Real-time monitoring of superoxide generation and cytotoxicity in neuroblastoma mitochondria induced by 1-trichloromethyl-1,2,3,4-tetrahydro-beta-carboline

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The potential neurotoxin 1-trichloromethyl-1,2,3,4-tetrahydro-beta-carboline (TaClo) has recently been suggested to be a causative factor in the clinical development of Parkinsonian symptoms after long-term exposure to precursor compounds such as the hypnotic chloral hydrate. TaClo is known to cause cell death in dopaminergic neuronal cells, however, the pathway and mechanisms remain undefined. This study reports for the first time that TaClo promotes cytotoxicity in SH-SY5Y neuroblastoma cells within 2 hours of initial exposure. TaClo also caused superoxide production from isolated mitochondria, which was comparable in response time and magnitude to production elicited by more established respiratory inhibitors such as rotenone and antimycin A. These findings present new evidence in support of TaClo-induced neuronal death via superoxide signalling and oxidative stress.

Keywords: Parkinson's disease, TaClo, Mitochondria, Complex I, Amperometric monitoring, Oxidative stress

Introduction

The mechanisms underlying the long-term progenesis of Parkinson's disease (PD) are not fully understood, although recent research has provided evidence for the involvement of a host of exogenous compounds and their endogenously generated metabolites.¹ A loss of dopaminergic neurons in the *substantia nigra pars compacta* is characteristic of the progressive neurodegeneration intrinsic to the PD phenotype.² It is now widely accepted that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), an impurity found in illicitly prepared caches of meperidine, can cause selective neural damage in the nigrostriatum.³ Selective uptake of the MPTP metabolite MPP⁺ by the plasma membrane dopamine transporter (DAT) concentrates the toxin within dopaminergic neurons,⁴ where it is capable of causing massive oxidative damage to DNA and cellular proteins via inhibition of complex I of the mitochondrial electron transport chain (mETC).^{5–7} This discovery promoted the investigation of chemically similar potentially neurotoxic

compounds in an attempt to establish a link between environmental toxin exposure and the PD phenotype. Chloral hydrate and trichloroethylene are two such compounds that were identified as having neurotoxic potential due to their metabolism to chloral after administration. Although the precise mechanism by which chloral induces central nervous system depression has not yet been fully elucidated, metabolic investigations have uncovered the generation of species with mitochondria-disrupting potential, one of which was 1-trichloromethyl-1,2,3,4-tetrahydro-beta-carboline (TaClo). TaClo is formed *in vivo* through the spontaneous reaction of endogenous tryptamine ('Ta') with chloral (trichloroacetaldehyde; 'Clo'). TaClo has been shown in many recent publications to inhibit complex I of the mETC in a manner comparable with MPP⁺^{8–11} and diphenylene iodonium (DPI),¹² a chemical to which TaClo bears striking structural similarity (Fig. 1), resulting in an increase in cellular damage through the generation of large amounts of reactive oxygen species (ROS). Complexes I and III of the mETC have previously been shown to generate superoxide (O₂^{•−}) immediately following exposure to known inhibitors rotenone and antimycin A.¹³ All

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Figure 1 Structural similarity between known complex I inhibitor DPI and proposed neurotoxin TaClo corroborated the ability of TaClo to act as an mETC inhibitor.

studies to date pertaining to the quantification of TaClo-mediated mitochondrial ROS generation have been reliant on endpoint studies of the effects of ROS on cellular components such as DNA, glutathione, and enzyme upregulation.¹⁴ These studies were limited by their inability to monitor the generation of potentially cytotoxic ROS directly and in real time making temporal resolution of their production very challenging. Additionally, traditional measurement techniques do not allow differentiation between the various ROS produced by the mitochondria.

The current study aimed to demonstrate whether TaClo was capable of eliciting measureable $O_2^{\bullet-}$ production in cultured neuroblastoma cells. The study focussed on changes in whole cell respiration as well as the real-time detection of $O_2^{\bullet-}$ flux from isolated mitochondria exposed to TaClo and other complex I inhibitors. Many studies cite the time scale of action of TaClo as being up to 72 hours post-administration before significant effects are observed¹⁴ however, other mETC inhibitors such as rotenone and antimycin A have been shown to act on the mitochondria within seconds.¹⁴ The vital dye resazurin was used in the current study to assess the ability of TaClo to affect whole cell respiration. Unlike more conventional reagents used in assessing cell respiration and viability (e.g. (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)), resorufin, the product of resazurin metabolism, is soluble and non-toxic. This eliminated the need for dye solubilization prior to reading and reduced result ambiguity caused by toxic intracellular dye generation.¹⁵ The use of resazurin thus provided an ideal method with which real-time cellular respiratory rates in intact cells could be measured.

Using an $O_2^{\bullet-}$ selective electrode it was also possible to monitor in real time the flux of $O_2^{\bullet-}$ generated from isolated mitochondria following treatment with TaClo. This technique has been previously used to monitor the ability of both rotenone and antimycin A to elicit $O_2^{\bullet-}$ production from human melanocyte-derived mitochondria.¹³ Electrochemical monitoring of $O_2^{\bullet-}$ offers many advantages over more indirect and non-specific methods, for example, the use of chemiluminescent dyes and end-point assays, which relate primarily to the high selectivity of the electrode for $O_2^{\bullet-}$.¹⁶ Unlike various luminescent and fluorescent probes, the electrode is capable of discriminating

between ROS and can only generate a measureable current through interaction with $O_2^{\bullet-}$. The functionalization of gold electrodes with the redox protein cytochrome *c* has been extensively used to monitor $O_2^{\bullet-}$ flux *in vitro*.^{13,17,18} Previous studies have attributed $O_2^{\bullet-}$ specificity to the site-directed immobilization of the protein and the low operating potential used to re-oxidize the cytochrome *c*.¹⁶

The current study used a vital dye based optical detection method to continuously monitor the short-term effects of TaClo on neuroblastoma whole cell respiration in conjunction with an amperometric $O_2^{\bullet-}$ detection technique that was used to selectively monitor TaClo-induced $O_2^{\bullet-}$ flux from isolated mitochondria. The integration of these data sets has provided new evidence for the involvement of TaClo in the cumulative oxidative damage observed in progressive neurodegenerative disorders.

Materials and methods

Reagents

Cell culture reagents were all purchased from PAA (Somerset, UK). The thiol cross-linker 3-3'-dithiobis (sulphosuccinimidylpropionate) (DTSSP) was supplied by Pierce (Chester, UK). TaClo was purchased from Exclusive Chemistry Ltd. (Obninsk, Russia). All other reagents were purchased from Sigma Aldrich (Poole, UK).

Cell culture

The three times cloned human neuroblastoma cell line SH-SY5Y was routinely cultured using antibiotic-free Dulbecco's Modified Eagles Medium supplemented with 10% fetal calf serum (FCS) and 1% non-essential amino acids (PAA Laboratories, Somerset, UK). Cells were maintained in a humidified environment containing 5% (v/v) CO_2 at 37°C throughout. Cells were grown to 80% confluence and were passaged five times over the course of experimentation, with mitochondrial isolation occurring at the third and fifth passage.

Real-time assessment of whole cell respiratory rate

Cells were seeded into black-walled, clear-bottomed 96 well plates at a density of 1×10^3 cells/well and allowed to attach overnight. The growth medium was subsequently replaced with either 0.001% (w/v) resazurin salt in phosphate buffered saline (PBS) or 0.001% (w/v) resazurin salt in PBS doped with 100 μM TaClo. An initial baseline fluorescence measurement was made immediately after medium replacement (λ_{ex} 530 nm and λ_{em} 590 nm) using an Infinite M200 fluorimeter (Tecan, UK) with associated Magellan control software. After 1-hour incubation, fluorescence measurements were taken using

the same parameters. This was repeated on an hourly basis for two further measurements. Cells were kept in the routine culture environment between readings.

Mitochondrial fraction preparation

Mitochondrial fractions were prepared as previously reported by Henderson *et al.*¹³ Briefly, cells grown to 80% confluence were trypsinized and washed in PBS. After re-suspension in sucrose-rich mitoprep buffer (250 mM sucrose, 2 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and 0.1 mM EGTA (ethylene glycol tetraacetic acid), pH 7.4), the outer cell membranes were disrupted using 20 passes with a power-driven teflon-glass homogenizer. The resulting homogenate was centrifuged at $450 \times g$ and 4°C for 10 minutes, after which the supernatant was decanted and further centrifuged at $11\,100 \times g$ 4°C for 10 minutes. The resulting mitochondria-containing pellet was re-suspended in fresh mitoprep buffer then snap frozen in liquid nitrogen and stored at -80°C in 20 μl aliquots until required. Prior to use, the protein concentration of the mitochondria-containing sample was determined using a Bradford assay (Bio-Rad, Hertfordshire, UK) as per the manufacturer's instructions. Mitochondria were kept in sucrose-rich buffer throughout all electrochemical experiments.

Preparation of $\text{O}_2^{\bullet-}$ -specific electrode

The electrode was functionalized as previously described.¹⁹ The surface of a 2-mm diameter gold electrode (BioAnalytical Systems, Cambridgeshire, UK) was polished using 0.02 μm aluminium oxide crystal slurry on a microcleaning cloth (BioAnalytical Systems). The electrode surface was further cleaned by sonication in 100% ethanol for 5 minutes. After rinsing with deionized water, the electrode was first incubated in 50 mM DTSSP for 5 minutes at room temperature, then thoroughly rinsed and incubated overnight in 2 mM cytochrome *c* at 4°C . After thorough rinsing to remove unbound protein, the electrode was ready for use.

For all experiments, the electrode was poised at an operating potential of +100 mV versus an Ag/AgCl reference electrode (Pierce), which also served as the counter electrode. A bespoke two-channel potentiostat was used and the data were acquired using PicoLog datalogging software.

The electrode was calibrated as previously reported using $\text{O}_2^{\bullet-}$ generated as a product of the catalytic decomposition of xanthine by xanthine oxidase.¹⁶

Amperometric monitoring of TaClo dose-response in isolated mitochondria

The protein concentration of the mitochondrial suspension was determined to be $3.45 \mu\text{g ml}^{-1}$. Aliquots of the thawed mitochondrial suspension were diluted

1:5 to $0.69 \mu\text{g ml}^{-1}$ in mitoprep buffer to ensure sufficient samples were available over the course of experimentation. A conical dish was used to present a 100 μl sample of the mitochondrial suspension to the $\text{O}_2^{\bullet-}$ electrode. Once a stable baseline current response was established, serial additions of 10 μM TaClo in mitoprep buffer were made to the cell. Any current change following addition was recorded for 1 minute before the next addition was made. Dosing continued until a steady-state response was achieved that remained unchanged following further TaClo addition.

Comparison of known mETC complex inhibitors to TaClo

Prior to use, 100 μM solutions of rotenone, antimycin A and TaClo were prepared separately in mitoprep buffer. Inhibitors were investigated in sequence, with only one inhibitor being used per mitochondrial sample. Aliquots of the mitochondrial suspension were diluted to $0.69 \mu\text{g ml}^{-1}$ in mitoprep buffer. The electrode setup was the same as used to investigate the TaClo dose-response. A 100 μl sample of mitochondrial suspension was introduced to the electrode and the current responses recorded. Once a stable baseline current response was observed, 10 μl of a particular inhibitor was added. The changes in current response were recorded for 3 minutes following inhibitor addition. Fresh aliquots of mitochondrial suspension were used for each inhibitor challenge with thorough cleaning of the electrodes and conical dish between tests. Three current traces were acquired for each inhibitor.

Results

Electrode calibration

The generation of $\text{O}_2^{\bullet-}$ by enzymatic conversion of xanthine to uric acid by xanthine oxidase has previously been used to calibrate the $\text{O}_2^{\bullet-}$ response of cytochrome *c*-functionalized gold electrodes. In the present study the current density at steady state was linearly dependent on the concentration of xanthine oxidase in the presence of excess substrate ($y = 21.504x$).

Effect of TaClo on mitochondrial function

After 1 hour incubation with resazurin, data from both TaClo-treated and untreated populations demonstrated that the fluorescence intensity at $\lambda_{\text{em}} 590 \text{ nm}$ was linearly dependent on incubation time (TaClo-untreated cells: $y = 0.4249x - 0.2803$, $r = 0.9993$, $n = 4$; cells treated with 100 μM TaClo: $y = 0.2063x - 0.0788$, $r = 0.9873$, $n = 4$). The rate of fluorescence intensity change was significantly higher in TaClo-untreated cells versus treated cells after 3 hours incubation, indicating that metabolic processes became significantly impeded in treated cells after just

3 hours exposure ($P < 0.05$ by one-way analysis of variance (ANOVA)) (Fig. 2).

Amperometric monitoring of $O_2^{\bullet-}$ generation by TaClo

A dose–response curve was generated by recording the current change produced by the cumulative addition of TaClo to isolated mitochondria suspended in sucrose buffer. The change in current was directly related to the flux of $O_2^{\bullet-}$ from the mitochondria, which occurred immediately following TaClo addition. Linear regression analysis of the peak current observed following cumulative TaClo additions within a given sample of isolated mitochondria ($\text{pA } \mu\text{M}^{-1}$) described the relationship as directly proportional to the equation $y = 12.384x + 0.6697$ ($r = 0.9929$, $n = 4$) where y = current change and x = TaClo concentration (Fig. 3).

Comparison of $O_2^{\bullet-}$ generation by known mETC inhibitors

All three inhibitors elicited $O_2^{\bullet-}$ flux from isolated mitochondria immediately following exposure, which was monitored directly and in real time using the cytochrome *c*-functionalized electrode. Fig. 4 describes the relative magnitudes of currents generated through the inhibition of the mETC with antimycin A, TaClo and rotenone, whereas Fig. 5 shows representative traces recorded following treatment with each of the mETC inhibitors. The complex III inhibitor antimycin A elicited the greatest current change of the three inhibitors studied, on average producing a peak current of 239.85 pA (± 24.65 pA, $n = 4$), whereas the complex I inhibitors TaClo and rotenone produced

Figure 2 TaClo-mediated changes in resazurin metabolism were monitored in real-time as an assessment of cell viability. Cells exposed to 100 μM TaClo exhibited lower fluorescence intensity at 590 nm following 3-hour incubation compared with control cells suggesting TaClo compromised the metabolic capacity of the cells within this timeframe. The data are presented $\pm$ SEM, $n = 4$, $P \leq 0.05$ as determined by one-way ANOVA after 3 hours.

Figure 3 Peak current responses of single isolated SH-SY5Y mitochondria samples to cumulative doses of TaClo. The data are presented $\pm$ SEM derived from three individual current traces.

Figure 4 Comparison of peak current responses generated by the addition of the mETC inhibitors TaClo, rotenone and antimycin A (10 μM in each case) to isolated mitochondria from SH-SY5Y cells. The data are presented $\pm$ SEM, $n = 4$; ***denotes $P < 0.005$ as determined by one-way ANOVA.

Figure 5 Comparison of respective traces elicited from the addition of 100 μM mETC inhibitor to the isolated mitochondria from SY5Y-SH cells. The traces shown are representative of a single-inhibitor challenge with a single sample of isolated mitochondria.

peak currents of 122.75 pA (± 20.49 pA, $n = 4$) and 118.23 pA (± 15.64 pA, $n = 4$), respectively. The peak current observed after addition of the complex III inhibitor (antimycin A) to the isolated mitochondria was significantly different from peak current generated by rotenone and TaClo addition, both of which are associated with complex I inhibition ($P < 0.05$ as determined by one-way ANOVA). There was distinct difference in the magnitude and time-course of $O_2^{\bullet -}$ current generated by the different mETC inhibitors as shown by the amperometric traces plotted in Fig. 5. Both of the complex I inhibitors generated an initial current peak lasting between 10 and 15 s which then decayed to the baseline current response that was recorded pre-stimulation. This did not occur for the complex III inhibitor which induced rapid current increases that repeatedly reached a plateau at a sustained mean value of +165 pA above the baseline response (± 56 pA, $n = 4$).

Discussion

TaClo is an endogenously generated metabolite of the hypnotic agent chloral hydrate. It has been described as a neurotoxin and has been reported as a causative factor in the degeneration of the *substantia nigra pars compacta*, which is a defining feature of PD.^{2,3,20,21} It has been proposed that the mechanism of TaClo-induced neurotoxicity involves increased ROS flux from the mitochondria over the course of TaClo accumulation.²⁰ All previous studies that have tried to assess the link between TaClo-induced cell toxicity and ROS production have exposed cells to TaClo for at least 72 hours before assessing cellular damage. It was, therefore, the primary aim of this study to show whether the complex I inhibitor TaClo was capable of eliciting acute cytotoxic effects in SH-SY5Y neuroblastoma cells, and to show with temporal resolution the real-time flux of $O_2^{\bullet -}$ from isolated SH-SY5Y mitochondria after TaClo exposure. The work presented in this paper has clearly shown, for the first time, that the generation of $O_2^{\bullet -}$ is an immediate consequence of mitochondrial complex inhibition by TaClo. This gives new insight into the time scale over which TaClo-dependent cell damage may occur.

Through continuous assessment using a resazurin-based cell viability assay it was found that TaClo was capable of significantly impeding cell respiration within 2 hours of incubation. When investigated using an $O_2^{\bullet -}$ -selective functionalized gold electrode, it was found that rapid flux of $O_2^{\bullet -}$ from the mitochondria was measurable in real time immediately following exposure to the inhibitor. The amperometric technique demonstrated that $O_2^{\bullet -}$ flux following TaClo treatment was comparable with that of two other known mETC inhibitors antimycin A and rotenone. The dynamics of the different responses

demonstrated that TaClo exerted an effect on complex I of the mETC and provided evidence that the toxic effects of the compound are mediated through the generation of ROS.

TaClo has been shown to cause considerable and significant cell death in many neuronal studies.^{8,10,14,22} The study by Bringmann *et al.*²² in particular suggested that despite TaClo being taken up into neuronal cells within 15 minutes of incubation, no measurable effect on cell viability could be observed. This may have been due to the trypan blue dye exclusion assay that the authors used to assess viability. Trypan blue assays rely on the loss of cell membrane integrity as a measure of damage, whereas resazurin assessment is a measure of the reductive capability of the intracellular environment.²³ Resazurin has been established as a tool for the assessment of cell cytotoxicity.^{15,24} The reduction of resazurin to fluorescent resorufin by metabolic activity, generally mitochondrial reduction reactions, allowed the comparative assessment of changes in metabolic function between populations of live cells. Unlike MTT assays, the resorufin product of resazurin metabolism is soluble, removing the need to lyse the cells and solubilize the product in order to read the assay outcome. This facilitated the measurement of cumulative resorufin product generation, therefore allowing the continuous assessment of any acute metabolic effects in response to TaClo exposure. The resazurin viability method is known to have inherent weaknesses which mainly relate to the non-specific reduction of the reagent in the cytosol; however compared with rate-to-reduction reactions carried out at the mitochondria, the non-specific cytosolic effects are thought to be negligible.¹⁵ The ability to continuously measure viability that was facilitated by the technique made it the most appropriate tool for monitoring the acute effects of the neurotoxin in these studies. The time scale over which TaClo was shown to exhibit significant effects on the cells was a novel observation. With resazurin, respiratory depression was observed within 2 hours, whereas many other studies incubate with TaClo for up to 72 hours before viability assessment begins. Owing to the length of time needed to employ more traditional cell viability techniques such as trypan blue, and coupled with the rapid onset of ROS generation and highly developed antioxidant processes, the acute effects of many mitochondrial inhibitors have not yet been fully investigated. The real-time and direct nature of monitoring $O_2^{\bullet -}$ generation from the isolated mitochondria with an electrode circumvented the time and selectivity complications surrounding other modes of ROS quantification such as the use of fluorescent probes dihydroethidium bromide (DHE) or dihydrodichlorofluorescein diacetate (DCF-DA).

The observation that antimycin generated the largest flux of $O_2^{\bullet-}$ of the three inhibitors investigated was in keeping with current literature regarding complex-specific $O_2^{\bullet-}$ generation.^{13,25} Inhibition of complex III is known to cause asymmetric generation of $O_2^{\bullet-}$ both into the intermembrane space where it can freely diffuse and interact with the electrode, and into the mitochondrial matrix where the charged molecule is trapped due to the relative impermeability of the inner mitochondrial membrane.²⁶ The $O_2^{\bullet-}$ flux observed from isolated mitochondria following treatment with rotenone may be caused by an interaction between TaClo and the NADH-ubiquinone oxidoreductase of complex I. Normal transfer between the N2 iron-sulphur cluster and ubiquinone of complex I is reported to be the site of unavoidable electron leakage from the mETC, causing local high $O_2^{\bullet-}$ flux.²⁷ TaClo is thought to inhibit electron transfer from complex I to ubiquinone through interaction with the quinone-binding site on the complex I oxidoreductase.¹² The inherent lipophilicity of TaClo would permit the molecules' access to this membrane-embedded site of the protein,²⁸ much like DPI.¹² The similarity between the modes of superoxide flux of TaClo and rotenone suggested that the compounds elicited effects by similar means, corroborating recent literature regarding TaClo-induced inhibition.²² Both inhibitors resulted in the current response decaying towards the baseline level following an initial increase, as shown in Fig. 5. This may have been due to the point at which the mETC inhibition occurred. It is known that mitochondria generate a basal level of $O_2^{\bullet-}$ as a consequence of the high turnover of electrons by the mETC. The baseline current at which amperometric monitoring started is representative of this basal $O_2^{\bullet-}$ production. When the mETC was blocked at complex I, the downstream flow of electrons was decreased, resulting in the turnover of electrons at complex III being much lower (despite electrons still transferring between complexes II and III). After initial high efflux of electrons from the mETC and subsequent bolus of generated $O_2^{\bullet-}$, the basal level of mitochondrial metabolism was decreased. Furthermore, $O_2^{\bullet-}$ generated by complex I is released to the matrix side of the inner membrane^{26,27} and, coupled with the reduced electron flow through the mETC, the outcome observed was a decrease in the $O_2^{\bullet-}$ available for detection.

In conclusion, this study provided evidence that TaClo is capable of eliciting changes in cell metabolism in less than 2 hours and that the inhibition of mETC by TaClo results in instant and reproducible flux of $O_2^{\bullet-}$ from isolated mitochondria. The rapid action with which TaClo affects the mitochondria is in line with other mitochondrial inhibitors such as rotenone and antimycin A, and further supports the

involvement of early-stage ROS signalling in the pathology of β -carboline precursor compounds. The large TaClo-induced $O_2^{\bullet-}$ flux observed from isolated mitochondria, coupled with a new insight demonstrated in this study regarding the short time scale over which TaClo can exert respiratory effects in whole cells, further implicate ROS as an important factor of its cytotoxic mechanism. A recent study suggested that at low doses TaClo had a stimulatory effect on tyrosine hydroxylase, the rate-limiting enzyme in neuronal catecholamine production.²⁹ The oxidation of excess catecholamines and the resultant increased production of intracellular ROS have been perhaps most prominently defined in the field of cardiac research.^{30–32} If at low doses TaClo acts as an mETC inhibitor while simultaneously boosting catecholamine (dopamine) production, it is possible that cytotoxic effects are exerted through an increase in oxidative stress from both increased dopamine oxidation and mitochondrial inhibition and, in the long term, promoting neuronal dysfunction as a consequence of destabilized cell metabolism. It has also been reported that ROS have profound effects on striatal neuronal ability to release and take up dopamine.^{33,34} This has been demonstrated by monitoring dopamine release following the inhibition of the mETC using rotenone.³⁴

There are many plausible routes by which TaClo has been reported to exert cytotoxic effects, many attributed to the increased generation of ROS^{9–11,22,35} which are known to be short-lived and highly reactive under certain conditions.³⁶ Recent studies have regarded an increase in ROS generation as secondary to 5-hydroxytryptamine (5HT) or DA release.^{35,37} The evidence presented by this study suggests that the uncontrolled production on $O_2^{\bullet-}$ by the mitochondria may be more central to the TaClo-induced pathogenesis of progressive neurodegenerative disorders than previously reported. In order to fully understand the roles of such molecules within the complex architecture of aerobic metabolism, real-time evidence is required that focuses directly on individual species at the time-point of generation such that inferred effects may be substantiated.

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