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Cisplatin induced Mitochondrial DNA Damage In Dorsal Root Ganglion Neurons

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Abstract

Cisplatin is a platinum-based chemotherapeutic agent that induces peripheral neuropathy in 30% of patients. Peripheral neuropathy is the dose limiting side effect, which has no preventative therapy. We have previously shown that cisplatin induces apoptosis in dorsal root ganglion (DRG) sensory neurons by covalently binding to nuclear DNA (nDNA), resulting in DNA damage, subsequent p53 activation and Bax-mediated apoptosis via the mitochondria. We now demonstrate that cisplatin also directly binds to mitochondrial DNA (mtDNA) with the same binding affinity as nDNA. Cisplatin binds 1 platinum molecule per 2166 mtDNA base pairs and 1 platinum molecule per 3800 nDNA base pairs. Furthermore, cisplatin treatment inhibits mtDNA replication as detected by 5-bromo-2'-deoxy-uridine (BrdU) incorporation and inhibits transcription of mitochondrial genes. The relative reduction in mtDNA transcription is directly related to the distance the gene is located from the transcription initiation point, which implies that randomly formed platinum adducts block transcription. Cisplatin treated DRG neurons exhibit mitochondrial vacuolization and degradation *in vitro* and *in vivo*. Taken together, this data suggests that direct mtDNA damage may provide a novel, distinct mechanism for cisplatin-induced neurotoxicity separate from the established nDNA damage pathway.

Keywords

Chemotherapy; Neuropathy; DRG Neurons; Cisplatin; Mitochondria: DNA and DNA damage

Introduction

Platinum-based chemotherapy (e.g. cisplatin, carboplatin and oxaliplatin) is used to treat various types of cancers including prostate, testicular, ovarian, and colon cancers. The dose limiting side effect of these drugs is peripheral neuropathy, which reduces the quality of life for many long-term cancer survivors. Cisplatin-induced peripheral neuropathy occurs in about 30% of patients with 20% being forced to discontinue treatment. Patients with cisplatin-induced peripheral neuropathy experience sensory loss, often accompanied by pain, starting in the distal extremities. Although cisplatin treatment is customarily discontinued once neuropathic symptoms begin, worsening of the symptoms may continue for weeks or

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months. This phenomenon, commonly known as “coasting,” is observed exclusively in platinum-based chemotherapy and suggests that there is ongoing neuronal damage after discontinuing cisplatin.

Cisplatin-induced nuclear DNA (nDNA) damage in dorsal root ganglion (DRG) neurons has been thoroughly investigated. Cisplatin covalently binds to nuclear DNA (nDNA) inducing DNA damage and apoptosis. Cisplatin-induced DNA damage up-regulates p53 and induces cell cycle changes in DRG neurons. Cell cycle changes include up-regulation of cyclin D1, activation of CDK4/6 complexes and phosphorylation of Rb. All of these events lead to Bax translocation to the mitochondria, cytochrome-c release, caspase activation and apoptosis. In culture, DRG neuron survival is dependent on maintenance levels of nerve growth factor (NGF). Increasing the concentration of NGF ten fold prevents both Bax translocation to the mitochondria and cell cycle changes observed in cisplatin treated DRG neurons.

Previous studies have shown cisplatin binds to mitochondrial DNA in liver and B50 cells. Mitochondrial DNA is a circular ~16,300 bp piece of DNA that encodes for 13 proteins essential for the various subunits of the electron transport chain including complex I (NADH dehydrogenase 1, 2, 3, 4, 4L, 5 and 6), complex III (cytochrome b), complex IV (cytochrome c oxidase 1, 2 and 3) and ATP synthase 6 and 8. Mitochondrial DNA also encodes for 22 tRNA and 2 rRNA that are important for synthesis of these proteins. Both replication and transcription are unidirectional from one start point.

The effects of cisplatin on DRG mitochondrial DNA (mtDNA) have not been investigated. Given that mtDNA differs from nDNA in several ways, it follows that cisplatin effects on mtDNA likely differ as well, and that these effects could contribute to cisplatin-induced neurotoxicity. Previous studies in neuronal systems have addressed total platinum-DNA adduct formation without making a distinction between nDNA and mtDNA. It has been shown that DRG neurons accumulate high levels of cisplatin-DNA adducts *in vitro* and *in vivo*. In nDNA, cisplatin-DNA adducts are removed and DNA is repaired by nucleotide excision repair (NER). Mitochondria do not have NER mechanisms for mtDNA.

We demonstrate that cisplatin forms adducts with mtDNA in DRG neurons, and that this alters mtDNA replication and transcription. The resultant cisplatin-induced mitochondrial dysfunction may lead to a novel mechanism of cisplatin neurotoxicity. Our studies were designed to quantitatively assess mtDNA binding and the resulting effect on mitochondrial transcription and replication. We have also assessed the effects of cisplatin on mitochondrial morphology and cellular viability.

Materials and Methods

Cell culture model

DRG from embryonic day 15 Sprague-Dawley rat were dissociated and treated with 20 μ M 2,5 Fluoro-2-deoxyuridine (Sigma, St. Louis, MO) and 20 μ M uridine (Sigma, St. Louis, MO) for 3–5 days to eliminate supporting cells via established methods. Cultures were maintained in EMEM containing 15% calf bovine serum (Hyclone, Logan, UT), 7mg/ml glucose (Sigma, St. Louis, MO) 1.2mM l-glutamine (Invitrogen, Carlsbad, CA) and 10 ng/ml NGF (Bioproducts for Sciences, Indianapolis, IN). For prolonged cisplatin exposure experiments, we utilized a DRG neuron culture system using increased NGF concentrations (100ng/ml) and the pan caspase inhibitor, zVAD-FMK (75 μ M) (Sigma, St. Louis, MO), to inhibit the apoptotic effects of cisplatin (2 μ g/ml) (Novaplus, Bedford, OH).

Survival Experiments

DRG neurons were cultured into poly-L-lysine (Sigma, St. Louis, MO) coated dishes etched with 1mm grids (Nunc, Rochester, NY). Dishes were marked to ensure counting of the same area with images taken at time 0 prior to treatment. Cells were treated over a period of 144 hours. Images were acquired every 48 hours of the same area taken at time 0 and live cells counted as previously described.

Long mtDNA PCR assay

The PCR assay was a modification of. A 13.4kb fragment, which spanned a large portion of the mitochondrial genome, was amplified and standardized against a 235bp mitochondrial fragment. PCR was run at 94° C for 4 min followed by 18 cycles at (94° C for 30 sec., 65° C for 12 min) and 72° C for 10 min using a Long Fragment PCR Kit (Roche Applied Science, Indianapolis, IN). The PCR product was run on a 1% agarose gel containing ethidium bromide and quantitated using a GelDoc (Bio-Rad, Hercules, CA) detection system. DNA loading was standardized by optical density measurements as well as amplification of a 235bp mtDNA fragment. The assay was standardized using liver mitochondrial DNA, reacting it with cisplatin *in vitro* and directly measuring DNA and platinum concentrations. Purified liver mtDNA was isolated using differential centrifugation in combination with a Genomic Tip 20G DNA isolation kit (Qiagen, Valencia, CA). DNA concentration was determined by optical density using a Nano Drop spectrophotometer (Bio-Rad, Hercules, CA). Liver mt-DNA was incubated overnight at 4° C, with cisplatin concentrations ranging from 1Pt molecule per 30 bp mtDNA to 1Pt molecule per 24,000 bp mtDNA. The number of platinum molecules bound to mtDNA was directly measured by inductively coupled plasma mass spectroscopy. PCR of the mtDNA, as previously described, was performed in parallel and PCR products expressed as a percent of control.

To measure adduct formation in DRG neurons we treated with cisplatin for 0, 48 and 144 hours. DRG neurons were harvested and total genomic DNA (gDNA) isolated using phenol-chloroform extraction. PCR was performed using 10ng of gDNA as previously described, PCR product calculated as a percent of control and adducts quantitated by comparison to the standard curve.

Bromodeoxyuridine (BrdU) mtDNA replication experiments

Neurons were plated onto collagen-coated dishes with glass cover slip bottoms (MatTek Corp., Bedford, OH). DRG neurons were treated with cisplatin for 48, 72, 96, 144 and 196 hours. Cells were then place in 10µM BrdU in medium for 24 hours, fixed with an ethanol-glycine fixative, stained with 1:10 anti-BrdU (Roche Applied Science, Indianapolis, IN) and detected with a 1:100 anti mouse FITC secondary antibody. Mitochondrial DNA quantitation was performed utilizing acquired z-stack images (100× magnification) of the entire cell, using laser scanning confocal microscopy (Carl Zeiss Inc., Germany). The intensity and area of BrdU fluorescence was measured within the cytoplasm by computer image analysis using KS-400 software (Carl Zeiss Inc., Germany). All images were measure against a baseline threshold. Mitochondria were labeled using a plasmid expressing DS red containing a mitochondrial targeting sequence. DRG neurons were transfected with a Lipofectamine 2000 transfection kit (Invitrogen, Carlsbad, CA), fixed with ethanol-glycine and stained for BrdU incorporation.

MtRNA transcription experiments

Dissociated DRG neurons were treated with cisplatin for 144 hours, harvested and total DNA/total RNA isolated using an All Prep DNA/RNA/Protein kit (Qiagen, Valencia, CA). RNA concentrations were determined by optical density on a nano-drop spectrophotometer

and PCR standardized by amplification of a 210bp genomic DNA fragment. Real time RT-PCR was performed with 100ng RNA per reaction using iScript One Step RT-PCR kit with SYBER Green (Bio-Rad, Hercules, CA) and a MyQ single color real time detection system (Bio-Rad, Hercules, CA). Primers were created to amplify 169–242 bp fragments along the length of the mitochondrial genome for ND1, COI, ATPase 6, ND4 and Cyt b.

Primer Pairs

ND1

Forward 5' GTTCTGCGAGGGTTGA 3'

Reverse 5' CCCAAACCATCTCTTACGA 3'

COI

Forward 5' GAGCAGGAATAGTAGGGACA 3'

Reverse 5' GTACAAGTCAGTTCCTGAAG 3'

ATPase 6

Forward 5' TTCTTCCCCATACATTTACCC 3'

Reverse 5' GCCTGCTGTAATGTTTGCTG 3'

ND4

Forward 5' AACCTCCTCACTCTTATTCTG 3'A

Reverse 5' ATAAGGATGATAGAGGGGTTTC 3'

Cyt b

Forward 5' ACTGCATTCATGGGCTATG 3'

Reverse 5' CGAAGAAGCGTGTAGGG 3'

In vitro and in vivo, morphological analysis

In vitro, DRG neurons were treated with cisplatin for 0, 48 and 144 hours. The cells were washed with PBS and fixed for transmission electron microscopy (TEM) in Trump's fixative consisting of 4% formaldehyde, 1% glutaraldehyde in PBS at pH 7.2. In vivo, adult male mice C57BL6J (n=4) were treated with two cycles of 5 days drug treatment followed by 5 days rest as previously described. Cisplatin (23mg/kg, cumulative) or vehicle (saline) were used to examine for possible effects of platinum drugs on ultrastructural changes in DRG in vivo. Mice were anesthetized with sodium pentobarbital and euthanized by intracardiac perfusion with phosphate buffered saline (PBS pH 7.2) followed by fresh Trump's fixative. Bilateral L4 and L5 DRG were carefully dissected out from each animal and immediately placed in Trump's fixative for 24 hours.

Transmission electron microscopy

After Trump's fixation, tissues and cell cultures were postfixed in 1% osmium tetroxide and stained *en bloc* with 2% uranyl acetate. The tissue was embedded in a mixture of Epon and araldite. All reagents were obtained from Electron Microscope Services (Ft. Washington, PA). Ultrathin sections (100 nm) were cut from the same blocks, mounted on 200 µm mesh copper grids, stained with lead citrate, examined and photographed with the operator unaware of the treatment condition using an FEI Technai 12 transmission electron microscope at 100 kV (Fei, Inc., Hillsboro, OR), equipped with a digital CCD camera (Advanced Microscopy Techniques, Danvers, MA).

Statistical Methods

Data was analyzed for means and SEM using one-way analysis of variance (ANOVA) in data with parametric distribution (Gaussian). Statistical significance was analyzed using Tukey-Kramer multiple comparison post-test. Percent mtDNA with “n” adducts was based

upon binomial distribution and a pre-specified probability of success/failure. Dose response relationships were analyzed using linear regression.

Results

Elevated NGF and zVAD-FMK inhibit cisplatin-induced apoptosis

As described previously, high levels of NGF (100ng/ml) plus the pan caspase inhibitor zVAD-FMK reduces acute cisplatin-induced neuron death. Cell survival was measured in cisplatin-treated (2ug/ml) DRG neurons at 10 ng/ml NGF or 100ng/ml NGF (Fig. 1). DRG neuron survival in 10ng/ml NGF was 101% at 48h, 103% at 72h, 97% at 96h and 97% at 144h, in 100ng/ml NGF was 94% at 48h, 94% at 72h, 94% at 96h and 95% at 144h. DRG neuron survival in cisplatin with 10 ng/ml NGF was 48% at 48h, 35% at 72h, 19% at 96h and 3% at 144h, in cisplatin with 100ng/ml NGF was 73% at 48h, 53% at 72h, 35% at 96h and 10% at 144h. Addition of both 100ng/ml NGF and 75 uM zVAD-FMK to cisplatin-treated DRG neurons maintained cell survival at 94% at 48h, 88% at 72h, 84% at 96h and 75% at 144h which was significantly increased compared to cisplatin-treated DRG neurons in 10ng/ml NGF alone ($p<0.001$) and was not significantly different from control DRG neuron survival. In summary, high levels of NGF delayed but did not prevent cell death. Cell death was further inhibited with the presence of a caspase inhibitor. The remainder of the experiments outlined utilize this culture system of cisplatin-induced apoptosis inhibition to specifically study cisplatin-mtDNA interactions.

Cisplatin binds mtDNA

A PCR assay, which amplified a 13.4kb mitochondrial DNA fragment, was used to measure the number of cisplatin-mtDNA adducts accumulated in cisplatin-treated DRG neurons, *in vitro* (Fig. 2A). Formation of a cisplatin-DNA adduct on mtDNA blocks PCR amplification reducing the amount of PCR product in the assay. The standard curve of the assay had a linear detection range from 1Pt molecule per 300 bp mtDNA to 1Pt molecule per 6,000 bp mtDNA ($r^2=0.9264$). (Fig.2B). We confirmed the dose response relationship of adduct formation, measured platinum levels and PCR inhibition using a Quant-IT™ PicoGreen assay (Molecular Probes, Eugene, OR) with similar results (data not shown). DRG neurons were exposed to cisplatin for 48 and 144 hours in the presence of 100ng/ml NGF and zVAD-FMK, and then total DNA was isolated and run in the PCR assay (Fig 2C). Ethidium bromide bands of PCR-amplified mtDNA were measured and expressed as a percent of control (Fig 2D). Cisplatin exposure decreased the amount of a 13.4 kb mtDNA PCR product to 50.6% of control at 48 hours and 21% of control at 144 hours, while a smaller 235 bp mtDNA PCR product was 92% of control at 48 hours and 108% of control at 144 hours. Cisplatin binding of the 13.4 kb mtDNA samples was calculated using the standard curve. The number of adducts at 48 hours was 1 platinum molecule per 2,166 bp mtDNA (7.5 platinum molecules per mitochondrial genome), which increased to 1 platinum molecule per 679 bp mtDNA (24 platinum molecules per mitochondrial genome) by 144 hours (Fig 2E).

Cisplatin inhibits mtDNA replication

Mitochondrial DNA replication was inhibited by cisplatin in dissociated DRG neurons as shown by 5-bromo-2'-deoxy-uridine (BrdU) incorporation. DRG neurons are non-dividing cells and BrdU is not incorporated into the nucleus. BrdU is incorporated into replicating mtDNA. To confirm BrdU incorporation was specific for mitochondrial DNA, we used a mitochondria-targeted plasmid expressing DS Red, which showed co-localization with BrdU staining (Fig 3A). DRG neurons were exposed to cisplatin with 100ng/ml NGF and zVAD-FMK for 0, 48, 72, 96, 144 and 168 hours and subsequently treated with BrdU for 24 hours. The amount of BrdU staining decreased after exposure to cisplatin. Both the intensity and

the area of BrdU staining was measured within the cytoplasm of each DRG neuron (Fig 3C). Intensity (total intensity/gray value) of BrdU incorporation was 822.1 in control that decreased to 330.2 at 48 hours, 548 at 72 hours, 545.8 at 96 hours, 181.5 at 144 hours and 134.4 at 168 hours. Area (green area/total area) of BrdU incorporation was 1.42 in control that decreased to 0.54 at 48 hours, 0.95 at 72 hours, 1.21 at 96 hours, 0.55 at 144 hours and 0.47 at 168 hours. Assuming that BrdU staining correlates with mtDNA replication, the mtDNA replication decreased 2.5 fold at 48 hours and 6 fold by 168 hours as determined by BrdU intensity ($p<0.001$). Similarly, mtDNA replication decreased 2.6 fold at 48 hours and 3 fold at 168 hours as determined by BrdU area ($p<0.001$) (Fig 3C).

Cisplatin-mtDNA adducts inhibit mtRNA transcription

Dissociated DRG neurons were treated with or without cisplatin, 100ng/ml NGF and zVAD-FMK for 144 hours followed by isolation of DNA/RNA for real time RT-PCR. The PCR was normalized using both optical density measurements and PCR amplification of a 210bp genomic DNA fragment. Selection of mitochondrial genes for the real time RT-PCR had increasing distances from the origin of transcription, ND1:3138bp–3290bp, COI:5382bp–5559bp, ATPase 6:7982bp–8224bp, ND4:11107bp–11332bp, and Cyt b:14499bp–14644bp (Fig 4A). Real time RT-PCR of cisplatin treated cDNA was run in parallel with and compared to control cDNA. The number of PCR cycles required to cross the PCR detection threshold increased the farther away from the transcription origin the gene was located (Fig 4B). There was a linear relationship between the increase in PCR cycles and the distance the gene was located from the origin of transcription ($r^2=0.7990$). Cycle number slightly decreased 0.27 cycles with ND1; however, this decrease was not significantly different from control. An increase in cycle number was observed with 1.21 cycles for COI ($p<0.05$), 1.48 cycles with ATPase 6 ($p<0.05$), 1.68 cycles for ND4 ($p<0.01$) and 2.21 cycles for Cyt b ($p<0.001$).

Cisplatin induces mitochondrial changes in vitro and in vivo

Dissociated DRG neurons were exposed to cisplatin in the presence of 100ng/ml NGF and zVAD-FMK for 0, 48 and 144 hours and morphology examined by transmission electron microscopy (TEM) (Fig 5). In controls, normal mitochondria are observed in the somata and neurites. After 48 hours cisplatin exposure, degradation of mitochondria was seen in both the somata and neurites. At 144 hours cisplatin treatment, the cells have decreased in size and extensive mitochondrial vacuolization was seen although the cell body and nucleus appear intact. Since this occurred in the presence of zVAD-FMK the observed mitochondrial changes are caspase independent. To further examine this phenomenon *in vivo*, DRG from adult mice that had been injected with cisplatin for 10 days were examined by TEM (Fig 6). DRG neurons from control mice had normal intact mitochondria. Cisplatin treatment induced mitochondrial degradation while the nuclear membrane remained intact.

Discussion

We have explored the mechanisms of cisplatin-induced peripheral neuropathy utilizing a rodent DRG neuronal model system. Previous work focused on cisplatin effects on nDNA and subsequent activation of apoptotic pathways. In this series of experiments, we have examined the effects of cisplatin on mtDNA, which may provide an alternate pathway for DRG cellular dysfunction. We have demonstrated that cisplatin forms adducts with mitochondrial DNA at a similar rate as nuclear DNA. We provide evidence that cisplatin-mtDNA adducts inhibit mtDNA replication, disrupt mtDNA transcription, and cause morphological changes within mitochondria. These findings are important because they provide an alternate pathway for DRG neuronal dysfunction, and a theoretical target for interventions to help prevent cisplatin-induced peripheral neuropathy. Furthermore, the

effects of cisplatin on mtDNA that we have demonstrated could produce gradual energy failure and prolong the neurotoxicity after the removal of cisplatin, which provides a plausible explanation for a unique feature of cisplatin-induced peripheral neuropathy known as “coasting.”

Cisplatin forms significant numbers of adducts with mitochondrial DNA

In previous studies, we measured platinum levels and adduct repair in total genomic DNA (gDNA), *in vitro* and *in vivo*. DRG neurons are non-dividing cells that repair cisplatin-nDNA adducts via the NER at the same rate as cancer cells; however, accumulation of adducts is higher in neurons. Platinum accumulation in total gDNA harvested from cultured DRG neurons was 1 platinum molecule per 3800 bp gDNA when treated for 48 hours with 2 μ g/ml cisplatin. Under the same conditions our PCR assay measured 1 platinum molecule per 2166 bp mtDNA. This indicates the rate of cisplatin binding in gDNA and mtDNA is similar; however, the mtDNA is without the NER DNA repair system. In contrast, *in vivo* studies where adult rats were given injections of 1mg/kg cisplatin over three weeks accumulated 1 platinum molecule per 30,400 bp gDNA in whole DRG (McDonald et al., 2005).

There are 2–10 copies of the mtDNA genome in each mitochondrion and thousands of mitochondria in each neuron. Therefore, the adduct levels measured in the mtDNA PCR assays are averages over millions of individual mitochondrial genomes. We have provided a table in order to better express the percentage of individual mtDNA strands that would contain cisplatin adducts as a function of varying adduct rate formation (Table 1). This calculation is based on the assumption that adducts are formed randomly around the mitochondrial genome. For example, if the measured mtDNA cisplatin adduct content was equal to [1] adduct per DNA target fragment (the highlighted column), 36.8% of strands would have no adducts, 36.8% would have 1 adduct, etc. In other words, in this example an estimated 63.2% of mtDNA strands would contain at least one cisplatin adduct. Using our actual data in this study, we measured 7.5–24 platinum adducts per mitochondrial genome *in vitro*, which would be estimated to affect >99.9% of the mtDNA in DRG neurons. Using the *in vivo* data from McDonald et al, we calculated there would be in the range of 0.5 – 2.0 platinum adducts formed per mtDNA genome with an estimated 39.4 – 86.5% of individual genomes containing at least one cisplatin adduct.

Adducts inhibit mtDNA replication and mtRNA transcription

Mitochondrial DNA is a circular, double stranded piece of DNA that is replicated and transcribed in one direction from a single start site. BrdU incorporation is a standard method for measuring nDNA synthesis *in vitro* and *in vivo*. It has also been used to identify new mtDNA synthesis in mammalian cell lines but not in primary neurons. In the present study, we were able to demonstrate inhibition of mtDNA replication in cisplatin treated DRG neurons that were associated with cell shrinkage and eventually cell death. Real time RT-PCR showed inhibition of mtRNA transcription. This effect of mtRNA transcription inhibition was significantly greater the further away the genes were from the transcription origin (cytB>ND4>ATPase6>CO1). This suggests that the transcription complex disengages when it meets randomly targeted platinum adducts. Interestingly, inhibition of mtRNA transcription was slightly (but not significantly) greater for ND1, which is the gene closest to the transcription origination site. We hypothesize that ND1 is increased both because the gene is less likely to be blocked and that the transcription machinery is constantly trying to re-engage on the mitochondrial genome. This reengagement of the mtDNA may also explain why we had an increase in BrdU incorporation at 72 and 96 hours cisplatin treatment, although this replicated mtDNA likely would not become a complete mtDNA genome.

Furthermore, these incomplete mtDNA fragments would likely be dysfunctional and subject to cellular degradation.

Inhibition of PCR product does not necessarily indicate a decrease in the number of mtDNA copies present in the cells but rather the reduction in available template because of adduct formation. Deficiencies in mtDNA would ultimately lead to mitochondrial dysfunction and is one explanation for the morphological changes we see by TEM. Morphological changes observed in cisplatin-treated cultured DRG neurons and adult mouse DRG, showed mitochondrial degradation in the presence of intact nuclei.

Mitochondrial DNA damage and “coasting” of cisplatin-induced peripheral neuropathy

Mitochondrial DNA damage may play an important role in cisplatin-induced peripheral neuropathy. A unique feature of cisplatin-induced peripheral neuropathy is that the neuropathy often continues to worsen for months after the discontinuation of the drug. This worsening of the neuropathy is termed “coasting,” and cisplatin damage to mtDNA as described in our study theoretically could help explain this troublesome clinical phenomenon. Cisplatin has been shown to inhibit fast axonal transport in both pigment cells and neuron like murine neuroblastoma cells in the absence of apoptosis. Since 95% of mitochondria reside in the axon, mitochondrial dysfunction would be predicted to cause disruption of axonal transport, which is an ATP-dependent process. Furthermore, both alterations of mitochondria (e.g. mitofusin 2) and axonal transport (e.g. KIF1A) are a known cause of inherited axonal peripheral neuropathies. With the high accumulation of cisplatin adducts in neurons compared to other organ types, the equivalent binding of cisplatin to mtDNA and nDNA and the absence of NER in the mitochondria, and the interrelatedness of mitochondria and axonal transport, it is a plausible idea that mtDNA damage and gradual attrition of mitochondria could play a role in a progressive and persistent neurotoxicity such as coasting.

Research Highlights

- Cisplatin binds mitochondrial DNA and the resulting adducts were quantitated by a novel PCR method.
- Cisplatin binding in mtDNA is similar to nuclear DNA binding.
- Adducts inhibit mtDNA replication and transcription.
- Cisplatin induces mitochondrial degradation in vitro and in vivo.

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Figure 1.

DRG culture system used to study cisplatin-mtDNA interactions. To inhibit cisplatin-induced apoptosis, we used 100ng/ml NGF to inhibit Bax translocation and cell cycle changes in combination with the pan-caspase inhibitor zVAD-FMK. Cell survival was determined in cultured DRG neurons treated with or without 2 μ g/ml cisplatin, 10 or 100ng/ml NGF, and with or without 75 μ M zVAD-FMK. Survival in DRG neurons treated with cisplatin, 100ng/ml NGF and zVAD-FMK was 94% at 48h, 88% at 72h, 84% at 96h and 75% at 144h (black arrow, n=3) and was not significantly different from control DRG neuron survival. Addition of 100ng/ml NGF and zVAD-FMK did however, significantly increase cell survival in cisplatin treated DRG neurons ($p < 0.001$).

Figure 2.

Cisplatin binds mtDNA and the resulting adducts can be quantitated. (A) A PCR assay using long PCR amplification of a 13.4 kb fragment of the mitochondrial genome was developed to quantitate the number of platinum-mtDNA adducts. Formation of a cisplatin-DNA adduct on mtDNA blocks PCR amplification reducing the amount of PCR product in the assay. (B) Standard curve using rat liver mtDNA exposed to cisplatin *in vitro* with PCR products and platinum-DNA binding measured in parallel. PCR samples were measured against the linear portion of the curve, 1Pt molecule per 300bp–6000bp ($r^2=0.9264$). (C) Ethidium bromide gel detection of mtDNA PCR products from cultured DRG neurons treated with 2 μ g/ml cisplatin, 100 ng/ml NGF and 75 μ M zVADFMK for 0, 48 and 144 hours. (D) Ethidium bromide gel quantitation of mtDNA PCR product from cisplatin treated DNA was expressed as a percent of mtDNA PCR product from non-treated DNA (time 0) Cisplatin induced a significant decrease in the 13.4kb mtDNA PCR product at both 48 and 144 hours (***- $p<0.001$, $n=3$) and no significant change with the 235bp mtDNA PCR product ($n=3$). (E) Calculation of cisplatin adducts compared to the standard curve.

Figure 3.

BrdU incorporation decreased in cisplatin-treated DRG neurons. (A) Co-localization of the BrdU incorporation into mtDNA (green) and a mitochondrial targeted plasmid, expressing DS-Red (red). (B) Representative images of BrdU incorporation into DRG neurons treated with cisplatin at 0 and 48 hours (100× magnification). BrdU incorporation observed at time 0 was decreased substantially by 48 hours cisplatin treatment. No BrdU incorporation is observed in the nucleus. (C) DRG neurons were exposed to 2μg/ml cisplatin for 0, 48, 72, 96, 144 and 168 hours in the presence of 100ng/ml NGF and 75μM zVAD-FMK. Both the intensity and area of BrdU fluorescence within the cytoplasm was measured. Cisplatin induced a significant decrease in BrdU incorporation at 48, 144 and 168 hours (***-p<0.001, n=38–77) and a less significant at 72 and 96 hours (*-p<0.05, ns, n=59–64). There appeared to be a recovery of BrdU incorporation at 72 and 96 hours, however, BrdU incorporation did not return to control levels.

Figure 4.

Cisplatin inhibited transcription of mitochondrial genes. (A) Primers were designed to amplify genes transcribed at various locations along the entire mitochondrial genome. (B) DRG neurons were exposed to 2 μ g/ml cisplatin, 100ng/ml NGF and 75 μ M zVAD-FMK for 144 hours, harvested, RNA isolated and real time RT-PCR performed. The number of cycles needed to cross the PCR threshold was compared to control cDNA. Changes in PCR cycles increased the farther away the gene was located from the transcription origin. The relationship of the cycle number to the distance from the transcription origin was linear ($r^2=0.7990$). Cycle number had a slight decrease of 0.27 cycles for ND1 (ns, n=5) and an increase of 1.21 cycles for CO1 (*-p<0.05, n=5), 1.48 cycles for ATPase 6 (**-p<0.01, n=5), 1.68 cycles for ND4 (**-p<0.01, n=5) and 2.21 cycles for Cyt b (***-p<0.001, n=5).

Figure 5.

Cisplatin induced mitochondrial degradation and vacuolization in cultured DRG neurons. TEM of intact (a, arrows) normal mitochondria and (b, *) nuclear membrane in control DRG neurons. DRG neurons treated with 2 $\mu\text{g/ml}$ cisplatin 100ng/ml NGF and 75 μM zVAD-FMK showed degradation of mitochondria (c) at 48 hours that increased (e) at 144 hours. The (d, f) nuclear membrane, however, remained intact.

Adult Mouse DRG

Figure 6.

Adult mice were treated with vehicle (saline) or cisplatin (23mg/kg, cumulative). DRG were harvested and examined by TEM. Control DRG treated with saline showed normal mitochondria (arrows) and intact nuclear membrane (*). Cisplatin treated DRG showed mitochondrial degradation (arrows) in the presence of intact nuclear membrane (*).

Table 1

Distribution of mtDNA genomes with a given (n) number of adducts when the average number measured is [0.5, 1, 2, 5 or 20] adducts per DNA molecule. Percent mtDNA with “n” adducts was based upon a binomial distribution and a pre-specified probability of success/failure.

Measured Platinum atoms/mtDNA					
[0.5]	[1]	[2]	[5]	[20]	
Distribution (%) of mitochondrial genomes with “n” adducts					“n” = 0 – 6
60.6	36.8	13.5	0.1	<0.1	0
30.3	36.8	27.1	3.3	0.3	1
7.6	18.4	27.1	8.4	0.9	2
1.3	6.1	18.2	14	1.8	3
0.2	1.5	9	17.6	4.4	4
<0.1	0.3	3.6	17.6	8.3	5
	<0.1	etc	etc	etc	6