The ying and yang of idebenone: not too little, not too much – cell death in NQO1 deficient cells and the mouse retina

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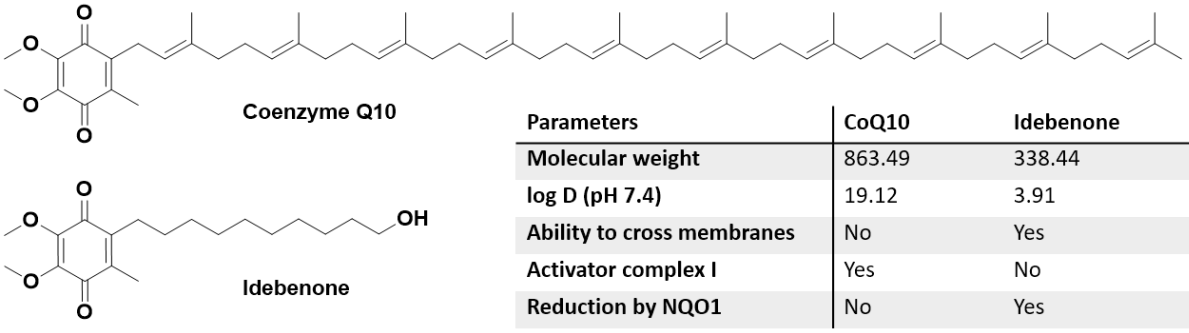
Abstract

Idebenone has recently been investigated as a drug therapy for Leber's hereditary optic neuropathy (LHON), a rare genetic mitochondrial disease that causes rapid and progressive bilateral vision loss. Although several studies have shown that idebenone can promote vision recovery in patients with LHON, the evidence for the efficacy of idebenone is still limited. Idebenone failed to demonstrate superiority over placebo in the primary end-points of the only published randomised, double-blind, placebo-controlled trial. There appears to be a patient-specific response to idebenone with high variability in therapeutic outcomes. A recent study suggested that the cytosolic enzyme NAD(P)H: quinone acceptor oxidoreductase (NQO1) is the major enzyme involved in the activation of idebenone, and the beneficial effects of idebenone are dependent on the expression of NQO1. Here, we confirm the NQO1-dependent activity of idebenone, but we also show, for the first time, that the cytotoxicity of idebenone is linked to cellular expression of NQO1. Upon idebenone administration, cells deficient in NQO1 show a marked decrease in viability in comparison to NQO1 expressing cells, with idebenone causing ROS production and deleterious effects on ATP levels and cell viability. In addition, our data highlights that only cells expressing NQO1 can significantly activate idebenone, indicating that other proposed metabolic activation pathways, such as complex II and glycerol-3-phosphate dehydrogenase, do not play a significant role in idebenone activation. Furthermore, we provide evidence of idebenone-induced toxicity in the retina *ex-vivo*, which can be explained by the variation of NQO1 expression between different cell types in the mouse retina. Idebenone mediated cell rescue in the rotenone *ex vivo* model also indicated that this drug has a narrow therapeutic window. These findings will help to guide the development of future therapies and drug delivery strategies including intra-ocular administration. The specific dependence of idebenone activity on NQO1 may also explain the variation in patient outcomes in clinical trials.

Introduction

Idebenone is a synthetic benzoquinone, initially selected from a medicinal chemistry program conducted in the 1980s by Takeda Pharmaceuticals, as a possible cure for Alzheimer's disease but with limited success [1]. In 2006, Santhera Pharmaceuticals started to investigate idebenone for the treatment of a number of different diseases, such as Duchenne muscular dystrophy [2], Leber's hereditary optic neuropathy (LHON) [3], and Friedreich ataxia for its reported antioxidant and ATP rescue capacity [4]. Initially, idebenone was simply considered as an analog of coenzyme Q10 (CoQ_{10}), also known as ubiquinone, a physiological and ubiquitous quinone present in all mitochondrial membranes [5]. Coenzyme Q10 is a component of the electron transport chain, and it functions as an electron carrier in aerobic cellular respiration, transporting electrons from complex I and II to complex III, consequently driving ATP production [5]. CoQ_{10} is a lipophilic molecule (logD 19.12), with a long isoprenoid tail and a hydrophilic quinone head [6]. These two features give the compound a unique ability to move inside cellular membranes and work as an electron donor and acceptor [7]. However, the high lipophilicity of CoQ_{10} presents a significant limitation for use as a therapeutic, in terms of low absorption and poor bioavailability [8]. Idebenone was first synthesised in order to improve the CoQ_{10} bioavailability, while retaining its pharmacological activity [9]. For this reason, idebenone possesses the same benzoquinone core of CoQ_{10}, but with a shorter, less lipophilic side chain: instead of ten repeats of isoprenoid elements, idebenone has a saturated ten carbon tail with a terminal hydroxyl group (Figure 1).



Parameters	CoQ10	Idebenone
Molecular weight	863.49	338.44
log D (pH 7.4)	19.12	3.91
Ability to cross membranes	No	Yes
Activator complex I	Yes	No
Reduction by NQO1	No	Yes

Figure 1 - Comparison of the two quinones: CoQ10 and idebenone

From these structural similarities, it was suggested that idebenone could emulate the same physiological mechanism as CoQ_{10}, but recent evidence suggests that there is a major difference between these two compounds. Idebenone is not such a suitable substrate for complex I as CoQ_{10} is; on the contrary, recent studies have demonstrated that a concentration of idebenone above 10 µM is enough to inhibit this enzyme instead, which can lead to superoxide generation [10][11]. On the other

hand, idebenone showed high affinity for the cytosolic enzyme NQO1 [12]. From this evidence, it was suggested that NQO1, rather than complex I, was responsible for the pharmacological activity of idebenone [12]. NQO1 is a cytosolic obligatory two-electron donor flavoenzyme which catalyses the direct reduction of quinones to hydroquinones [13]. It is expressed in different tissues, particularly present in the liver for its detoxification activity, but it is at lower expression levels in other tissue such as the brain [14]. It was assumed that idebenone itself is metabolized/activated by NQO1: once idebenone is reduced by this enzyme, it becomes hydrophilic enough to traverse the cytosol, but also lipophilic enough to cross the mitochondrial membrane and interact with complex III, bypassing complex I or complex II [12](Figure 2).

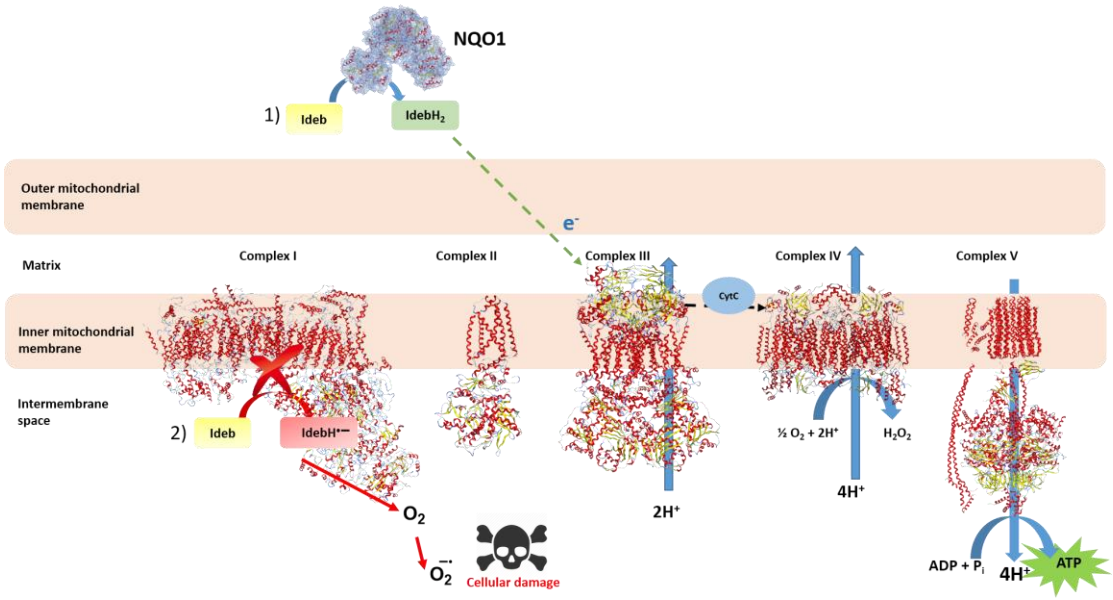


Figure 2 - Postulated mechanisms of idebenone action. The two possible pathways that idebenone (Ideb) may take within cells. In pathway 1 idebenone is reduced by NQO1 in the cytoplasm into idebenol ($IdeH_2$, the active form), which can mediate electron transfer to complex III in the mitochondrial inner membrane, reactivating the electron flow. Pathway 2 shows the interaction between idebenone and complex I: idebenone can act as an inhibitor and can be reduced to a semi-quinone (IdebH•−), which can generate ROS.

This complex I-bypass activity was recently described in detail by Haefeli *et al.*, who demonstrated that idebenone was able to restore the ATP levels under conditions of impaired complex I function, thus providing a strong rationale for the use of idebenone in mitochondrial complex I deficiency disorders such as LHON [12]. Despite the positive results obtained *in vitro* using different cell lines, the results *in vivo* have not always been consistent [15][16]. Recent research conducted by Smith *et al*. demonstrated an accumulative deleterious effect of idebenone due to oxidative stress in healthy wild type mice, resulting in an increase of NQO1 enzyme in response to this oxidative environment [17].

This negative effect could be due to the impairment of complex I, or due to superoxide generation caused by the interaction between idebenone and the hydrophilic binding site within complex I (Fig.2). The duality of idebenone: antioxidant/pro-oxidant, complex I inhibition/complex III stimulation may explain the contradictory results obtained in clinical trials [15]. Critically, the putative pro-oxidant activity of idebenone in tissues showing lower expression of NQO1 and the consequent cytotoxic effect has not yet been clarified.

The aim of this paper was to analyse the role of NQO1 in idebenone bioactivity and toxicity *in vitro*, and approximate a therapeutic window for idebenone in complex I compromised mouse retinal explant culture. We hypothesized that by using three different cell lines, with different expression levels of NQO1, the effect of NQO1 on idebenone-mediated activity/toxicity could be elucidated. Furthermore, by using mouse retinal explant culture, we could investigate the activity/toxicity of idebenone on a heterogeneous population of cell types, where the expression of NQO1 is variable from cell to cell. In this study, we provide evidence of idebenone toxicity correlated to NQO1 activity in cells, highlighting a potential detrimental effect of idebenone.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich (Gillingham, UK) unless specified otherwise. Idebenone was kindly supplied by Santhera Pharmaceuticals (Pratteln, Switzerland). All culture media, buffers and cell culture supplements were obtained from Gibco Laboratory (Gibco Invitrogen Corp, Paisley, UK). CellTiter-Glo® Luminescent Cell Viability, CellTiter-Blue® Cell Viability Assay and ROS-Glo™ H_2O_2 Assay were purchased from Promega (Southampton, UK). For all assays described, organic compounds were dissolved in 100% dimethylsulfoxide (DMSO) as a stock solution and sequentially diluted in the medium for a maximum of 0.1 % DMSO concentration.

Cell culture

R28 cells were purchased from KeraFAST (Boston, MA, USA). HepG2 cell line was kindly provided by Prof. Karl Hoffmann's lab, IBERS, (Aberystwyth University), while SH-SY5Y was provided Dr. Emma Kidd's lab (Cardiff University). Cell lines were cultivated under normal culture conditions (37 °C, 5% CO_2, and 90% relative humidity. HepG2 and R28 were cultivated in Dulbecco's Modified Eagle's Medium (DMEM), while SH-SY5Y were cultivated in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12). All media were supplemented with antibiotics (100 units/mL penicillin, 1

µg/mL streptomycin), 2 mM Glutamax, 1 g/L glucose and either 10% or 2% v/v fetal bovine serum (FBS).

Western blot analysis

The quantification of NQO1 protein expression was determined using western blots analysis. Briefly, cells were lysed in a RIPA lysis buffer (50 mM Tris pH 7.4, 1% NP-40, 150 mM NaCl, 0.25% sodium deoxycholate, 1 mM EDTA), supplemented with protease inhibitors and centrifuged for 15 min at 12,000g. Proteins were separated by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (10%) and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). Membranes were washed in TBST (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Tween 20) and after incubation for one hour in blocking buffer (5% milk powder in PBS), membranes were subjected to primary antibodies in cold blocking buffer overnight at 4 °C. The dilution of primary antibody was 1:1000 and 1:2000 for NQO1 (Anti-NQO1, Abcam, Cambridge, UK) and actin (Anti-beta actin, Abcam) respectively. The following day, the membranes were washed with TBST before secondary antibodies were added in blocking buffer for one hour. The relative amount of protein in different bands was analysed by enhanced chemiluminescence (ECL), quantified by densitometry (Image Lab Software 6.0.1, (Bio-Rad, Hercules, CA) and normalized against actin.

Determination of quinone reduction in cells

The intracellular reduction of quinones was determined using the water-soluble tetrazolium salts ((2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-1, Santa Cruz Biotechnology, Heidelberg, Germany). The conversion of salt into the corresponding formazan dye, upon reduction by hydroquinones, was followed by reading the increase of absorbance at 450 nm using the CLARIOstar plate reader (BMG Labtech, Ortenberg, Germany). The assay was performed as described by Tan and Berridge [18]. Briefly, the day prior to the assay, cells were seeded in 96-well plates (1.5 x 10^4 cells per well) in the corresponding culture media containing 2% FBS. The day after, the media was replaced with Hanks' Balanced Salt Solution (HBSS) containing 450 µM WST-1 and 25 µM of testing compounds, with or without dicoumarol (20 µM). Cells were incubated at 37°C and WST-1 reduction was followed over a period of 8 hours. The menadione-dependent WST1 reduction in presence of recombinant NQO1 was used as positive control [19]. The mean background value from cell-free wells incubated with dye was subtracted from signals.

Cell viability analysis

Cell viability was determined using two different assays: CellTiter-Glo Luminescent Cell Viability and CellTiter-Blue Cell Viability Assay, measuring ATP level and reduction of resazurin, respectively. In both

assays, the cells were seeded in 96-well plates (1 x 10^4 cells per well) in the corresponding culture media containing 2% FBS, one day before the assay. The day after, the media was replaced with fresh media (2% FBS, 1 g/L glucose) which either contained 5-100 µM of idebenone, or a vehicle (matched DMSO content (maximum 0.1%v/v)) alone control without idebenone. Cells were incubated at 37 °C for 24 hours, after which CellTiter-Blue or CellTiter-Glo reagents were applied according to the manufacturer's instructions. In brief, 20 µL of CellTiter Blue reagent was added to each well (100 µL) and incubated for 4 hours at 37 °C. Following this period, the fluorescence was measured using a CLARIOstar plate reader using excitation/emission wavelengths of 560/590 nm. In the case of CellTiter-Glo, 100 µL of the CellTiter-Glo reagent solution was added to each well (100 µL), and the luminesce signal was measured using CLARIOstar plate reader. Data were normalized to vehicle control-treated cells.

NQO1-dependent rescue of ATP levels under complex I inhibition by rotenone

To assess the ability of idebenone to rescue ATP levels under complex I inhibition, the cells were treated with 25 µM of rotenone in the absence of glucose for a determined time, in the presence/ absence of idebenone. The cells were seeded in 96-well plates (1 x 10^4 cells per well) in the corresponding media containing 2% FBS, one day before the assay. The day after, the media was replaced with fresh media (2% FBS, 0 g/L glucose) containing the rotenone solution (25 µM) with or without idebenone. Cells were incubated at 37 °C for 2 hours. Subsequently, the cellular ATP levels were quantified using CellTiter-Glo® Luminescent Cell Viability Assay. Data were normalized to vehicle control-treated cells in the absence of rotenone (untreated).

Determination of H_2O_2 level

The reagent ROS-Glo™ H_2O_2 was used to quantify cellular H_2O_2, and thus as an indicator of ROS generation. The ROS-Glo™ H_2O_2 assay was carried using the manufacturer's protocol. The day prior to the assay, cells were seeded in 96-well plates (1 x 10^4) in the corresponding media containing 2% FBS. On the day of the assay, the media was replaced with 80 µL of a fresh medium containing 25 µM of menadione or tested compounds, followed by 20 µL of H_2O_2 substrate dilution buffer (containing 125 µM of H_2O_2 substrate). The plates were incubated for a 6h, then 100 µL of ROS-Glo detection solution was added to the plates. The plates were incubated at room temperature for another 20 minutes. Subsequently, the luminescence signals were measured immediately using the CLARIOstar plate reader. The mean background value from cell-free wells incubated with the reagent was subtracted from luminesce signals.

Activity and toxicity analysis in the retinal explant

Wild-type, 6-8 month old, female C57 BL/6J mice were used as the source of retinal explants. Mice were kept in a 12-hour light-dark cycle with food and water available ad libitum. Maintenance and all experimental procedures were carried out in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Home Office, UK.

Mice were sacrificed by cervical dislocation. Eyes were immediately enucleated, transferred to a culture dish with ice cold HBSS (Gibco, Invitrogen Corp, Paisley, UK)). The retina was dissected and divided into four radial sections, and each was flat mounted onto a 0.4 µm pore PTFE membrane culture plate insert Sigma-Aldrich (Gillingham, UK) with the ganglion cell layer facing up.

The inserts were placed in a 6-well plate containing idebenone at various concentrations for toxicity tests, or idebenone plus 100 µM rotenone for activity tests, solubilised in 0.1% DMSO and pre-warmed Neurobasal A media (Invitrogen) supplemented with 2% B27, 1% N2 and 1% PenStrep Glutamine 100X, and transferred to an incubator for 24 hours at 37 °C, 5% CO_2. The sections were fixed in 4% PFA, permeabilised in 0.1% TritonX for 1 hour, blocked in 10% FBS for 3 hours, and incubated with the anti-RBPMS primary antibody (1:200) overnight at 4 °C. They were then incubated with the secondary antibody - goat anti-rabbit IgG H&L (Alexa Fluor 488) (1:500) at room temperature for 4 hours, and stained with Hoechst 33342 nuclear stain (1:1000) for 30 minutes. Images (425 μm^2) were acquired as described in Figure 3 with 488 nm excitation and band pass (BP) 500-530 nm emission filter for Alexa Fluor 488, and 350 nm excitation and BP 411-480 nm for Hoechst 33342. RBPMS positive cells and nuclei in the retinal ganglion cell layer were counted using QuPath [20]

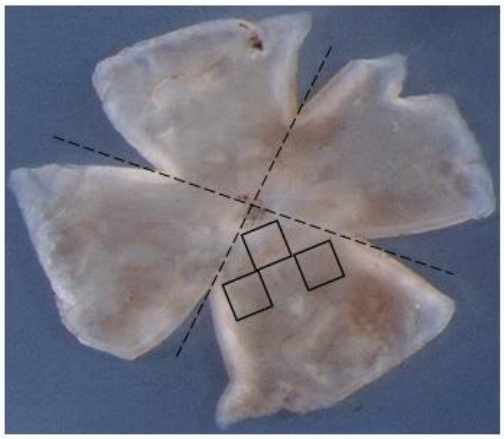


Figure 3 - The retinal flat mount imaged using a light microscope with objective magnification of 8x. Broken black lines represent the cuts made to create four radial sections. Each quarter was allocated to a different experimental group. The black rectangular boxes show the locations on each quarter where three images (425 μm^2) were acquired with a Zeiss LSM 510 confocal microscope (Carl Zeiss, Ltd, UK) using a 20X (0.8 NA) objective lens.

NQO1 expression in retina

Eyes from 2 months old female C57BL/6J mice were enucleated immediately after culling and transferred to 4% PFA and left overnight at 4 °C before cryopreservation. Sagittal sections of 10 µM were blocked with 10% FBS in 0.1% Triton-Tween for 1 hour, incubated with anti-NQO1 in 0.1% Triton-Tween overnight at 4 °C, incubated with goat anti-rabbit IgG H&L (Alexa Fluor 488) (1:500) at room temperature for 2 hours, and stained with Hoechst 33342 nuclear stain (1:1000) for 10 minutes. Images were acquired using an upright fluorescent microscope (Leica WETZLAR DM6000B) with a DFC350 FX camera and 20X objective lens with a selective FITC filter set (excitation/emission 495/519 nm), and a DAPI filter set (excitation/emission 359/461 nm).

Results

Idebenone NQO1-dependent reduction

Since the expression of NQO1 enzyme may limit the potential therapeutic effect of idebenone, we firstly investigated the NQO1 level in the three different cell lines used throughout this study: HepG2, SH-SY5Y and R28. In agreement with previous work [21][22], the western blot analysis revealed that the expression levels of NQO1 was high in HepG2 cells but almost absent in SH-SHSY and R28 cells, as shown in Figure 4.

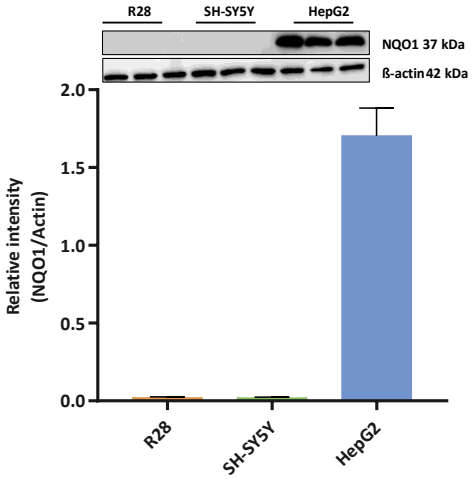


Figure 4 - Protein expression of NQO1 in R28, SH-SY5Y and HepG2 cell lines. The NQO1 protein was quantified by densitometry (n=3, error bars represent mean ± SEM).

We hypothesized that this diversity of NQO1 level between the cell types may lead to a different equilibrium between the different oxidation states of idebenone within the cells: i.e. idebenone would mainly be present in the hydroquinone form (idebenol) in HepG2, but instead in the oxidized form (idebenone) in SH-SY5Y and R28 cells. In order to study the redox cycling of idebenone in the cell lines, it was of interest to monitor indirectly the reduction of idebenone by NQO1 in living cells.

In the cell, oxidized electron cyclers, such as quinones, can undergo a series of reduction/oxidation cycles by cellular electron sources, such as NADH or oxygen. In the last few years, this redox capacity of living cells has been used as a rapid and sensitive assay to detect viable cells. One of the most common redox cycler compounds used for the quantification of cell viability is WST1, a water-soluble cell-permeable tetrazolium dye. This tetrazolium salt requires an electron coupling reagent (i.e. 1-methoxy-PMS) and is cleaved to a formazan dye by the mitochondrial succinate reductase which exists

in the mitochondrial respiratory chain and is active only in viable cells [23]. However, Tan and Berridge reported the use of WST-1, in the absence of 1-methoxy-PMS, to monitor quinone reduction by NQO1 in cells [18]. Under this condition, WST-1 is converted into the corresponding formazan dye upon reduction by hydroquinones (i.e. idebenol), which mediates the WST-1 reduction by electron donation and re-oxidation into the corresponding quinone (idebenone). This capacity of hydroquinone to reduce WST-1 was used to also assess whether idebenone can be reduced by other oxidoreductases except for NQO1 in cells by testing it in the presence or absence of dicoumarol (NQO1 inhibitor [24]). The idebenone NQO1-dependent reduction was initially tested in HepG2 cells, where NQO1 is highly expressed, which was about 5-fold greater in the presence of idebenone than in its absence (Figure 5a). These results suggested that idebenone is highly metabolised by NQO1. In contrast, in the presence of dicoumarol, WST-1 was not reduced significantly more by the idebenone-treated cell in comparison to the control cells. The assay was repeated in the SH-SY5Y cell line, where no substantial conversion of WST-1 to formazan was detected, confirming that the reduction of idebenone is dependent on NQO1 (Figure 5b).

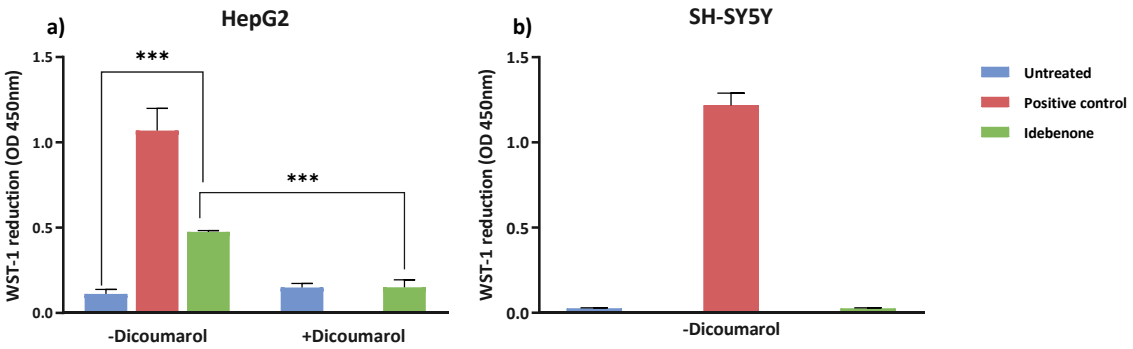


Figure 5 - (a) NQO1-dependent reduction of idebenone in HepG2, (b) No idebenone reduction detected in SH-SY5Y. Idebenone reduction was measured using the WST-1 assay. **5a** the absorbance detected in HepG2 cells after the incubation with 450 µM of WST-1, the NQO1 inhibitor dicoumarol (20 µM) was added to confirm the specificity of idebenone reduction by NQO1. **5b** the absorbance detected in SH-SY5Y after the incubation with 450 µM of WST-1in the presence or absence of idebenone (untreated). Menadione in the presence of recombinant NQO1 in a free-cell environment was used as a positive control in all experiments. Data were analysed using 2-way ANOVA, Tukey multiple comparison test (***$p \leq 0.001$).

Idebenone and NQO1-dependent ATP rescue

It has previously been suggested that the hydroquinone form of idebenone (idebenol) is responsible for ATP rescue activity, through the NQO1-complex III pathway: idebenol is able to bypass

mitochondrial complex I and complex II by shuttling electrons from NQO1 to complex III [12]. Having established that HepG2 cells contain NQO1 and were capable of reducing WST-1 in the presence of idebenone, this cell type was selected to analyse whether ATP rescue could be achieved via the presence of idebenone. Firstly, treatment of these cells with rotenone (a complex I inhibitor) drastically reduced ATP levels to 8% residual ATP in glucose free medium (Figure 6). However, the addition of idebenone to this rotenone model caused ATP rescue in a dose-dependent manner, with almost complete rescue at 10 µM (Figure 6a). In order to elucidate the role of NQO1 in the ATP rescue activity of idebenone, the experiment was repeated in the presence of dicoumarol (Figure 6b). The results indicated that the effect of idebenone was completely lost when the function of NQO1 was inhibited by dicoumarol, suggesting that idebenone could affect the mitochondrial metabolism only if the active enzyme is available (Figure 6b).

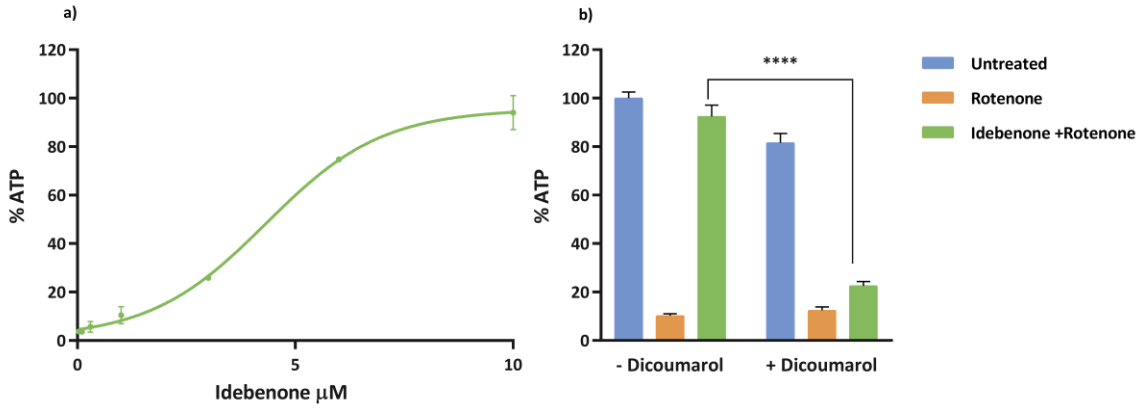


Figure 6 - (a) Idebenone mediated ATP rescue in HepG2 cells is dependent of NQO1 activity, (b) Influence of dicoumarol on the idebenone activity. HepG2 cells were incubated with 25 µM rotenone in presence or absence of 10 µM dicoumarol and/or 10 µM idebenone for 2h. ATP levels are expressed as a percentage of ATP in control cells in the absence of rotenone (untreated). Error bars represent mean ± SEM of three independent measurements. Data shown in panel b were analysed using 2-way ANOVA, Tukey multiple comparison test **** = $p \leq 0.0001$

In accordance with this experimental setup for HepG2, the idebenone was tested on SH-SY5Y and R28 cell lines, in order to exclude any possible dicoumarol effect (other than its inhibition of NQO1) being responsible for the observed abolition of idebenone activity, hence confirming the importance of NQO1. In the SH-SY5Y and R28 cells, the idebenone treatment, under complex I impairment, was ineffective even at the highest concentration (Figure 7). The failure of restoring ATP levels was attributed to the poor NQO1 expression in these cells, as confirmed via our western-blot analysis (Figure 4).

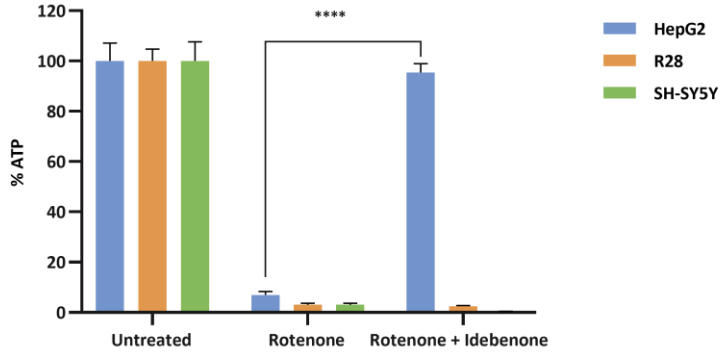


Figure 7 - ATP rescue by idebenone activity in rotenone treated cells is cell specific. Three cell lines were treated with rotenone (25 µM) in the presence or absence of idebenone (10 µM). ATP levels are expressed as a percentage of ATP in the absence of rotenone (untreated). Bars represent mean ± SEM of three independent measurements. Data were analysed using 2-way ANOVA, Tukey multiple comparison tests, *P* values were calculated versus rotenone treated cells (****p ≤ 0.0001).

Idebenone cytotoxicity in NQO1-deficient cell lines

Previous studies reported that idebenone can inhibit mitochondrial complex I function and concomitantly, promote superoxide production [11]. The majority of these studies were conducted on isolated complex I proteins or mitochondria, so it was of interest to evaluate a possible cytotoxic effect of idebenone in our cell lines. Since, idebenone failed to rescue the ATP level in the SH-SY5Y and R28 cells due to the low level of NQO1, it was expected that idebenone could be accumulated in these cells. This is in contrast to HepG2 cells, where idebenone is mostly present in the reduced form as idebenol due to the high presence of the NQO1 enzyme. Consequently, the accumulation of idebenone might promote a cytotoxic effect in the SH-SY5Y and R28 cells due to the potential interaction with complex I. All three cell lines were therefore incubated with a range of idebenone concentrations for 24 h, and the cell viability was evaluated using two different methods: the resazurin reduction by living cells (CellTiter blue) and the quantification of total ATP level in cells (CellTiter Glo). In both assays, a significant reduction of cell viability was evident in a dose-dependent manner. Cells exposed to 15 µM of idebenone exhibited some reduction of cell viability in R28 cells, but less so in SH-SY5Y; however, in both cell lines, a concentration of idebenone above 25 µM showed a remarkably toxic effect (Figure 8). Concentration-response studies showed a half maximal inhibitory concentration (IC_{50}) of 16 (± 0.09) µM and 29 (± 0.54) µM for R28 and SH-SY5Y cells, respectively.

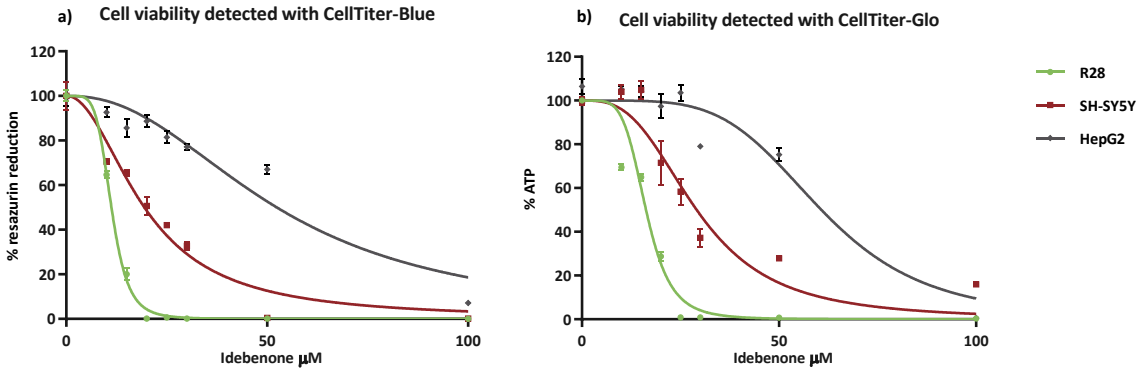


Figure 8 - The toxicity caused by idebenone is cell type specific. Dose dependent curve of idebenone (10-100 µM) treatment on R28, SH-SY5Y and HepG2 for 24 h. The graphs show the % cell viability detected with CellTiter-Blue (% rezofurin reduction) (a) and CellTiter-Glo respectively (% ATP level) (b). Each value is normalised to control (untreated) cells. Error bars represent mean ± SEM of three independent measurements.

Interestingly, the idebenone-induced toxicity was substantially lower in HepG2 (IC_{50}=60 ± 2.68 µM). These results suggest that the accumulation of the oxidative form of idebenone is correlated with its toxicity and potential complex I inhibition.

Pro-oxidant effect of idebenone in NQO1 deficient cell lines

Idebenone complex I inhibition can cause an accumulation of excess superoxide that is, in turn, converted to H_2O_2 by superoxide dismutase in the cytoplasm (CuZnSOD, SOD1) or in mitochondria (MnSOD, SOD2)[25]. Non-physiologically high levels of H_2O_2 induce the loss of mitochondrial membrane potential, cytochrome c release, and cause caspase-3 activation resulting in cell death[25]. To determine whether the hydrogen peroxide was involved in the cytotoxic effect of idebenone, the H_2O_2 cell level was studied in R28 and SH-SY5Y cells after exposing them to different idebenone concentrations. In both cell lines, a significant increase of H_2O_2 was evident after 6h of treatment with idebenone. The level of H_2O_2 in SH-SY5Y and R28 cells exposed to 25 µM idebenone was significantly higher ($P \leq 0.01$) when compared with the untreated control cells, while the treatment with 10 µM did not show a significant difference (Figure 9). As expected, idebenone did not show a substantial pro-oxidant effect on HepG2 at concentrations lower than µM, in line with the low cytotoxicity of idebenone detected in these cells (Figure 9).

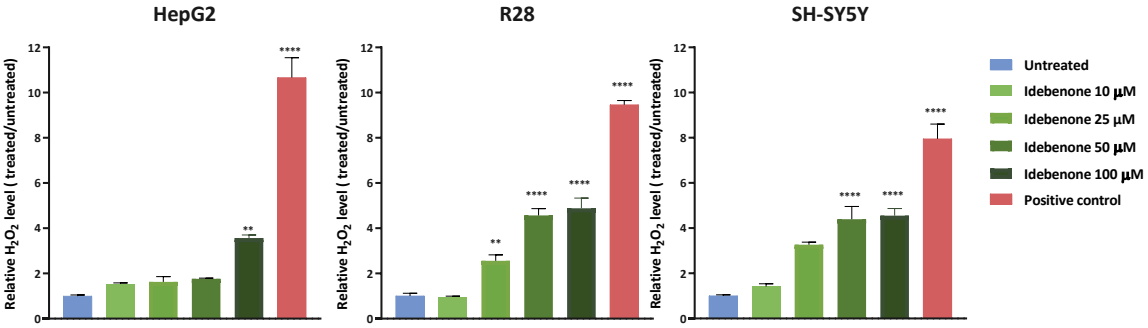


Figure 9 - Idebenone increases the H_20_2 production in NQO1-deficient cell lines compared to in HepG2 cells. Relative H_2O_2 level detected in R28 and SH-SY5Y respectively. Menadione (25 µM) was used as positive control. Error bars represent mean ± SEM of three independent measurements. Data were analysed using 1-way ANOVA, Tukey multiple comparison test, *P* values were calculated versus untreated cells (**p ≤0.01, ****p ≤ 0.0001).

NQO1 expression in retinal layers

From our experiments in cell lines, we found that the therapeutic effect of idebenone is dependent on its conversion to idebenol by the enzyme NQO1. Since the conflicting evidence surrounding the clinical efficacy of idebenone has been found in diseases characterised by a loss of retinal ganglion cells [17][26], we looked at the expression of NQO1 in the retina. We found that NQO1 is expressed in the mouse retina, with the highest level expression seen in some areas of the ganglion cell layer (Figure 10).

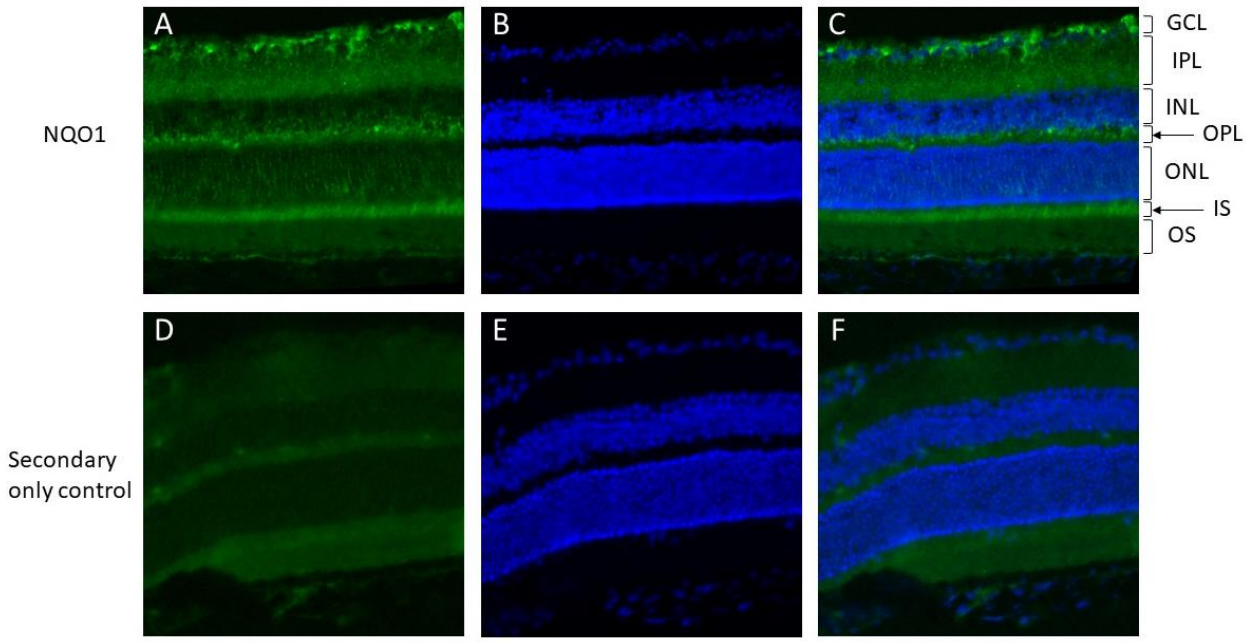


Figure 10: NQO1 is expressed in the retinal ganglion cell layer (GCL) of the mouse retina. The expression of NQO1 can be seen across all layers of the mouse retina, mostly in the inner segment (IS) layer of photoreceptors, outer and inner plexiform layers (OPL and IPL, respectively) where neurons form synaptic connections, with the highest expression seen by some but not all cells in *the* ganglion cell layer (A&C). Relatively low expression of NQO1can be seen in the outer segment (OS) layer, and in the outer and inner nuclear layers (ONL and INL, respectively). For comparison a secondary only control is shown (D-F). Hoechst nuclear stain was used to identify the retinal layers (B&E).

Idebenone rescue and toxicity threshold in the ganglion cell layer of the mouse retina

Due to the expression of NQO1 in the ganglion cell layer, idebenone can be reduced to idebenol, providing a potential treatment for diseases associated with retinal ganglion cells (RGC) loss caused by complex I dysfunction. However, RGCs are not the only cells present in this layer, and clearly there are cells present with very low, if any, expression of NQO1 (Figure 10). Due to the toxic effects seen with higher concentrations of idebenone in cell lines, we first looked at the effect of increasing the concentration of idebenone above 10 µM on cells in the ganglion cell layer in the mouse retinal explant. Explants were incubated with 10, 20, 50 or 100 µM of idebenone for 24 hours and the effect on cell loss after 24 hours was compared to the effect of 100 µM rotenone. There was no cell loss in the ganglion cell layer with 10 µM of idebenone, but significant toxicity was seen at 20 µM and all higher concentrations tested, and was similar to the toxicity induced by 100 µM rotenone (Figure 11).

To test the hypothesis that idebenone provides protection against RGC loss caused by complex I dysfunction, we incubated retinal explants with 100 µM rotenone for 24 hours and co-incubated with various concentrations of idebenone which are below its toxic dose. We found that rotenone induced loss of 41 % of cells whereas idebenone provided partial protection against RGC loss caused by complex I dysfunction (Figure 12). The efficacy of idebenone in complex I compromised RGCs by rotenone, increased with increasing concentration, but a statistically significant rescue from rotenone induced cell loss is seen only with 10 µM where it increased cell survival by 33% (Figure 12).

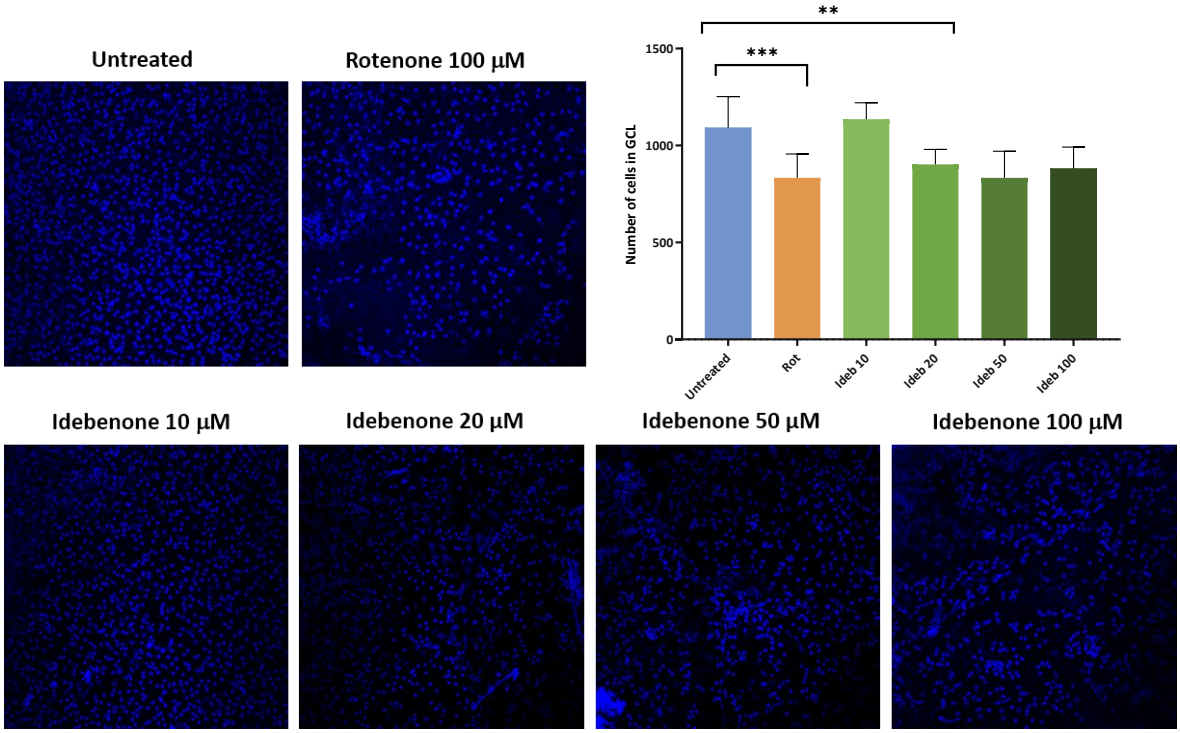


Figure 11: High concentrations of idebenone cause cell loss in the ganglion cell layer of the retinal explant. (A) Representative *en face* images of Hochest nuclear stain in the ganglion cell layer from the central retina of explants incubated with 10, 20, 50 or 100 µM idebenone (Ideb), or 100 µM rotenone (Rot) for 24 hours. (B) Mean number of cells per area in the ganglion cell layer (GCL) of the central retina after 24 hours *ex vivo*. No cell loss is seen after 24 hours *ex vivo* with 10 µM idebenone. Significant cell loss is seen with 20, 50 and 100 µM idebenone. Error bars represent ± SEM. Data were analysed using 1-way ANOVA, Tukey multiple comparison test, *P* values were calculated versus untreated cells (DMSO vehicle) (**p ≤0.01, ***p ≤ 0.001).

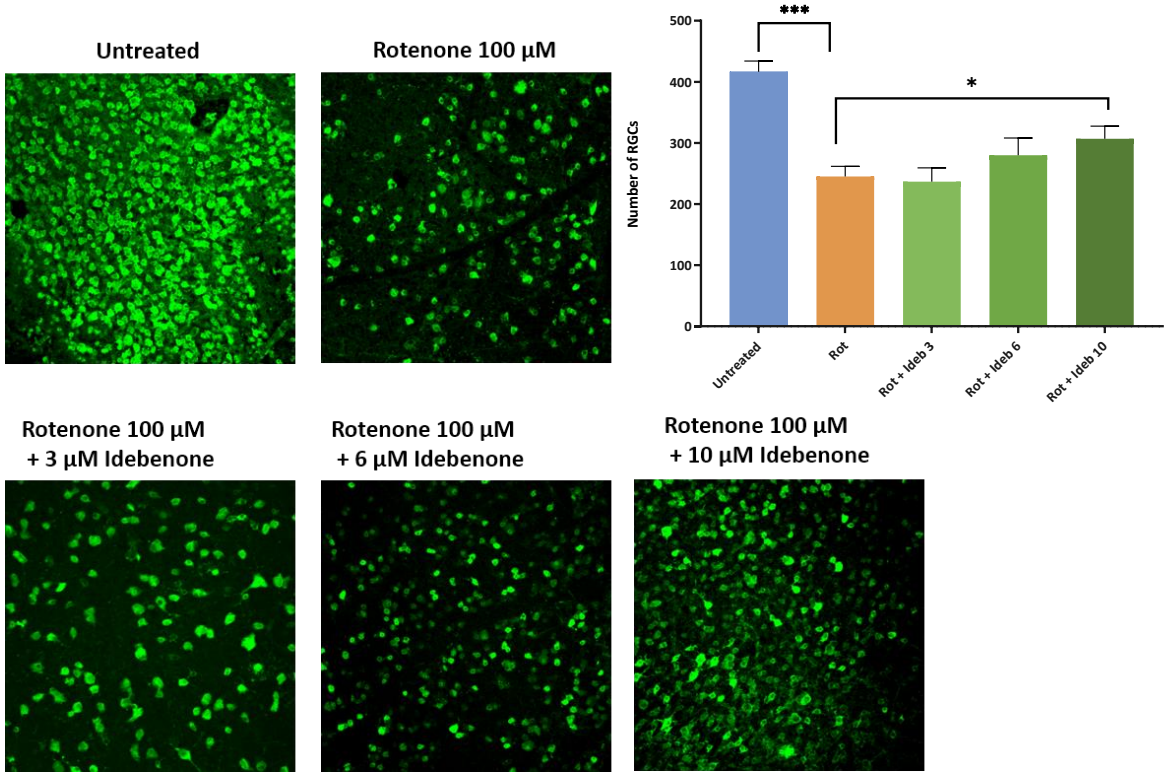


Figure 12: Idebenone rescues rotenone induced RGC loss at 10 µM in the retinal explant. (A) Representative *en face* images of RNA-binding protein with multiple splicing (RBPMS) positive cells in the central retina incubated with 100 µM rotenone and either 0, 3, 6 or 10 µM of idebenone. Representative images from control groups are included: 100 µM rotenone after 24 hours *ex vivo*. (B) Mean number of RGCs in the central retina after 24 hours *ex vivo*. There is a significant reduction in the number of RGCs in retinae treated with 100 µM rotenone compared to those treated with the 0.01% DMSO vehicle (untreated). The number of RGCs is significantly higher in explants treated with 100 µM rotenone and 10 µM idebenone compare to those treated with 100 µM rotenone only. Error bars represent ± SEM. Data were analysed using 1-way ANOVA, Tukey multiple comparison test, *P* values were calculated versus untreated cells (0.01% DMSO) (**p ≤0.01, ***p ≤ 0.001).

Discussion

Idebenone is the only drug approved for the treatment of LHON [3]. However, there are still questions regarding the efficacy and specificity of the drug. In fact, although idebenone is generally well-tolerated in patients, different studies have reported evidence of potential adverse effect such as the impairment of complex I [10], superoxide generation [11], opening of the permeability transition pore complex [27], inhibition of a calcium-activated chloride channel [28] and induced apoptosis in cells. Here, we highlight the importance of the NQO1 enzyme for the activity of idebenone and the potential toxic effect in cells with low NQO1 expression. Although, there is evidence from several studies that idebenone can be bio-activated from other enzymes such as Complex II and glycerophosphate (G3PDH) shuttle[29],[30], here we have instead demonstrated that specifically NQO1 plays the crucial role in idebenone activity. Our results indicate that idebenone is not reduced in cells with low NQO1 expression, so the potential bio-activation from Complex II or glycerophosphate (G3PDH) shuttle can be considered only marginal in comparison with NQO1. Moreover, in NQO1-deficient cell lines, idebenone is not able to reverse the rotenone-induced ATP depletion even at higher concentrations, and, on the contrary, concentrations above 25 µM showed critical cytotoxicity.

Unlike NQO1, the mitochondrial enzymes are ubiquitously present in all cells. The lack of idebenone activity in SH-SY5Y and R28 cells suggests the complex II and glycerophosphate (G3PDH) shuttle are not able to metabolise idebenone at the same rate as NQO1, consequently, in these cells, the oxidised state of idebenone is the major species. The accumulation of the quinone form could promote the idebenone side effects reported in the literature. Here, we focused on the pro-oxidant activity of idebenone, investigating the increase of H_2O_2 level within cells. It is not uncommon for a classic antioxidant substance to possess pro-oxidant characteristics, depending on the concentrations and the cell environment [31]. In our study, idebenone was able to quickly (within six hours) promote the generation of H_2O_2 in NQO1 deficient cell lines, thereby triggering a deleterious oxidative stress to the cells. A potential pro-oxidant pathway is through the inhibition of complex I[11], which is one of the main sites of superoxide radical production in the respiratory chain, but other pro-oxidant pathways cannot be excluded, and the increase of H_2O_2 level is most likely due to multiple causes. The pro-oxidant activity of idebenone was initially suggested by Tai et al., in 2001. They found that exposure of the dopaminergic neuroblastoma SH-SY5Y cells to 25 µM or higher concentrations of idebenone for 72 h, significantly reduce the cell viability and increase the caspase-3 activity in the cells [32]. In our study, we confirmed the pro-oxidant characteristic of idebenone by directly detecting the H_2O_2 level in the cells, and we found that idebenone drastically reduced cell viability after only 24h of incubation. This difference in idebenone toxicity might be found in the different experimental conditions

employed in the studies: higher serum concentration (10%), which reduce the drug toxicity [33], and different methods for detecting changes in cell viability [34].

The findings in the retinal explant provide further support to the theory that idebenone can switch from an antioxidant to a pro-oxidant, depending on idebenone concentration and NQO1 expression. While no toxicity was seen with 10 µM of idebenone, 20-100 µM caused a similar level of toxicity in the ganglion cell layer to that seen with 100 µM of the complex I inhibitor rotenone. Furthermore, when tested at safe concentrations, there was an increase in RGC rescue with increasing idebenone concentration, but only the highest safe concentration of 10 µM showed statistically significant effect by increasing cell survival from 41 to 73%. These findings suggest that sufficient NQO1 is available in the ganglion cell layer to reduce idebenone, but it might be mainly localised in the retinal ganglion cells, which represent 43% of the cell population in the mouse ganglion cell layer [35]. The remaining cell populations in the ganglion cell layer, which consist of amacrine cells, astrocytes and microglia cells, might show lower or no NQO1 presence, making these cells more vulnerable to idebenone mediated toxicity, as suggested by our results (Figure 11). This theory is also supported by the findings of Smith *et al*., whereby the beneficial effects of idebenone were limited in mice with complex I dysfunction, and RGC dendropathy was reported in wild-type mouse retina [17]. In addition, the expression level of NQO1 varies in the different tissues of the human eye, subepithelial conjunctival tissue, corneal stroma, and the pial septa of the optic nerve show a low NQO1 expression [36], that may further limit topical eye administration of idebenone.

Although idebenone showed overall positive results in a subgroup of treated LHON patients [37], it failed to ameliorate the disease course in Friedreich ataxia patients [38]. In this study we showed that the different NQO1 expressions in the tissues, combined with the potential complex I inhibition, might explain the contradictory results obtained in these disease models and in patients. In fact, our *in vitro* and *ex-vivo* data indicate a cell-specific response to idebenone, with a loss of activity in NQO1-deficient cell lines and a significant increase in toxicity. Altogether, we have demonstrated that Idebenone has a very narrow therapeutic dose before becoming either ineffective or toxic.

Acknowledgements

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