Inhibition of mitochondrial protein synthesis with doxycycline induces biosynthesis of OXPHOS Complex V

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By Ulliana Savitskaya

Background

A mitochondrion is an organelle present in nearly all eukaryotic organisms, with every human cell containing from 1000 to 2500 mitochondria (Gahl, 2019; Pizzorno, 2014). Scientists believe that mitochondria have evolved from a prokaryotic cell, also known as a bacterium, due to its similar internal structure and the possession of its own mitochondrial DNA, granting it an ability to synthesise its own proteins (Cavalier-Smith, 2006). Its overall structure includes a double-membrane system, consisting of an inner and outer mitochondrial membrane, separated by inter-membrane space (Figure 1). The matrix is enclosed by the inner mitochondrial membrane, housing ribosomes, various enzymes and most importantly, mitochondrial DNA (mtDNA). MtDNA is a double-stranded, circular molecule containing genes responsible for the protein synthesis of subunits of enzyme complexes that are part of the oxidative phosphorylation (OXPHOS) system. OXPHOS occurs in the inner mitochondrial membrane and is one of the steps of cellular respiration, the main goal of which is to produce adenosine triphosphate (ATP). ATP is an energy carrier molecule that travels all around the body providing energy for various processes such as muscle contraction and nervous impulses (Deshpande & Mohiuddin, 2020).

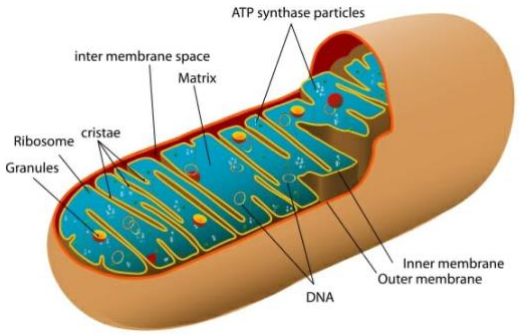


Figure 1: inner structure of a mitochondrion (Wikipedia, 2022)

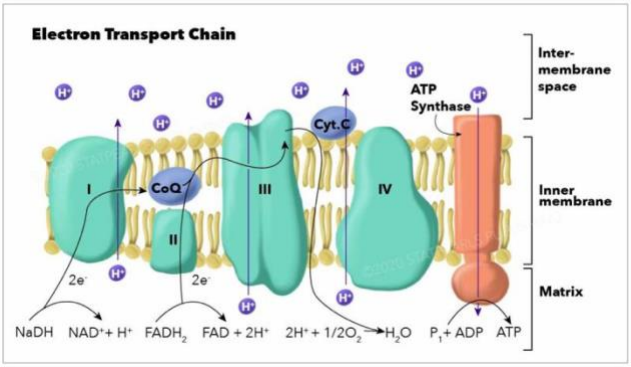


Figure 2: Oxidative Phosphorylation (OXPHOS) system (Ahmad et al., 2021)

The OXPHOS system consists of five complexes (I-V), four that make up the electron transport chain (ETC). As electrons donated by NADH and succinate travel through the ETC, electron carriers in the complexes (iron-sulfur clusters and hemes) are reduced and oxidised, releasing energy that is used to pump protons across the inner mitochondrial membrane from the matrix into the inter-membrane space (Figure 2). With the accumulation of protons in the inter-membrane space, there is an electrochemical gradient across the membrane, driving ATP synthase (complex V) to produce ATP and pump protons back into the matrix. All five of these complexes are imbedded into the mitochondrial inner membrane and are composed of subunits coded for either by nuclear DNA (nDNA; complex II), or nDNA as well as mtDNA (Complex I, III, IV and V) (Scarpulla, 2013).

Mitochondrial diseases are a group of genetic disorders that affect the mitochondria usually due to mutations in nDNA or mtDNA (Philadelphia, 2014). They are very hard to diagnose and treat due to the wide range of symptoms associated with them, depending on the proportion and location of mitochondria affected. There is no single test for a mitochondrial disorder and diagnoses are usually made in specialised mitochondrial labs after lengthy investigations of the patient's genome (Khan et al., 2015). Treatment usually includes symptom management and various supplements, without the ability to eliminate the root cause of the disorder. Therefore, with an estimated 1 in 5000 people suffering from

a mitochondrial disorder, an increasing number of researchers are looking into the nature of mitochondrial mutations and potential treatments for the disruptions in cellular respiration (Cleveland Clinic, 2018).

One such branch of research relates to the effects of antibiotics on mitochondria. Due to the prokaryotic origin of mitochondria, some antibiotics act the same way on mitochondria as they do on bacteria, disrupting them in a variety of different ways (Boguszewska et al., 2020). Doxycycline is an antibiotic from the group of tetracyclines, which inhibit protein synthesis, deeming the bacterium incapable of growth and multiplication (Patel & Parmar, 2021). By extension, we expected doxycycline to have the same effect on mitochondrial protein synthesis, however Dr Taanman and his team found that doxycycline actually induced biosynthesis of 2 mtDNA-encoded subunits of the OXPHOS system, MTATP8 and MTATP6, while inhibiting the synthesis of the other 11 mtDNA-encoded subunits (Figure 3). However, this only occurred after long-term treatment with doxycycline, as short treatment resulted in inhibition of all subunits (unpublished data, host lab). I spent the 6 weeks of my research project conducting experiments which looked into how doxycycline affects the expression of various mtDNA-encoded subunits in A549 cancer cells in order to better establish the correlation between the treatment and expression of these proteins.

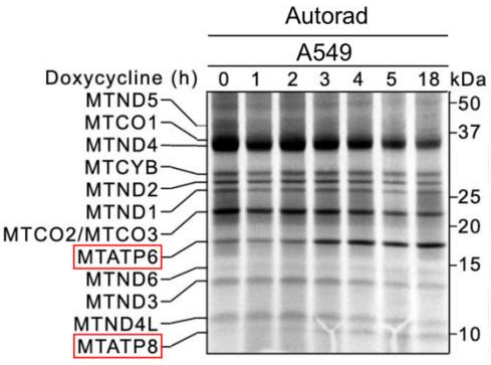


Figure 3: A549 cultures treated with 10 µg/ml of doxycycline for 0-18h (unpublished data, host lab)

Methodology

The first week of my research project consisted of growing A549 cells in petri dishes (Figure 4) and treating them with doxycycline for a different number of days from 0 to 7 days. A549 cells are lung carcinoma epithelial cells meaning, due to their cancerous origin, they grow and multiply faster than regular cells, which is the reason we used this cell line (Steubing, 2023). Each day consisted of checking the petri dishes for any signs of contamination with bacteria under the microscope and changing the medium that the cells grew in making sure they had enough glucose to carry out respiration and to prevent the accumulation of waste products such as lactate. We also included A549 rho0 cells which do not contain any mtDNA as a control so no protein synthesis can occur inside the mitochondria.

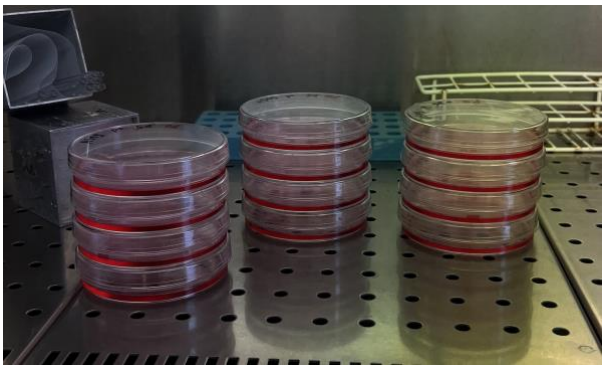


Figure 4: A549 cell cultures in petri dishes

Once the cells have reached confluency, which is the stage at which the cells have covered the maximum amount of petri dish surface available, I harvested them. This included washing the petri dishes with phosphate buffered saline (PBS), adding trypsin to detach the cells from the dish and collecting the solution into the tube. After multiple rounds of centrifugation at increasing speeds, I collected the final pellet which contained all of my cells. After this step all of my samples, proteins and cells were stored at -80ºC when not in use.

After lysing the cells with the detergents n-dodecyl-β-D-maltoside and adding protease inhibitors to prevent damage to the mitochondrial proteins, I centrifuged the samples to separate the proteins from the cell debris. This was followed by a BCA protein assay to analyse the protein concentration in each sample to ensure equal loading of proteins in the future experiments (Figure 5).

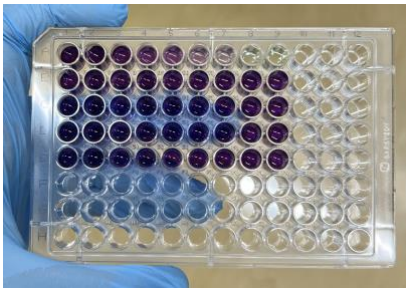


Figure 5: loaded BCA protein assay

Over the next 4 weeks I carried out various experiments using Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), blue native (BN-PAGE) and clear native (CN-PAGE) gels to analyse the protein and complex expression in my samples. I used SDS gels to analyse individual proteins synthesised by the mitochondria, and native gels to analyse whole OXPHOS complexes.

Even though the process was slightly different for the three different types of gels, the overall technique involved:

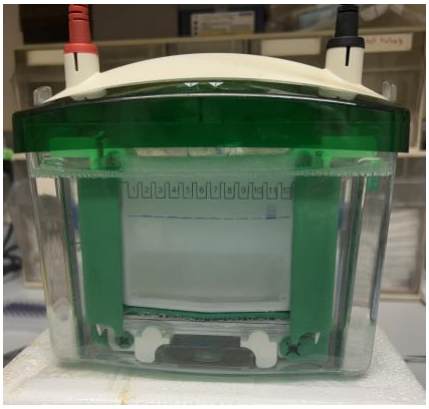


Figure 6: Running SDS-PAGE

1.Loading the negatively charged protein sample into wells in the gel, corrected to the concentration of protein obtained from the BCA protein assay;
2.Running the gels by using a cathode and an anode to provide a potential difference (Figure 6), resulting in movement of negatively charged proteins towards the other (anode) end of the gel, separating them based on charge;
3.Blotting the pattern from the gel onto the filter paper and saturating all protein binding sites on the paper with milk;
4.Probing the blots by adding primary antibodies specific to the protein or complex we wanted to investigate, followed by secondary antibodies;
5.Developing the blot using the BioRad Imager.

For CN-PAGE and BN-PAGE we poured our own gels due to them being more reliable and cheaper. Often, we also reprobed the gel blots with another primary antibody if the loading for that particular gel was to a high standard and there was little background on the blot. High background refers to a high signal/noise ratio which is often a problem. The reasons often include poor sensitivity of primary antibodies, small amount of protein in the sample or poor washing technique with a lot of secondary antibodies left over on the paper after probing.

I also used native gels to check in-gel activity of various enzymes present in oxidative phosphorylation which is useful in figuring out not just what concentration of this enzyme is present in the sample but also how effective it is in real time. I finished my 6 week placement off with a spectrophotometric assay to measure how doxycycline affects the activity of Complex V ATPase.

Results and discussion

After making up the samples using equal concentrations of proteins in each sample calculated from the BCA assay results, I started off with testing these calculations by probing an SDS gel with antibodies for SDHA and beta-tubulin. SDHA corresponds to the number of mitochondrial proteins encoded by mtDNA, whereas beta-tubulin shows the number of all proteins in those cells. The results showed that my sample of cells that have been treated with doxycycline for 1 day has a lower concentration of mitochondrial proteins compared to the other samples. This is visible in Figure 7, which is a compilation containing all of the SDS western blots I developed over my 6-week project. The third band from the left, corresponding to 1 day of treatment in the SDHA blot is weaker than the other 6. This will manifest in all of the other blots; however, it can be corrected by comparing the ratio of the intensities of the protein band and the SDHA band.

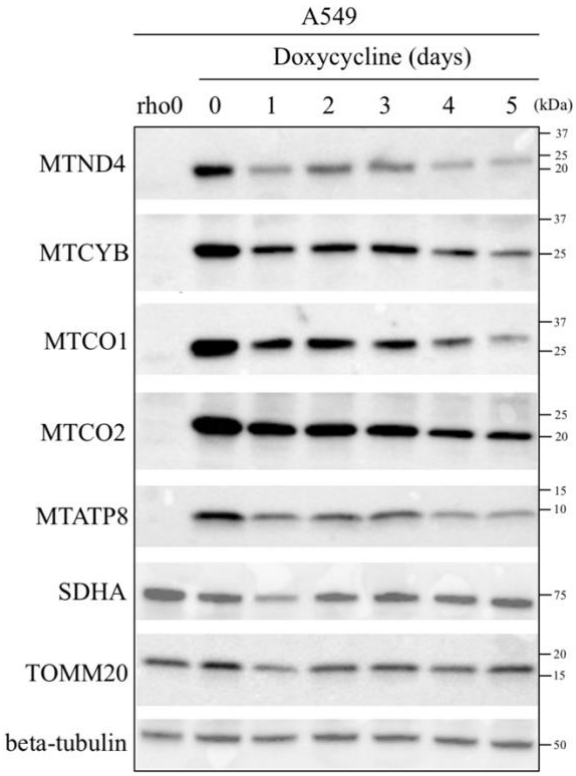
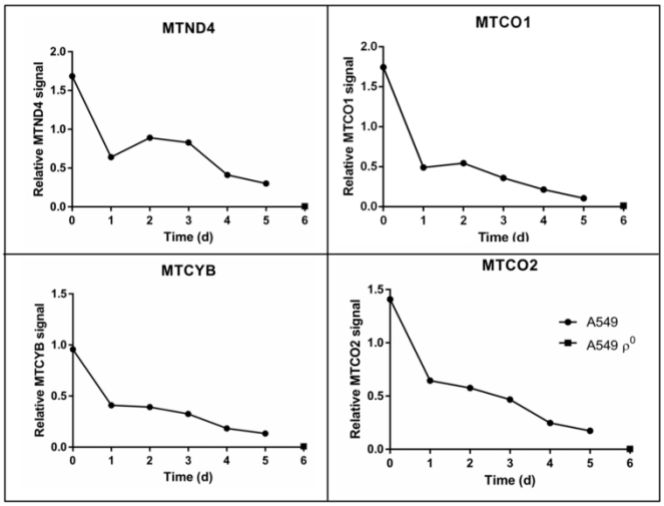


Figure 7: Western blots of SDS-PAGE

Following the test, I ran SDS gels over the course of 2 weeks, probing them with all proteins that Dr Taanman investigated in his metabolic labelling work, except for MTATP6 as it usually manifests in western blots with a lot of background that are difficult to analyse. My results mostly follow the pattern that Dr Taanman and his team observed before with MTND4, MTCYB, MTCO1 and MTCO2 all steadily going down with longer doxycycline treatment (Figure 7). Particularly in the western blot of MTND4, the low concentration of mitochondrial proteins in the 1 day sample is very obvious, with the band being a lot less intense than the bands for days 2 and 3. The quantification graphs with corrected protein intensity values for MTND4, MTCYB, MTCO1 and MTCO2 all show a steady decline in protein concentrations with prolonged doxycycline treatment (Figure 8). In MTND4 there is a drop at 1 day of doxycycline treatment, but I believe that can still be attributed to the SDHA result for that sample.

Figure 8: Relative protein signal and days of doxycycline treatment



MTND4 is a protein that is found in Complex I, MTCYB in Complex III, MTCO1 and MTCO2 in Complex IV of the oxidative phosphorylation system (Signes & Fernandez-Vizarra, 2018). This suggests that doxycycline works as expected on these complexes by inhibiting the synthesis of these proteins, hence preventing the electron transport chain from functioning.

The western blot for MTATP8, even though not as evident, shows the concentration of the protein not going down as much as the other proteins during days 2 and 3 of treatment. As expected from Dr Taanman's results, MTATP8 concentration decreases at first, then increases after a few

days of doxycycline treatment and then decreases again in days 5 and 6. MTATP8 and MTATP6 are both proteins found in Complex V, suggesting that protein synthesis only increases in Complex V. Dr Taanman and his team suggest that this may perhaps be a compensatory mechanism where ATP Synthase reverses its function becoming ATPase in an attempt to maintain the proton gradient across the inner membrane. However, it is just a theory that needs to be understood better before becoming the accepted mechanism of action.

SDS-PAG denature OXPHOS Complexes allowing us to look at singular proteins, whereas Native Gels show complexes as a whole. After carrying out Blue Native and Clear Native gels, I found that my results are also close to what we were expecting (Figure 9). The concentrations of Complexes I, III and IV decreased significantly upon doxycycline treatment, which is in line with the results from SDS western blots discussed above. All of the proteins making up Complex II are encoded by nuclear DNA so doxycycline does not affect its concentration as it inhibits only mitochondrial protein synthesis. Lower intensity of the band at day 1 reflects the low mitochondrial protein concentrations discussed above, as expected. I believe that the western blot of Complex V shown in Figure 9 reflects the most important finding of this project, that has already been discussed above – inhibition of mitochondrial protein synthesis by doxycycline induces the biosynthesis of complex V. The intensity of the bands doesn't change significantly, except for day 1 for the reason already discussed, which can be seen in Figure 10. ATP5A is one of the proteins present in Complex V, primary antibody for which we added during probing the western blot.

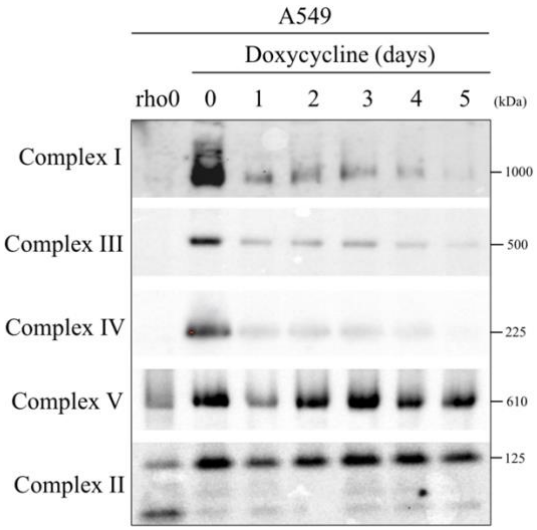


Figure 9: Western blots of BN-PAGE and CN-PAGE

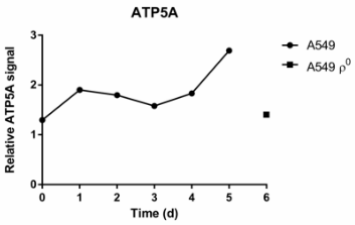


Figure 10: Relative Complex V signal and days of doxycycline treatment

In addition, I measured in-gel activity of Complexes II and V which, according to the theory discussed above, are both supposed to stay relatively constant. This can be seen in Figure 11. Obtaining similar results using various different methods (denatured isolated proteins, whole complexes and in-gel activity) adds reliability to my findings and decreases the probability of Complex V concentration increasing after doxycycline due to chance.

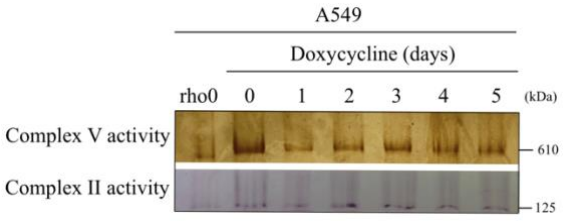


Figure 11: In-gel enzyme activity

Finally, I have measured the activity of Complex V ATPase using a spectrophotometric assay, however after analysing the data it didn't have any trends or associations. The method included measuring the activity after adding ATP into the sample followed by adding oligomycin and measuring the activity again. Oligomycin inhibits ATPase so by finding the difference between the two activities, we would isolate the activity of Complex V only. However, this involved calculating the gradient of the line on the graph (Figure 12) before and after adding oligomycin, which proved difficult due to a large range of values. Dr Taanman believes this might be due to the low concentration of proteins in the sample, making the fluctuations in the values too high to yield any useful calculations out of the results. We concluded that the method needs to be refined in order to achieve useful outputs from the assay.

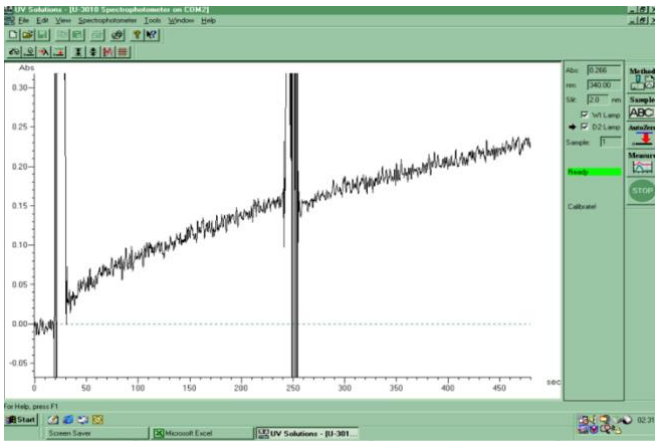


Figure 12: A graph to show the signal detected before and after adding oligomycin in the shape of a spike at 250 seconds

The main finding of this project is that the inhibition of mitochondrial protein synthesis by doxycycline appears to induce biosynthesis of the oxidative phosphorylation Complex V. This is a very exciting finding, as with more in-depth research it may potentially lead to understanding the pathological mechanism of mitochondrial diseases better – an advancement in a field that urgently requires one.

Reflections

Throughout my first year at university, I have grown to understand that excitement and anxiety are often two sides of the same coin, so I went into the lab on my first day with the appreciation and acceptance for my worries without realising just how much I am about to learn.

During a reception evening earlier in the year when I got the opportunity to meet my supervisor, Dr Taanman, he mentioned the tough competition for his project – 28 people for just one place. In addition to my lack of any previous experience in a laboratory, I felt the most severe impostor syndrome I've felt thus far. For the first 2 weeks coming into the Royal Free Hospital, the main goal I had subconsciously set for myself was not to disappoint, not to make mistakes, and to go above and beyond. As beneficial as it is to aim high and set high goals for oneself, I was putting immense unnecessary pressure to excel in an unknown to me environment, too scared to ask for help or admit that I didn't understand something. My fear of disappointing everyone who had a role in selecting me was so overbearing that a few weeks into the project my hands started shaking whenever I would carry out an important and meticulous task, which lead to me accidentally aspirating half of the cells in one of the samples. As terrifying as it was in that moment, I knew that the right thing for me to do would be to tell Dr Taanman that I made the mistake in hopes that we can fix it before it's too late. To my surprise, he simply smiled and said: "Let's go have a look then". This was the first moment during the 6 weeks that made me realise – perhaps it is okay to make mistakes.

This was also the time I understood what it means for 'science to be unpredictable'. My cells grew at a slower rate than expected and had different protein concentrations when probed with SDHA and beta-tubulin, so I had to use other samples to repeat the BCA protein assay with sample calculations. Some of the western blots and a Complex I in-gel enzyme activity didn't work with no obvious explanation. As frustrating it was in the moment, it was equally as important for me to realise that more

often than not experiments don't work as expected, which is not a reflection of my abilities but instead an opportunity to grow and analyse my method.

A few weeks into project, I was speaking with one of the PhD students working on his own experiments in the lab who reassured me that no one in the lab was expecting me to have all of the skills and knowledge required to be a research assistant, but instead to be enthusiastic about the project and willing to learn from my mistakes. Understanding this brought me a lot of peace for the last 2 weeks of my project during which I throughly enjoyed the experiments I was carrying out without checking every measurement time and time again and taking on a more proactive role in them.

In addition to feeling a lot more competent and at ease in the lab, I felt a lot more confident to be authentic to myself. Having conversations on topics not related to science without the anxiety of appearing lazy, unfocused and uncaring made the last few weeks so much more enjoyable and allowed me to foster friendships that I have kept beyond this project. Coming into a well-established team is always tough but as long as I stayed authentic to myself and influenced the energy in the room instead of adapting to the existent one, the team dynamic shifted to allow for me to swiftly join in. This is one of the most important lessons that I have learnt during these 6 weeks which I will forever cherish and take further into my academic and social endeavours in the future.

Overall, I am so grateful for the invaluable experience to not just learn more about mitochondria and wet lab research but also about myself, my capabilities and the people around me.

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