their mitochondrial rescue effect through activation of the Akt pathway rather than Akt activation merely being associated with the rescue effect of our top compounds. As predicted, pretreatment with either Akt inhibitor LY29400 (a phosphatidylinositol 3-kinase inhibitor) or triciribine (a selective inhibitor of cellular phosphorylation/activation of Akt) abolished the rescue effect of ursocholanic acid and DUA on cellular ATP levels in *parkin*-mutant fibroblasts (Fig. 5B).

Neither DUA nor ursocholanic acid are FDA-licensed drugs; little information is available on their bioavailability and safety in humans. In contrast, the chemically closely related bile acid ursodeoxycholic acid has been in clinical use as treatment for primary biliary cirrhosis for >30 years. Its clinical pharmacokinetics are well characterized (Ward *et al.*, 1984). The chemically closely related ursolic acid is a naturally occurring compound present in many plants. Based on their structural similarities, we hypothesized that both ursolic acid and ursodeoxycholic acid may have a similar mitochondrial rescue effect as DUA and ursocholanic acid. Indeed, both ursolic acid and ursodeoxycholic acid normalized intracellular ATP levels similar to the effect observed for DUA and ursocholanic acid (Fig. 6).

Effect in Parkin-deficient neuronal model system

We next assessed the rescue effect of ursocholanic acid and ursodeoxycholic acid in a neuronal cell culture model. Small interfering RNA mediated knockdown of *parkin* resulted in a reduction of Parkin protein levels by 80% in cortical mouse neurons as shown by western blotting and a decrease in cellular ATP levels by 40%. Treatment with 10 pM ursocholanic acid or 10 pM ursodeoxycholic acid rescued the cellular ATP loss in these Parkin-deficient neurons (Fig. 7). Thus, ursocholanic acid and ursodeoxycholic acid have a rescue effect on mitochondrial dysfunction not only in *parkin*-mutant fibroblasts but also in parkin-deficient neurons.

Rescue effect in *LRRK2*^{G2019S} mutant patient tissue

We finally determined whether ursocholanic acid and the chemically related and FDA-licensed drug ursodeoxycholic acid also have a mitochondrial rescue effect in other forms of familial

Table 1 Positive hits of the primary screen, and results from stage 1 and 2 of the drug screen

Drug name	Stage 1			Stage 2		
	Cell free	Toxicity	EC50 MMP	ATP recovery	Dose response curve	EC50 ATP
Podophyllotoxin acetate	✓	✓	Ambiguous	X	X	X
2,6-Dimethoxyquinone	✓	✓	1 μM	X	X	X
Ginkgolic acid	✓	✓	1.6 mM	✓	✓	250 nM
2′,Beta-dihydroxychalcone	✓	✓	200 nM	✓	✓	250 nM
Gatifloxacin	✓	✓	100 nM	✓	✓	250 nM
Amlodipine besylate	✓	✓	1 μM	✓	✓	250 nM
Simvastatin	✓	✓	1 μM	X	X	X
Hydroquinone	✓	✓	1 μM	X	X	X
7-Methoxychromone	✓	✓	100 nM	X	X	X
Perindopril erbumine	✓	✓	12 μM	✓	✓	150 nM
Ceftibuten	✓	✓	1 μM	✓	✓	250 nM
Cefdinir	✓	✓	25 μM	✓	✓	350 nM
3Alpha-hydroxy-3-deoxyangolensic acid methyl ester	✓	✓	100 nM	✓	✓	150 nM
Dibenzothiophene	✓	✓	600 nM	X	X	X
Clonidine hydrochloride	✓	✓	1 μM	X	X	X
Desipramine hydrochloride	✓	X	X	X	X	X
Ginkgolide a	✓	✓	100 nM	✓	✓	150 nM
Sericetin	✓	✓	158 nM	✓	✓	150 nM
Friedelin	✓	✓	1 μM	✓	✓	150 nM
3Beta,7beta-diacetoxydeoxodeacetoxydeoxydihydrogedunin	✓	✓	240 nM	X	X	X
Oleanolic acid acetate	✓	X	X	X	X	X
Pristimerol diacetate	✓	✓	631 μM	✓	✓	125 nM
Khellin	✓	✓	6 μM	✓	✓	250 nM
Khivorin	✓	✓	6 μM	X	X	X
Allopurinol	✓	✓	1 μM	X	X	X
Menthone	✓	X	7 mM	X	X	X
Acetylcholine	✓	X	60 μM	X	X	X
Probenecid	✓	X	13 μM	X	X	X
Enalapril maleate	✓	X	2 μM	X	X	X
Acivicin	✓	X	31 mM	X	X	X
Ephedrine (1 R,2S) hydrochloride	✓	✓	Ambiguous	✓	X	X
Propylthiouracil	✓	✓	Ambiguous	✓	X	X
Clobetasol propionate	✓	✓	10 μM	✓	✓	1 μM
Santonin	✓	✓	125 nM	X	X	X
Ursocholanic acid	✓	✓	1 μM	✓	✓	350 nM
Methylergonovine maleate	✓	✓	Ambiguous	✓	X	X
Androsterone sodium sulfate	✓	✓	5 mM	✓	✓	350 nM
Dehydro (11,12)ursolic acid lactone (no longer available)	✓	✓	100 μM	✓	✓	350 nM
Cholest-5-en-3-one	✓	✓	1 μM	✓	X	X
Fluorometholone	✓	✓	350 nM	✓	X	X
Prazosin hydrochloride	✓	✓	250 nM	✓	✓	150 nM
Narasin	✓	X	X	X	X	X
Cedryl acetate	✓	X	X	X	X	X
N-benzyltropan-4-ol	X	✓	X	X	X	X
Naproxol	X	✓	X	X	X	X
Hydroxychloroquine sulphate	✓	✓	1.2 mM	✓	✓	1 μM
11-Oxoursolic acid acetate (no longer available)	✓	✓	0.1 nM	✓	✓	150 nM
Prednisolone	✓	✓	446 mM	✓	✓	350 nM
Ebselen	✓	✓	100 nM	✓	✓	1 μM
Racephedrine hydrochloride	✓	✓	5 μM	X	X	X
Snap (*S*-nitroso-*N*-acetylpenicillamine)	✓	✓	200 nM	X	X	X
3-Amino-beta-pinene	✓	✓	10 μM	✓	✓	1 μM
Benzalkonium chloride	✓	✓	12.5 nM	✓	✓	1 μM
Melezitose	✓	✓	1 μM	✓	✓	1 μM

(continued)

Table 1 Continued

Drug name	Stage 1			Stage 2		
	Cell free	Toxicity	EC50 MMP	ATP recovery	Dose response curve	EC50 ATP
3-Oxoursan (28-13)olide	✓	✓	1 μM	X	X	X
Budesonide	✓	✓	390 nM	✓	✓	1 μM
Prednisolone acetate	✓	✓	150 nM	✓	✓	1 μM
Furegrelate sodium	✓	✓	3.9 nM	X	X	X
Tamoxifen citrate	✓	✓	1 nM	✓	✓	1 μM
6,7-Dichloro-3-hydroxy-2-quinoxalinecarboxylic acid	✓	✓	Ambiguous	✓	X	X

This table details the positive hits of the primary screen and the results from each part of stage 1 and stage 2 of the drug screen. ✓ indicates that the compound fulfilled the necessary criteria and went through this particular stage; X indicates it did not and was therefore not taken any further. 'Cell free' indicates whether the compound reacted with the fluorescent dye tetramethylrhodamine methyl ester (TMRM) in a cell free assay. The 'Tox' column provides information on possible toxicity of the respective compound. 'EC50 MMP' indicates the EC50 concentration of the compounds in the mitochondrial membrane potential assay. 'ATP recovery' indicates if the compounds were also effective in recovering the ATP levels in *parkin*-mutant fibroblasts. 'Dose response curve' indicates whether the compounds displayed a known characterised dose response curve shape. 'EC50 ATP' provides information about the EC50 of the compounds in the cellular ATP assay.

Table 2 Top 15 hits that rescued the mitochondrial membrane potential and cellular ATP levels in all four patients and had drug-like dose response curves

Drug name	Compound origin	Steroid like structure	Additional comments
Gatifloxacin	Synthetic	X	Antibiotic with negative effect on glucose homeostasis and neurological function *in vivo*
Amlodipine besylate	Synthetic	X	Ca-antagonist, concerns about side-effect profile (including oedema, insomnia, dizziness, depression)
3Alpha-hydroxy-3-deoxyangolensic acid methyl ester	Natural	X	No information on use in humans or rodents
Ginkgolide a	Natural	X	Previous studies have given inconsistent results for neuroprotective effect of ginkgo in neurodegenerative disease and related model systems
Pristimerol diacetate	Semi synthetic	X	No information on use in humans or rodents
Ephedrine (1R,2S) hydrochloride	Natural	X	Sympatomimetic amine, intolerance and drug interaction likely in Parkinson's disease
Ursocholanic acid	Natural	✓	Taken forward
Androsterone sodium sulphate	Semi synthetic	✓	Steroid, excluded due to likelihood of side effects on long term treatment
Dehydro (11,12)ursolic acid lactone	Natural	✓	Taken forward
Cholest-5-en-3-one	Semi synthetic	✓	cholesterol, excluded due to likelihood of side effects on long term treatment
Hydroxychloroquine sulphate	Synthetic	X	Inhibitory effect on mitophagy
11-Oxoursolic acid acetate	Natural	✓	Unable to obtain more of the compound
Budesonide	Semi synthetic	✓	Steroid with high-first pass effect, excluded due to likelihood of limited biological availability
Prednisolone acetate	Semi synthetic	✓	Steroid, excluded due to likelihood of side effects on long term treatment
Tamoxifen citrate	Synthetic	X	Can cause cognitive impairment and other major side effects

Additional information is provided on origin of compound, the presence of a steroid-like structure as well as justification for not taking the majority of these compounds forward. The two compounds taken forward are the chemically related substances ursocholanic acid and dehydro (11,12) ursolic acid lactone. Additional information on those compounds that have been excluded from further analysis is provided in the Supplementary material.

Parkinson's disease. We therefore investigated the effect of these compounds on cellular ATP levels in *LRRK2*^{G2019S} mutant patient tissue. Treatment of *LRRK2*^{G2019S} mutant fibroblasts from three different patients with Parkinson's disease carrying this mutation with 10 nM of ursocholanic acid or ursodeoxycholic acid for 24 h resulted in complete rescue of cellular ATP levels (Fig. 8), similar to the effect observed in *parkin*-mutant patient tissue. Therefore, the beneficial effect of these compounds does not appear to be limited to *parkin*-associated Parkinson's disease.

Discussion

The strong evidence of mitochondrial dysfunction in both sporadic and familial Parkinson's disease suggests targeting mitochondria as a promising strategy for disease-modifying therapy in Parkinson's disease (Meissner *et al*., 2011; Schapira, 2012). We had previously demonstrated a complete rescue of mitochondrial dysfunction in *parkin*-mutant patient tissue using the glutathione precursor

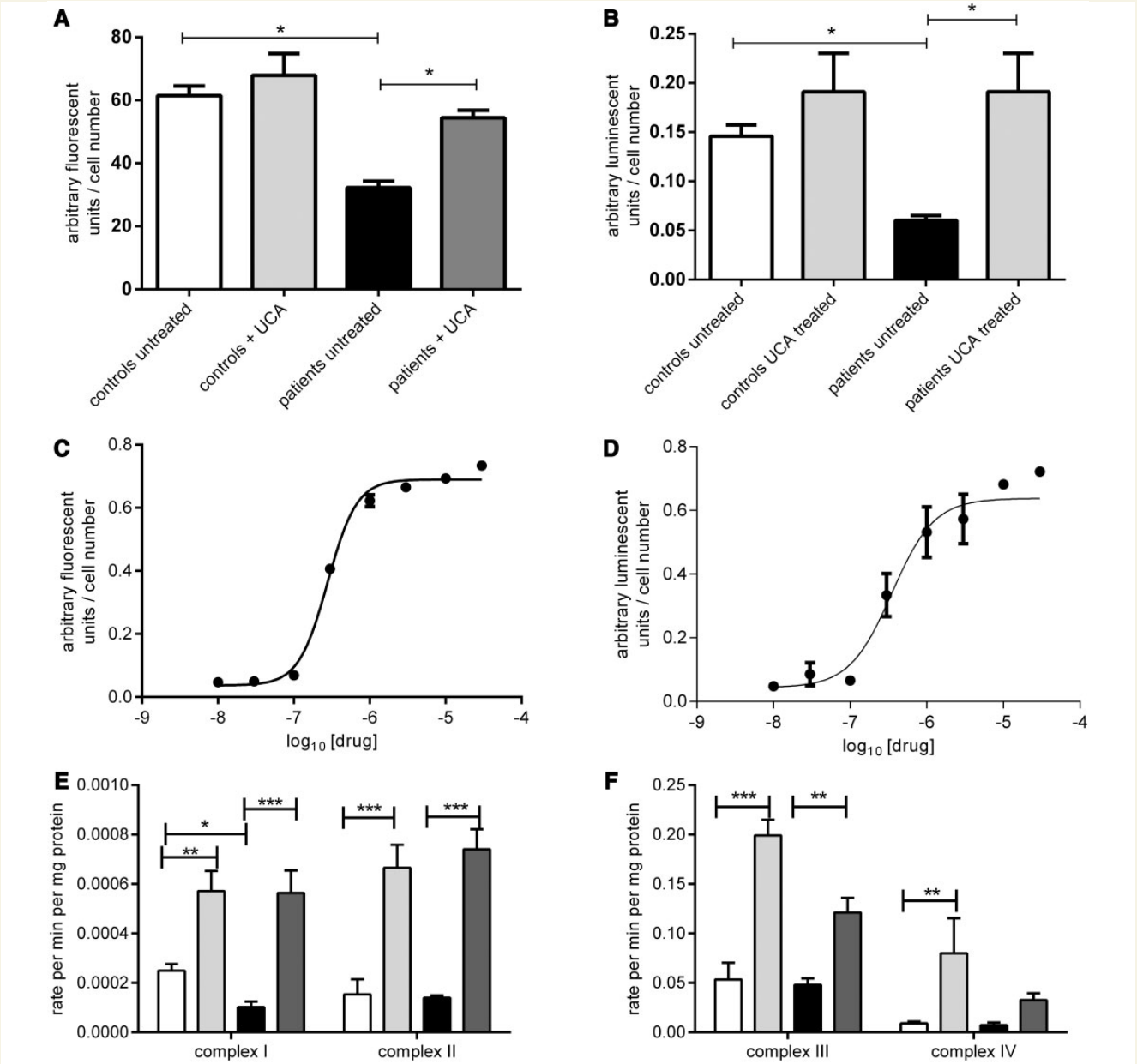

Figure 2 Rescue of mitochondrial function in *parkin*-mutant fibroblasts by treatment with 100 nM ursocholanic acid (UCA) for 24 h. (**A**) Mitochondrial membrane potential and (**B**) cellular ATP levels are decreased in untreated fibroblasts of patients with *parkin* mutations compared with untreated controls ($P < 0.05$), treatment with ursocholanic acid results in normalization of mitochondrial membrane potential and ATP levels ($P < 0.05$). (**C**) Mitochondrial membrane potential and (**D**) cellular ATP levels after treatment with increasing concentrations of ursocholanic acid for 24 h, reflecting a sigmoidal dose response curve. (**E** and **F**) Activity of each of the individual respiratory chain enzymes are increased by treatment with ursocholanic acid in both control and *parkin*-mutant fibroblasts. Data presented are corrected to protein levels $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

L-2-oxothiazolidine-4-carboxylic acid (OTCA) and also a mild partial rescue effect on mitochondrial function after rapamycin treatment (Mortiboys *et al*., 2008; Tain *et al*., 2009). Based on these 'proof of principle' data, we have now undertaken the first drug screen in Parkinson's disease patient tissue and identified a group of chemically-related compounds with marked rescue effect on mitochondrial function. Our data are in keeping with previous studies that reported a protective effect of the taurine conjugate of ursodeoxycholic acid (TUDCA) against mitochondrial toxins in *parkin*-deficient *Caenorhabditis elegans* (Ved *et al*., 2005). Recently, an Akt-mediated, partial neuroprotective effect of TUDCA on MPTP-induced dopaminergic cell death has been observed in a mouse model of Parkinson's disease (Castro-Caldas *et al*., 2012). Our data strongly suggest a class effect for bile acids and their derivates such as DUA, ursocholanic acid and ursodeoxycholic acid and the natural pentacyclic triterpenoid

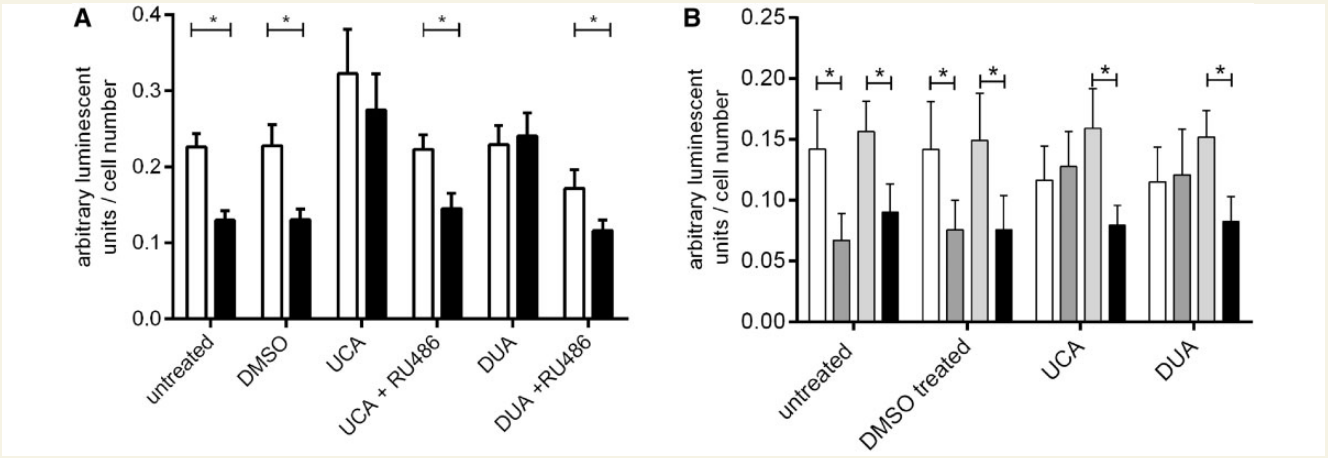

Figure 3 Inhibition or knockdown of the glucocorticoid receptor abolishes the rescue effect of ursocholanic acid (UCA) and DUA in *parkin*-mutant fibroblasts. (**A**) Cellular ATP levels are reduced in *parkin*-mutant patient fibroblasts (black bars) compared with controls (white bars) and recovered to normal levels after treatment with 100 nM ursocholanic acid or DUA for 24 h. This rescue effect is completely abolished by pretreatment with 1 μM RU486 (glucocorticoid receptor antagonist) for 4 h. (**B**) Cellular ATP levels are reduced in parkin mutant patient fibroblasts transfected with either scramble small interfering RNA (dark grey bars) or glucocorticoid receptor small interfering RNA (black bars) compared with control fibroblasts transfected with scramble small interfering RNA (white bars) or glucocorticoid receptor small interfering RNA (light grey bars) $*P < 0.05$. Treatment with 100 nM ursocholanic acid or DUA completely rescues this defect in *parkin* mutant fibroblasts transfected with scramble small interfering RNA (white and dark grey bars) but not in *parkin* mutant fibroblasts transfected with glucocorticoid receptor small interfering RNA treatment with ursocholanic acid and DUA (black bars) compared with controls also transfected with glucocorticoid receptor small interfering RNA (light grey bars). DMSO = dimethylsulphoxide.

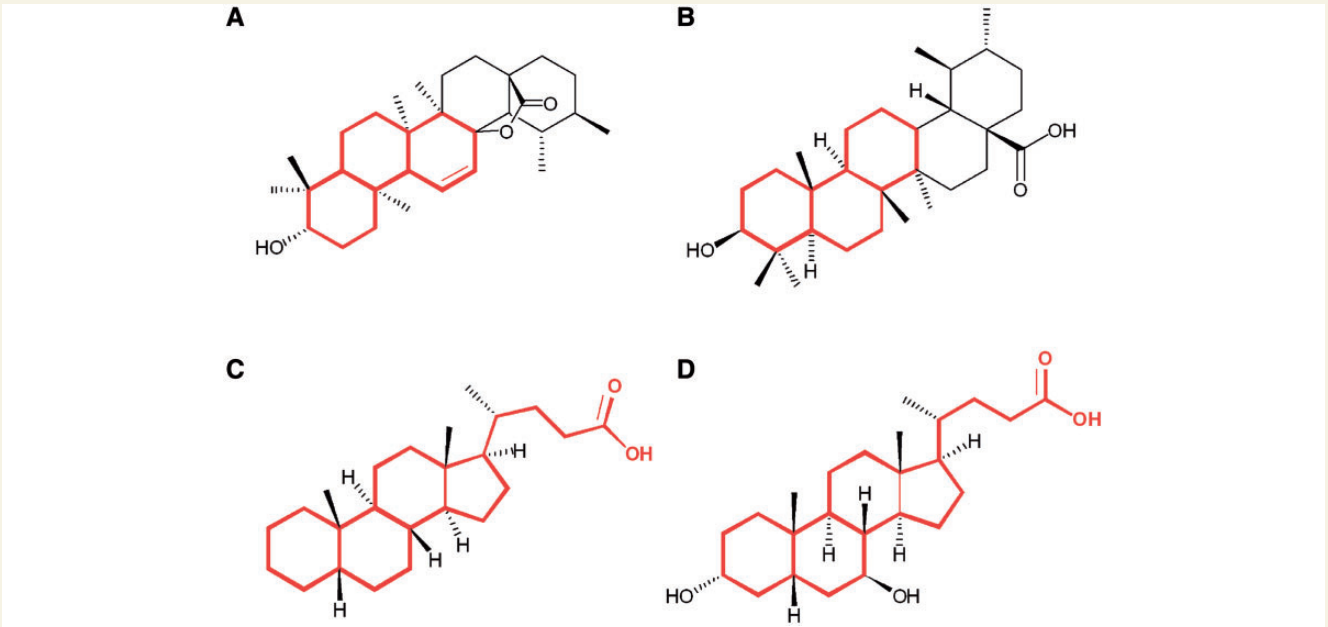

Figure 4 Structures of the *top* two compounds identified from the original drug screen, namely (**A**) dehydro (11,12) ursolic acid lactone and (**C**) ursocholanic acid and two further compounds which are structurally similar, namely (**B**) ursolic acid and (**D**) ursodeoxycholic acid. The structural similarities are highlighted in red. The structures are represented in standard chemical format displaying the 3D orientation of groups. Where no group is specified a methyl group is attached. Hydrogens are only shown if they affect the 3D orientation of the molecule.

ursolic acid. The bioavailability of ursolic acid and its dose-dependent increase in brain tissue of mice has been well characterized (Yin *et al*., 2012). A beneficial effect of both ursolic acid and ursodeoxycholic acid or TUDCA has also been described in different *in vitro* and *in vivo* model systems for other neurodegenerative conditions, including Alzheimer's disease, Huntington's disease and stroke (Keene *et al*., 2002; Rodrigues *et al*., 2003; Ramalho *et al*., 2008; Wilkinson *et al*., 2011).

Of note, 7 of 15 of the compounds that rescued both the mitochondrial membrane potential and cellular ATP levels as well as

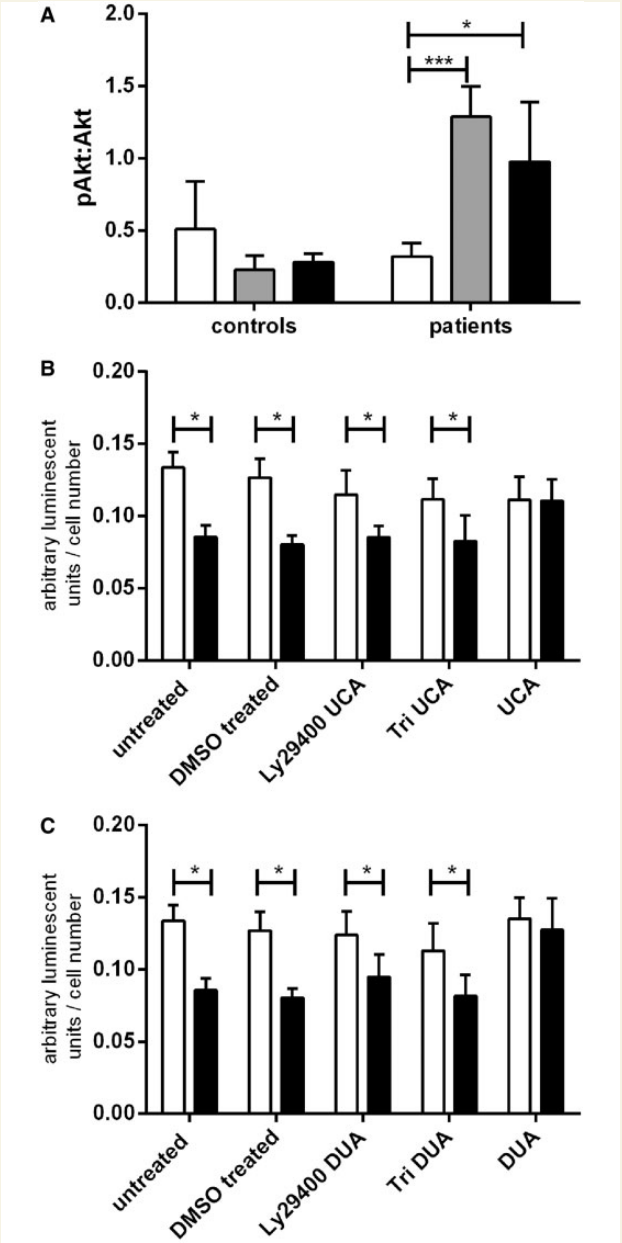

Figure 5 The rescue effect of ursocholanic acid and DUA is Akt mediated. (**A**) pAkt^{Ser473} protein levels as a ratio to total Akt protein levels as measured by ELISA. pAkt^{Ser473} levels are increased in *parkin*-mutant patient cells after treatment with both ursocholanic acid (grey bars) and DUA (black bars) (***$P < 0.001$, *$P < 0.05$). (**B** and **C**) Cellular ATP levels in control fibroblasts (white bars) and *parkin*-mutant fibroblasts (black bars). Pretreatment with the phosphatidylinositol 3-kinases (PI 3-kinase) inhibitor LY29400 or triciribine, which selectively inhibit the cellular phosphorylation/activation of Akt, abolish the rescue effect of both ursocholanic acid (**B**) and DUA (**C**) (*$P < 0.05$). DMSO = dimethylsulphoxide.

having drug-like dose response curves had a steroid-like structure. Lim *et al*. (2012) reported independently a neuroprotective effect of the chemically closely related sterol biosynthesis intermediate lanosterol. Both ursolic acid and lanosterol induce mild mitochondrial uncoupling that has been proposed as a promising strategy for disease modification in Parkinson's disease (Liobikas *et al*., 2011; Ho *et al*., 2012; Lim *et al*., 2012).

The inhibition of the mitochondrial rescue effect of DUA and ursocholanic acid after pretreatment with RU486 is in keeping with previous observations on glucocorticoid receptor-mediated biological activity of ursolic acid or ursodeoxycholic acid (Tanaka and Makino, 1992; Sharma *et al*., 2011). However, genome-wide gene expression studies did not reveal any relevant and consistent changes in *parkin*-mutant fibroblasts after treatment with ursolic acid or DUA (data not shown). In particular, there was no effect on messenger RNA levels of mitochondrial master regulators such as *PGC1alpha* (now known as *PPARGC1A*) or mitochondrial uncoupling proteins. The biological function of glucocorticoids encompasses both genomic and non-genomic effects, including direct binding to the mitochondrial membrane, which can lead to partial uncoupling of oxidative phosphorylation (Haller *et al*., 2008).

We appreciate that our work largely focused on assessing the effect of compounds in *parkin*-mutant Parkinson's disease patient tissue. However, the beneficial effect of the lead compound, ursocholanic acid and the chemically related licensed drug ursodeoxycholic acid were also clearly apparent in *LRRK2*^{G2019S} mutant fibroblasts. Ten per cent of all sporadic and ~30% of familial Parkinson's disease can be due to the *LRRK2*^{G2019S} mutation in Ashkenazi Jewish patients with Parkinson's disease (Ozelius *et al*., 2006). The prevalence may be even higher in other populations (Lesage *et al*., 2006). The mitochondrial phenotype is generally accepted to be correct for *PARK2* but additional work is needed to determine whether rescue of mitochondrial function will result in at least partial rescue of neuronal dysfunction and cell loss in *LRRK2*^{G2019S}-mutant model systems. If this was to be the case, then our lead compounds or structurally related drugs may already have a beneficial effect in a significant number of patients with Parkinson's disease even if their effect was limited to *parkin*- and *LRRK2*^{G2019S} mutant patients with Parkinson's disease only.

Mitochondrial dysfunction was first implicated in the pathogenesis of Parkinson's disease when drug abusers developed parkinsonism after accidental exposure to the complex I inhibitor MPTP (Abou-Sleiman *et al*., 2006; Schapira, 2008). Subsequently, several groups reported independently decreased complex I activity in Parkinson's disease (Mizuno *et al*., 1989; Parker *et al*., 1989; Schapira *et al*., 1989). It is now widely accepted that mitochondrial dysfunction and impaired morphology play a crucial role in the pathogenesis of early-onset Parkinson's disease due to mutations in *parkin* (*PARK2*), *PINK1* or *DJ1* (*PARK7*) (Cookson and Bandmann, 2010). Mitochondrial dysfunction has also been observed in patient tissue (see above) or model systems of late-onset Parkinson's disease due to mutations in *LRRK2* or alpha synuclein (*SNCA*) (Loeb *et al*., 2010; Hindle *et al*., 2013). Akt, a protein kinase with multiple targets, is activated by successive phosphorylation at two sites. Failure of Akt signalling has been described as the 'common core' underlying neuronal degeneration and cell death in both familial and sporadic Parkinson's disease (Greene *et al*., 2011). Akt phosphorylation is reduced in dopaminergic neurons of sporadic Parkinson's disease (Malagelada *et al*., 2008; Timmons *et al*., 2009). Both increased expression of alpha synuclein (SNCA) and *SNCA* mutations lead to reduced Akt

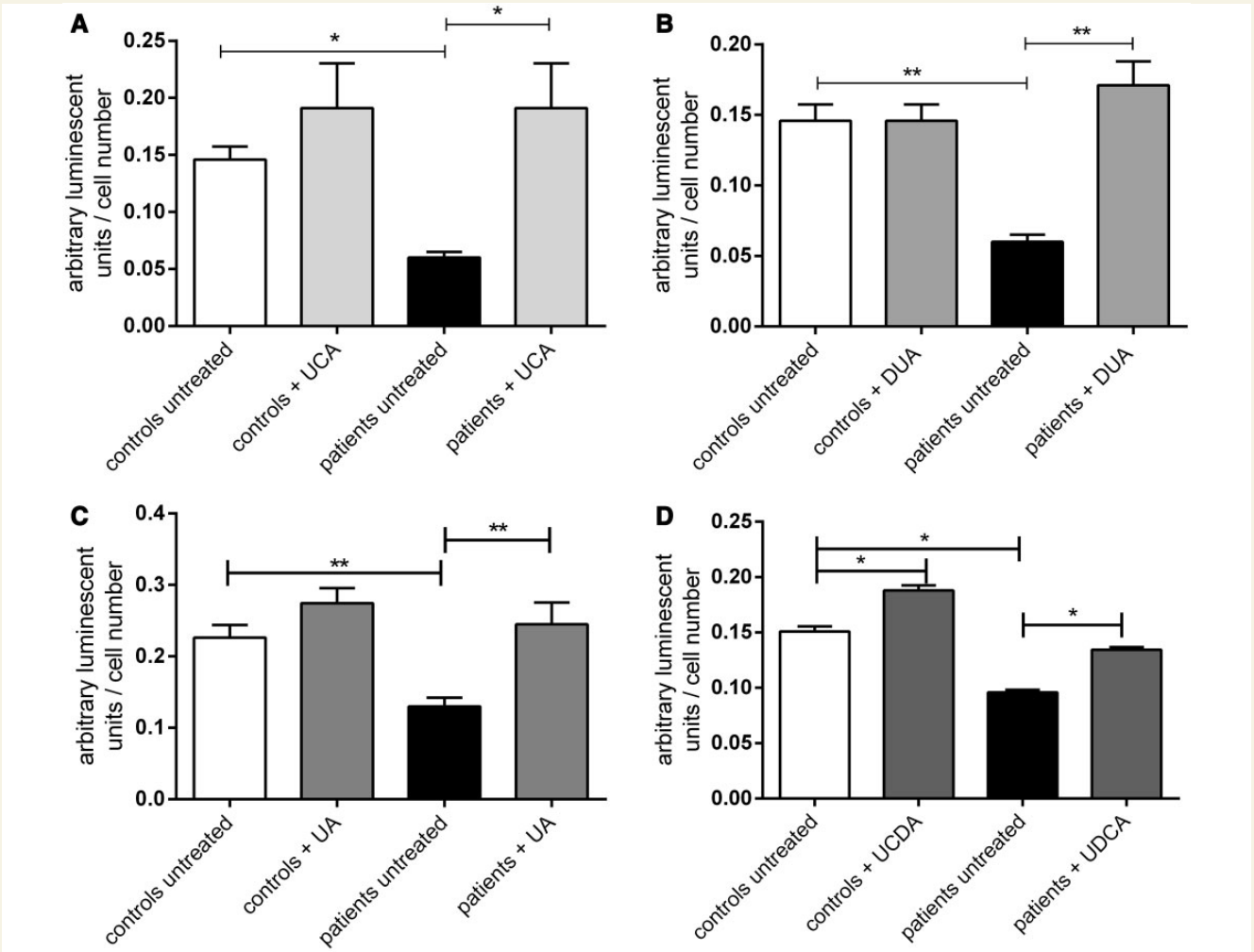

Figure 6 Rescue of cellular ATP levels by 24-h treatment of *parkin*-mutant fibroblasts with 100 nM ursocholanic acid (UCA, **A**), DUA (**B**), ursolic acid (UA, **C**) and ursodeoxycholic acid (UDCA, **D**). Cellular ATP levels are significantly reduced in untreated *parkin*-mutant patient fibroblasts ($*P < 0.05$) but significantly increased after treatment with any of these four respective drugs ($*P < 0.05$, $**P < 0.01$).

activation (Chung *et al*., 2011). Similarly, *LRRK2* mutations (in particular G2019S) as well as Parkin, PINK1 and DJ1 deficiency result in decreased Akt phosphorylation (Yang *et al*., 2005; Fallon *et al*., 2006; Murata *et al*., 2011; Ohta *et al*., 2011). In contrast, the protective effect of beta-synuclein is mediated by increased Akt phosphorylation and increased *parkin* expression normalizes reduced Akt phosphorylation in MPTP-treated mice (Hashimoto *et al*., 2004; Yasuda *et al*., 2011). Further work is needed to determine whether the mitochondrial rescue effect and increased Akt phosphorylation at Ser^{473} after treatment with ursocholanic acid and DUA (as observed in our *parkin*-mutant fibroblast model) can also be observed in other forms and model systems of Parkinson's disease.

Ursodeoxycholic acid has been licensed for the treatment of patients with primary biliary cirrhosis since 1980. It is typically used at a dose of 10 mg/kg of body weight per day in patients with primary biliary cirrhosis but Parry *et al*. (2010) also reported 'excellent' safety and tolerability of ursodeoxycholic acid in patients with motor neuron disease at 15 mg, 30 mg and 50 mg/kg per day. There was a significant correlation between serum and CSF concentrations of ursodeoxycholic acid. There is therefore good rationale to assume that ursodeoxycholic acid may also be well tolerated in Parkinson's disease and cross the blood–brain barrier. Drug repositioning of FDA-licensed drugs such as ursodeoxycholic acid is a promising strategy to save time and costs but Parkinson's disease-specific, reliable data on safety, tolerability and CSF penetration of ursodeoxycholic acid will nevertheless be of paramount importance before ursodeoxycholic acid can be taken into clinical trials to assess its putative disease-modifying effect in Parkinson's disease.

Dopaminergic neurons derived from inducible stem cells have already been used to assess compounds for their putative rescue effect on crucial pathogenic mechanisms for Parkinson's disease and other conditions (Cooper *et al*., 2012). However, the inducible stem cells-based approach, although in many ways exciting and promising, is also costly and not without inherent problems. Our study demonstrates that a step-wise strategy, encompassing an initial screen in Parkinson's disease patient fibroblasts but

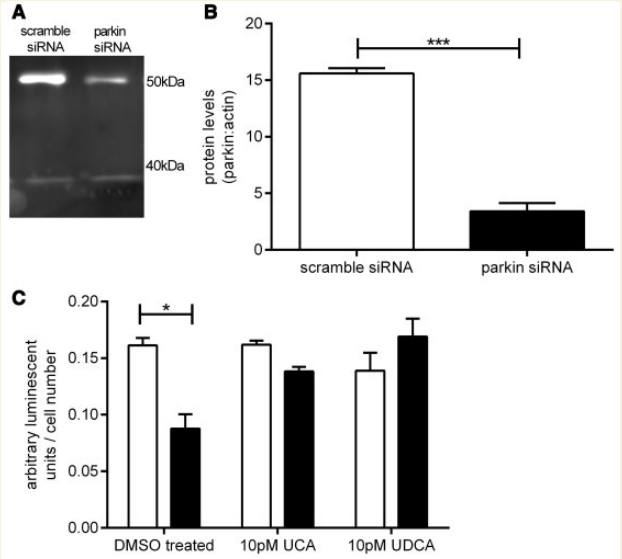

Figure 7 Ursocholanic acid (UCA) and ursodeoxycholic acid (UDCA) rescue effect in cortical neurons with small interfering RNA mediated *parkin* knockdown. (**A**) Western blot showing Parkin band at ~50 kDa and actin band at ~40 kDa in scramble small interfering RNA and *parkin* small interfering RNA transfected cortical neurons. (**B**) Parkin protein levels are reduced by 80% in *parkin* small interfering RNA knockdown cortical neurons as assessed by western blotting (***$P < 0.001$). (**C**) Cellular ATP levels in cortical neurons at 9 days in culture transfected with either scramble small interfering RNA (white bars), or *parkin* small interfering RNA (black bars). There is a reduction of 43% in cellular ATP levels in the *parkin* small interfering RNA transfected cells, (**$P < 0.01$), which is rescued by treatment with 10 pM ursocholanic acid or ursodeoxycholic acid. DMSO = dimethylsulphoxide.

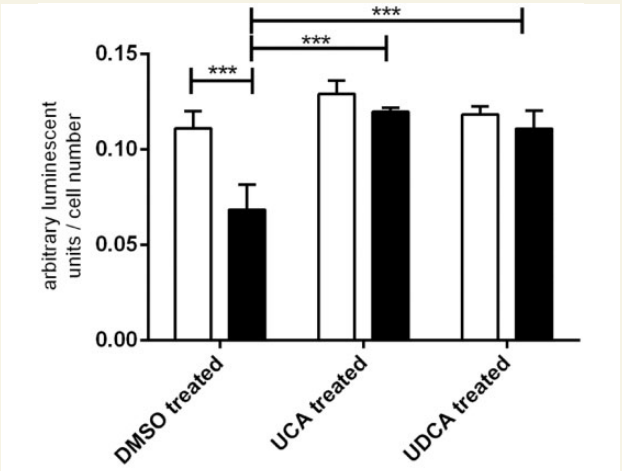

Figure 8 Cellular ATP levels are reduced in fibroblasts from three *LRRK2*^{G2019S} mutant patients (black bars) compared with controls (white bars) *$P < 0.05$. There is complete recovery of ATP to normal levels after treatment with 10 nM ursocholanic acid or 10 nM ursodeoxycholic acid for 24 h. DMSO = dimethylsulphoxide.

subsequent confirmation of top hits in a neuronal model system may be a less costly and more robust strategy.

Previous studies investigating the potential rescue effect of pharmacological compounds in model systems of early onset Parkinson's disease have concentrated on a hypothesis-driven approach testing individual compounds rather than assessing a compound library in a hypothesis-free approach. Vitamin K(2) acts as a mitochondrial electron carrier that rescues mitochondrial dysfunction in pink1-deficient *Drosophila* (Vos *et al*., 2012). However, it is unclear whether vitamin K(2) also rescues mitochondrial dysfunction in Parkin deficiency. The disaccharide trehalose increases the removal of abnormal proteins through enhancement of autophagy. Trehalose treatment ameliorates tau pathology but fails to revert the loss of dopaminergic neurons in a mouse model of tauopathy with parkinsonism, overexpressing human mutated tau protein with deletion of *parkin* (Rodriguez-Navarro *et al*., 2010). Co-enzyme Q10 reduces the vulnerability of inducible stem cell-derived, *PINK1* mutant neural cells to the lowest, but not to high concentrations of valinomycin and concamycin A, rapamycin did not reduce lactate dehydrogenase release after exposure to these toxins. In contrast, both rapamycin and the LRRK2 inhibitor GW5074 reduced the production of mitochondrial reactive oxygen species in *PINK1* mutant neural cells exposed to valinomycin. However, none of these compounds were assessed for their rescue effect on baseline mitochondrial (dys)function in *PINK1* mutant model systems before toxin exposure (Cooper *et al*., 2012). Future drug screens may be preceeded by *in silico* screens assessing compounds for their likely effect on enhancing the biological activity of proteins such as Parkin or PINK1, but also on other proteins such as thioredoxin with a reported rescue effect in Parkin-deficient *Drosophila* (Umeda-Kameyama *et al*., 2007; Trempe *et al*., 2013). Other therapeutic approaches include the overexpression of enzymes bypassing complex I activity such as the *Saccaromyces cerevisiae* enzyme *Ndi1p* (Vilain *et al*., 2012).

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Suppplementary material

Supplementary material is available at *Brain* online.

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