Cadmium mediated changes in mitochondrial metabolic function of a renal epithelial cell line (A6), evaluated with the MTT-assay

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Master project in Molecular Biology
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Roskilde University (RUC)
(2008)

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This is the Master Thesis by Claes Schütt at the department for Science, Systems and Models (NSM), at Roskilde University (RUC).
I wish to thank my supervisor Henning F. Bjerregaard for help and assistance and insightful observations.
I would also like to say thank you to dr. Pierre Bouchelouche for his help and comments.

Roskilde 2008
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Abstract

Cadmium is an industrial and environmental pollutant implicated in several pathologies, including cardiac, renal and hepatic toxicity. Cadmium accumulates in the liver and kidneys, with resulting lesions leading to organ dysfunction. The *Xenopus laevis* A6 cell line is used as a cell-model of human kidney distal tubuli. The distal tubuli are often a secondary target of in vivo cadmium toxicity, it may however be an important one due to its role in regulating calcium homeostasis.

Measuring the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT), is a commonly used method in determining cell proliferation and in evaluating toxicity of a wide variety of compounds. This project describes a novel way of using the classic MTT viability assay, to estimate acute changes in mitochondrial activity.

The project examined and found discrepancies between cell viability, when this was determined by the Trypan Blue Exclusion assay or the MTT assay, after a three hour cadmium (0-200µM) exposure period. The MTT assay underestimated cell viability, possibly due to cadmium acting as an inhibitor of the enzymes responsible for MTT reduction.

In order to evaluate the MTT assays dependence on mitochondrial enzymes, a series of experiments examined the effects of mitochondrial inhibitors on reduction of the MTT substrate. The following agents were examined: Electron transport chain inhibitors rotenone (complex I inhibitor), antimycin a (complex III inhibitor), sodium azide (complex IV inhibitor), oligomycin (ATP-synthase) and CCCP (an uncoupler of Oxidative Phosphorylation). Rotenone, sodium azide and oligomycin decreased MTT reduction relative to the controls. Antimycin a and in particular CCCP increased MTT reduction. The experiments confirmed that the MTT assay measures the combined activity of reductases associated with the mitochondrial electron transport chain.

The activity of mitochondrial enzymes is under regulation by calcium. The effect on MTT-reduction from altering mitochondrial calcium levels was examined using calcium transport inhibitors (Thapsigargin and Ruthenium Red) or calcium ionophores (Ionomycin). Decreasing calcium levels decreased activity, while increasing calcium increased activity relative to the controls.

The MTT assay was then used to asses the effects of cadmium in combination with the previously examined compounds, in order to investigate cadmiums effect on MTT reducing enzymes. The experiments indicate that cadmium may inhibit the mitochondrial ATP-synthase, in addition to reductases associated with the electron transport chain.

Cadmium is known to sometimes induce cell-death through apoptosis pathways, by initiating the mitochondrial permeability transition event. It was therefore examined if Cyclosporin A, an inhibitor of the Mitochondrial Permeability Transition Pore, could protect against the cadmium mediated disruption of MTT reduction. Cadmium may also initiate cell-death pathways by activating members of the protease family, Calpains. Calpain Inhibitors I+II were used to estimate if inhibition of Calpains could ameliorate cadmium toxicity.

The effect of cadmium and the various other inhibitors used in this project, on the morphology and membrane integrity of A6 cells, was examined using visual identification using light microscope imaging. This in conjunction with the Trypan Blue exclusion assay used to describe cadmium induced cell-death, established that the decrease in MTT reduction seen with the various inhibitors were not principally due to cell death. It also showed how the electron transport chain uncoupler CCCP could protect against cadmium induced cellular damage.
Abstract (dansk)

Cadmium er et industri- og miljø forurenende stof indvolveret i flere typer af pathologier inklusiv toksiske skader i hjerte, lever og nyrer. Cadmium akkumuleres i lever og nyre, hvilket kan føre til organskade og dysfunktion.


En ofte anvendt colorimetrisk metode til at måle celle-død og viabilitet er ved at følge den enzymatiske reduktion af 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT). Denne metode anvendt i MTT-assay anvendes til at bestemme toksicitet af mange forskellige stoffer.

Dette projekt beskriver en måde at bruge det klassiske MTT assay på til at måle ændringer i aktiviteten af mitokondrielle reductase enzymer.

Cadmium toksicitet bestemt ved et Trypan Blå assay og MTT assayet viste stor diskrepans, efter en 3 timers eksponeringstid for cadmium (0-200µM). MTT assayet underestimerede celle viabilitet i forhold til denne bestemt ved trypan blå assay. Denne forskel skyldes at cadmium fungerer som en inhibitor af enzymerne ansvarlig for at reducere MTT.

For at bestemme MTT assayets mulige forbindelse til mitokondrielle enzymer, blev effekten på reduktion af MTT undersøgt, ved inkubering med inhibitorer af den mitokondrielle elektron transportkæde (ETC). Følgende stoffer blev benyttet i eksperimenterne: Inhibitorer af ETC, rotenone (kompleks I), antimycin a (kompleks III), natrium azid (kompleks IV), oligomycin (ATP synthase) og CCCP (afkobler af oxidativ phosphorylering). Rothenone, azid og oligomycin hæmmede aktiviteten af MTT reducerende enzymer. Antimycin a og i særdeleshed CCCP øgede aktiviteten af MTT reducerende enzymer, i forhold til kontrol prøverne.

Eksperimenterne bekræftede derved at MTT assayet måle r den samlede aktivitet af en gruppe reductase enzymer, hvoraf nogle er associeret med ETC.


MTT assayet blev derefter benyttet til at undersøge cadmiums effekt i kombination med de forskellige mitokondrielle inhibitorer for at belyse cadmiums effekt på MTT reducerende enzymsystemer. Eksperimenterne indikerer at cadmium formentlig hæmmer den mitokondrielle ATPsynthase i kombination med en eller flere reductaser på ETC.

Cadmiums celle-toksiske effekt har ofte været sat i forbindelse med induktion af apoptose ved aktivering af den mitokondrielle permeabilitets transitions pore (MPTP). Det blev derfor undersøgt om hæmning af cyclosporin a hæmmende af MPTP kunne beskytte mod cadmiums cytotoksiske effekter, målt som en hæmning af ETC aktivitet vha. MTT assayet. Cadmium kan også aktivere apoptose mekanismer via calpain systemet, der også vil have en effekt på mitokondri aktiviteten. Calpain Inhibitor I og II blev benyttet til at undersøge om hæmning af calpain aktivitet kunne beskytte mod cadmiums effekt på mitokondri enzymsystemer.

Cadmiums og de forskellige ETC inhibitorers samt de andre benyttede stoffers toksiske effekt på A6 cellerne blev undersøgt ved visuel identifikation af morphologiske ændringer i cellemembran struktur og integritet. Denne metode blev benyttet til at fastslå at de målte ændringer i aktiviteten af MTT reducerende enzymer ikke primært skyldes celle-død. Disse forsøg bekræftede desuden at ETC afkobleren CCCP beskyttede mod cadmiums toksiske effekter.
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Abreviations
ΔΨm Mitochondrial membrane potential
AIF Apoptosis Inducing Factor
ANT Adenine Nucleotide Translocator
Apaf-1 Apoptotic Protease Activating Factor-1
ATP Adenosine Triphosphate
Bax Proapoptotic Bcl-2
Bcl-2 B-cells Lymphoma gene 2
BHT Butylhydroxytoluene
Bid Proapoptotic Bcl-2
CAD Caspase Activated DNAse
CARD Caspase Activating Recruitment Domain
Caspase Cysteinyl Aspartate-Specific Protease
Cd Cadmium
CFTR Cystic Fibrose Transmembrane Conductance Regulator
CsA Cyclosporin a
CsR Calcium Sensing Receptor
Cyp-D Cyclophilin-D
Cyt-b Cytochrome b
Cyt-c Cytochrome c
DD Death Domain
DED Death Effector Domain
DISC Death Inducing Signal Complex
DNA Deoxyribonucleic Acid
ER Endoplasmic Reticulum
FADD Fas Receptor Associated Death Domain
Fas FS7-associated Surface Antigen (Apo-1)
FasL Fas Ligand
GSH Gluthathione
ICE Interleukin-1β Converting Enzyme
IMM Inner Mitochondrial Membrane
IMS Inner Mitochondrial Space
IP1 Inositol 1,3,4 Triphosphate
IP3-R Inositol 1,3,4 Triphosphate Receptor
MPT Mitochondrial Permeability Transition
MPT-P Mitochondrial Permeability Transition Pore
NEM N-ethylmaleimide
NO Nitric Oxide
OMM Outre Mitochondrial Membrane
OxPhos Oxidative Phosphorylation
p53 Human Tumor Supressor
PARP Poly(ADP-ribose) Polymerase
PCD Programmed Cell Death
ROS Reactive Oxygen Species
RR Ruthenium Red
RyR Ryanodine Receptor
SERCA Sarco-Endoplasmic Reticulum Calcium ATPase
Smac/ DIABLO Second Mitochondria-derived Activator of Caspases
SOC Store Operated Calcium channel
SR SarcoPlasmic Reticulum
tBid Truncated Bid
TER Trans Epithelial Resistance
TNF Tumour necrosis Factor α
TG Thapsigargin
TRAIL TNF-Related Apoptosis-Inducing Ligand
VDAC Voltage Dependent Anion Channel
1. Introduction

This project had two main objectives. One of the goals was to describe a novel way of utilizing the MTT viability assay originally described by Mossman, to estimate changes in mitochondrial function[116]. Secondly, this modified assay will then be used to describe aspects of cadmium induced injury of a cell line modelling human kidney distal tubuli. The following chapters will give an overview of the theoretical background for the experiments and the knowledge necessary for evaluating the MTT assays effectiveness in assessing cadmium induced injuries in A6 cells, a model of human kidney distal tubuli.

Cadmium mediated cellular injuries, due to the multi-target nature of the toxic heavy-metal, necessitates the introduction of a broad spectrum of subjects, some of which will be described in more details than others. The following chapters should be seen as introductions, presenting enough knowledge allowing for an understanding and evaluation of the data obtained from experiments with the modified MTT assay.

The first chapters will present an introduction and description of the A6 cell line from *Xenopus laevis*, used as a model for human nephronic distal tubuli. Cadmium exposure may induce cell death through various pathways including programmed cell death (apoptosis) or necrosis. The cell death pathways, both in terms of regulation and “execution”, is very complex, requiring the correct timing of and function of a large cellular machinery. Calcium is a ubiquitous cellular messenger, regulating numerous processes including apoptosis induction, to the function and activity of mitochondria. Eventually leading to an introduction to the mitochondria, calcium homeostasis and cellular signaling will be described in some detail. Calcium channels and receptors are sometimes implicated in cadmium toxicity and will therefore be described in greater detail.

The bio-reduction of MTT to its formazan product, the basis of the MTT assay, has often been associated with dehydrogenase/reductase activity of the mitochondria. Mitochondria are thus integral to the two major topics of the project, cadmium toxicity and the MTT assay, and accordingly will receive some attention. The inhibitors and toxicants used to evaluate the modified assay will be described. Compounds assayed ranged from inhibitors of the electron transport chain, an inhibitor of the mitochondrial ATP-synthase complex, to calcium ionophores and calcium channel blockers. These will be briefly described before a longer introduction to cadmium toxicity.

Lastly an overview of the classic MTT assay will be presented. While the MTT assay remains ubiquitous in its use, several important facts about its actual mechanism, the bio-reduction of MTT to MTT-formazan, remains unresolved. Reduction of MTT was originally used to estimate cell death, since it was thought to be associated with the activity of mitochondria of living cells only. Later evidence suggests that it may sometimes give an overestimation of cell death, as changes in mitochondrial function is not always correlated with cell death, if this is determined as disruption of plasma membrane integrity. Thus the MTT assay is now primarily and often used as an assay for cell viability.

This project will present evidence for its possible use in evaluating changes in mitochondrial activity, and potentially the activity of electron transport chain components. The original assays are optimized for use as viability assays, with longer exposure periods (24-48 hours) and maximum
output of tests. This project will use a modified assay for use with free cells in suspension, rather than cells settled in 96 well microtiter plates. The modified assay will be changed for use in assaying acute or shorter exposure periods of cadmium and other test reagents, of 0-3 hours prior to the 3 hour incubation period with the MTT reagent.

Exposure to toxic compounds, such as cadmium and the electron transfer chain inhibitors used in this project, will affect the cells morphology and cause some cell-death in addition to affecting the function of mitochondria. The project will use light-microscopy imaging as a visual identification of changes in A6 cell morphology after exposure to the chemicals. This technique enables one to monitor the progressive effects from toxic exposure, and can be a valuable tool as an addition to the MTT assays endpoint-only measurements.
2. A6 Cells

The A6 cell line is derived from the kidney of the South African clawed toad *Xenopus laevis*. Ion transport in these cells is similar to that found in human nephronic distal tubuli, and it has been proposed as a suitable model for studies of ion transport in these tissues [23;50;51;142]. When grown on a supporting substrate A6 forms a confluent polarized epithelium, with the apical side oriented towards the growth medium and the basolateral side towards the supporting surface. A6 cells at confluence show a high electrical resistance (5000 Ω cm²) and a large mean transepithelial potential difference, established by strong bonding and connection of cells by tight junctions, and asymmetrical distribution of transport proteins in the apical and basolateral membranes[136].

Sodium is transported actively from the apical to the basal side entering passively through a channel in the apical membrane and released primarily through the Na⁺/K⁺-ATPase inserted in the basolateral membrane. K⁺ entering through the Na⁺/K⁺-ATPase exits the cells passively and is recycled through a potassium channel in the basal membrane [136].

Active electrogenic chloride transport is achieved by the Na⁺/2Cl⁻/K⁺-symporter and driven by the sodium gradient established by the Na⁺/K⁺-ATPase. Inserted in the basolateral membrane it transports Cl⁻ ions into the epithelial cells from the basal medium. Cl⁻ ions are secreted passively to the apical space through Ca²⁺ or cAMP controlled channels [5;194].

When grown on a non-permeable surface, sodium absorption, chloride secretion and vectorial water flow to the basal side induces “dome”-formation or haemocyst-like growths, upwelling of the epithelial monolayer by the pressure of water on the basolateral membrane.

Calcium homeostasis is tightly regulated in all tissues. In this cell line calcium is secreted to the basal side through a Ca²⁺/Na⁺ exchanger driven by the established Na⁺ gradient. Intracellular calcium is thus kept at a low level compared to extracellular concentrations [136].

![Fig.2.1](image)

Fig.2.1 A6 cells grown in culture flasks shown at three different stages of cell density: a) single cells as after a recent subcultivation, b) at confluent growth, tight-junctions on the apical side binds the epithelial cell layer together, preventing movement of ions or water between the cells c) fully differentiated cells in the confluent monolayer transport salts and associated water across the plasma-membrane, from the apical (towards the medium) to the basolateral side (towards the growth surface). The net transport of water builds a pressure that causes the epithelium to release from the growth surface in dome-like formations [136].

Cadmium (Cd²⁺), a heavy metal toxicant, may affect a number of Ca²⁺ sensitive functions in A6 cells, presumably due to the closeness of crystal ionic radii of the two divalent cations. Cd²⁺ ions
are presumed to bind to and activate a cation-sensitive receptor (CSR) on the plasma membrane [51]. The receptor belongs to the superfamily of G-protein-coupled receptors. Sensitive to extracellular levels of calcium, as well as other cations (eg. Mg\(^{2+}\), Cd\(^{2+}\), Gd\(^{3+}\)), the CSR’s normal function is thought to be involved in the regulation of cellular calcium levels through emptying of intracellular calcium stores. The CSR is integral to the maintenance of normal mineral ion homeostasis, through its role in regulating kidney function, and therefore an important factor in maintaining a near-constant [Ca\(^{2+}\)] in the blood [6;28;70].

Binding of Cd\(^{2+}\) to the receptor activates phospholipase C, resulting in a breakdown of polyphosphoinositides and accumulation of two second messenger molecules, inositol-3-phosphat (IP\(_3\)) and diacylglycerol (DAG). DAG activates protein kinase C. IP\(_3\) empties cellular calcium stores through binding to and activation of the IP\(_3\)-receptor channel on the endoplasmic reticulum. The increase in cytosolic calcium affects a number of cellular functions[12;64;163;168;169].

**Fig.2.2** A Schematic model of ion transport responsible for movement of Na and K in *Xenopus laevis* A6 cells. The most prominent channels and transporters are shown along with their respective inhibitors. Active transport of ions is only possible through transcellular movement, across the cell membrane. The original schematic is from Rehn et al., [136]The CFTR channel has been added from Ling et al.[98].
3. **Apoptosis and Necrosis.**

Regulated cell death has long been recognized as an essential event in the normal functioning of multi-cellular organisms. The programmed and timed disappearance of tissue structures during embryogenesis has been known for centuries, although the concept of cells was first introduced in 1839 by Schleiden and Schwann. The phenomenon of programmed cell death (PCD) was examined for a century before explanations of its function was proposed. Research into the diverse mechanisms was made difficult due to the inherent differences in the morphological characteristics described in different model-systems: Researchers using embryogenesis as a model described one set of characteristics (apoptosis) while models utilizing pathological insult showed other characteristics (necrosis). It was first in 1972 that a theory distinguishing between these two forms of PCD was put forth by Kerr, Wylie and Currie[78]. Other forms of PCD has since then been described (eg. Autophagy). Refer to the Nature article by Vaux for a brief but comprehensive review, of the timeline of PCD and apoptosis research [170].

Different types of apoptosis have been classified, based on morphological alterations during cell death, such as nuclear events, protease activation or organellar rearrangements. Current research into the different forms of PCD has indicated an inherent interaction rather than autonomy of the programmed events, perhaps limiting the usefulness of some older classification systems, until these mechanisms are better understood [170].

![Fig.3.1](image)

**Fig.3.1** A generalized scheme of distinct morphological characteristics and changes in cells undergoing cell-death by necrotic or apoptotic pathways. Necrotic cells may swell in size as ATP production is stopped and water enters the cell by diffusion. The membrane integrity is eventually compromised resulting in a disordered cellular breakdown. Apoptosis represents the ordered cell-death pathway where ATP production is maintained until the cell eventually is eventually terminated and packaged into small apoptotic bodies [140].

Apoptosis is the best characterized form of programmed cell-death. It is an evolutionary well conserved mechanism, important in a number of diverse cellular functions, such as embryogenesis, normal tissue development and the immune response. It represents a mechanism for removal of dead, dying or unwanted cells. Disruption of the normal regulation of apoptosis can lead to a variety of pathological conditions, and has been associated with carcinogenesis, chemoresistance (in cases of enhanced protection against Apoptosis), and diseases such as AIDS and a number of neurodegenerative disorders[39;60;183].
Apoptosis is a genetically controlled, ordered breakdown of cells. The defining morphological characteristics are the controlled breakdown of cellular organelles, plasma-membrane remodelling and "blebbing", DNA condensation and fragmentation (often by formation of ladder-like structures) and cell shrinkage with cytoskeletal rearrangements. The externalisation of phosphatidylserine on the plasma-membrane is another hallmark and marker of apoptosis. The end product, formation of apoptotic bodies are usually removed through phagocytosis. ATP-formation is often maintained throughout the apoptotic process, as many of the biochemical functions for correct execution of the programmed cell-death are energy requiring[145;178].

At least two other forms of cell-death are usually characterized as types of programmed cell-death, necrosis and autophagy. Necrosis represents an unordered cellular breakdown, usually the result of a pathological stimulus or injury. Toxic injuries to the cell lead to membrane damage and disruption, resulting in massive swelling and a disordered cellular breakdown. DNA fragmentation is unordered, without the ladder-like formations often observed in apoptosis. ATP-formation is usually severely disrupted or terminated, an event that separates necrosis from apoptosis [39]. Recently another type of PCD mechanism has been described called Caspase Independent Cell Death (CICD) [34]. It is thought to be a form of cell death induced by pro-apoptotic factors, but proceeding through a caspase independent pathway due to genetic disruption or inhibition of Caspases. This pathway would have distinct morphological characteristics distinguishing it from apoptosis. The existence and any cellular and in vivo relevance of this type of PCD is being debated, since experimental verification has been difficult[34].

Apoptosis in mammals is induced through several different pathways, depending on the method of induction, but principally through either the “extrinsic” or death-receptor induced pathways and/or the “intrinsic” or mitochondrially mediated pathway[79;158]. Both apoptotic pathways result in the activation of a cascade of executioner caspases (cysteinyl, aspartate-specific proteases). The two mechanisms of activation are not likely to be isolated systems. Instead there seems to exist a level of crosstalk and biofeedback between the two, dependent on the mechanism and source of induction, ensuring a high degree of regulation of the apoptotic machinery[25;36;81;95;158].

The “extrinsic” pathway of apoptosis is activated by binding of ligands, such as TNF, FasL and TRAIL to a receptor on the plasma membrane, from the TNFα-superfamily of receptors, usually called death-receptors [40]. Extracellular regions of the death-receptors aggregate at the cell surface, subsequent to the binding of cytokine ligands. This results in the recruitment of adaptor molecules to an intracellular globular domain on the death receptor, termed death domains (DD). One of the adaptor molecules recruited is Fas Associated Death Domain (FADD). It contains a death-domain that binds to the DD on the death-receptor, either directly or through other adaptor molecules. FADD also contains a region called the death effector-domain, which binds the weakly active zymogen ProCaspase-8. This multi-protein complex is termed the Death Inducing Signalling Complex or DISC[79;80;183]. Binding of ProCaspase-8 results in a proximity induced activation and rapid autoproteolysis of the zymogen, ending in formation of the active Caspase-8. Caspase-8 is either released to cellular compartments or functions bound to the DISC. Active Caspase-8 activates downstream executioner/effecter caspases, such as Caspase-3, resulting in DNA degradation and cellular morphological changes as described earlier [120].

This direct route of apoptosis activation is an evolutionary highly conserved form of PCD. The mechanistic details are found to be very similar between otherwise very diverse organisms, such as
humans and *C. Elegans* (where it was first described). In mammalian systems the extrinsic pathway of apoptosis is associated with a select number of cell types (called Type I), including cells of the immune system [145].

![Fig.3.2](image)

**Fig.3.2** The structure of the Death-Receptor complexes of the extrinsic pathway of apoptosis induction. The figure shows the composition of the Death-Inducing-Signalling Complex (DISC) downstream of three death receptors TNFR1, CD95 and DR4/5.[39].

Activation of the “Intrinsic” pathway of apoptosis induction is, unlike the “Extrinsic” pathway, principally associated with the mitochondrion [197]. This pathway has no requirement for an external activation factor, but may be activated by a diverse set of stress signals, including UV radiation, viral factors, some tumor suppressor genes and most chemotherapeutic agents[57;60;140]. These signals are decoded and interpreted by a diverse set of cytosolic or intra-organelar molecules that relay the signal to the mitochondrion. The intrinsic pathway is the most common method for apoptosis induction, and cell types expressing this mechanism are termed type II[145].

The mitochondrion, apart from being the “powerhouse” of the cell, by being the center for oxidative phosphorylation, is also heavily involved in the regulation of apoptosis. Mitochondria contain a large number of apoptosis inducing elements, that are released from the mitochondrion upon disruption of it’s membranes integrity, a key event in the intrinsic pathway[65;127;135]. As the integrity of the inner mitochondrial membrane (IM) is important for the oxidative phosphorylation by maintaining a proton gradient, disruption of this membrane will negatively affect ATP-production. The morphological changes in MK membrane structure and integrity observed during apoptosis is affected and possibly regulated in several ways, including through calcium signalling, ROS generation and activity of electron transport chain components[37;66;86;129].
Disruption of the mitochondrial membrane during apoptosis, is often associated with formation of large pores through both inner and outer membranes, the mitochondrial permeability transition pores (MPTP) [60]. Formation of the pore results in an uncoupling of the oxidative phosphorylation through depolarisation of the mitochondrial membrane potential, $\Delta \Psi_m$. Although mitochondrial permeability transition is considered a significant event in apoptosis signalling, the actual molecular composition and mechanistic details of the permeability pore is still being widely debated and investigated. [123;197].

3.1. Cytochrome-C mediated cell death

The alteration of the mitochondrial membrane releases a variety of proteins, some of which activates the executioner/effect or caspases. The molecules released through disruption of mitochondrial membrane integrity are collectively known as apoptogenic factors[127]. Among the most important to be released is cytochrome c, but also other apoptotic factors including Smac/DIABLO are released from the outer membrane space [25]. Alterations of the inner membrane integrity is associated with release from the mitochondrial matrix of Apoptosis Inducing Factor (AIF) and Endonuclease G (endoG)[80].

Cytochrome c released from the MK after an MPT event, complexes with apoptosis protease activating factor–1 (APAF-1), along with ATP/dATP and procaspase-9 to form the apoptosome. This large complex can activate downstream effector/executioner caspases. It is inhibited by inhibitors of apoptosis proteins (XIAP, IAP), which are in turn under negative regulation by Smac/DIABLO, a protein released from MK during permeabilisation of the outer membrane[112]. Once activated the apoptosome in turn activates downstream caspases-3 and -7. These caspases can mediate the apoptotic signal by cleaving ICAD/DFF45. ICAD is a strong inhibitor of the potent DNase CAD/DFF40. Cleavage of the tightly bound inhibitor releases it from the ICAD/CAD complex, activating DNAses resulting in degradation of chromosomes into nucleosomal fragments[39]. Another nuclease, Endo G, released from the mitochondrion during apoptosis, also participates in the degradation of DNA[127].
Fig. 3.3 An example of Cross-talk between the two major apoptotic pathways, the "extrinsic" or death-receptor controlled and the "intrinsic" mitochondria associated pathway. Convergence or cross-talk between the two pathways is regulated by various pro- or antiapoptotic molecules including caspases, Bcl-2 proteins and calpains. Activation of the membrane-bound death receptor activates the proenzyme Procaspase-8 to its active zymogen form of Caspase-8. Caspase-8 cleaves the Bcl-2 protein Bid to its truncated form tBid. tBid mediates the oligomerisation of Bax, that translocates to the mitochondria activating the mitochondrial permeability transition pore (MPTP), initiating the permeability transition event. Opening of the MPTP releases apoptogenic factors from the mitochondria including cytochrome c (CytC) and Smac/Diablo. CytC and Caspase-9 integrates into the Apoptosome complex activating the executioner Caspase-3. Smac/Diablo released from the mitochondria suppresses activity of XIAP that otherwise would inhibit the Apoptosome and Caspase-3[158].

3.2. Mitochondrial Permeability Transition

The permeabilisation of the MK outer membrane appears to be integral to many models of apoptosis induction, though a number of different theories have been proposed to account for the phenomenon. Many models explaining the permeabilisation of mitochondria describe the opening of a pore, through both mitochondrial membranes. The mitochondrial permeabilisation transition pore (MPTP) is thought to be composed of pre-existing proteins in the MK, including the Voltage Dependent Anion Channel (VDAC), the Anion Nucleotide Translocator (ANT) and Cyclophilin D (Cyp-D). This “minimum requirement” model of MPT induction is still being debated, as it has been problematic to verify the model due to the technical difficulties in isolating an intact functional membrane bound MPTP[66;69;197].

The MPT pore is triggered by pathologically high levels of Ca^{2+} as well as other stimuli including oxidants and depletion of adenine nucleotides. Inhibitors may include antioxidants, low pH and molecules affecting the function of the pores different elements (eg. cyclosporin a binding to Cyp-D) [60;81;135].
Members of the Bcl-2 family, a group of proteins often associated with regulation or initiation of apoptotic events, have been implicated in MPT regulation. The Bcl-2 protein Bax has for example been shown to sometimes regulate the interaction of ANT and VDAC. The induction of mitochondrial permeability transition (MPT) has likewise been linked to t-Bid interaction with VDAC[197].

The importance of the level of MPT induction is also being investigated, as it is possible that a low level of activation (MPTP “flickering” on and off) is of relevance in certain types of apoptosis, while a full scale activation could presumably lead to a total disruption of MK function and instead necrosis [69].

![Diagram of the Mitochondrial Permeability Transition Pore (MPTP)](image)

**Fig.3.4** The components of the Mitochondrial Permeability Transition Pore (MPTP). The putative components of the pore include the VDAC, ANT and Cyp-D. The pore is under regulation by a number of other factors including \( \text{Ca}^{2+} \), \( \text{H}^+ \) and ROS. The exact arrangement and stoichiometry of pore components is unknown. The image shows 3-D structures from the Protein Database of some components: the structure shown for cyclophilin-D (Cyp-D) is of Cyp-A. ANT, adenine nucleotide translocase; PBR, peripheral benzodiazepine receptor; VDAC, voltage-dependent anion channel; RSH, reduced thiols; RSSR, thiol disulfide [27].

### 3.3. Bcl-2

The release of apoptogenic factors from mitochondrion during apoptosis is heavily regulated by the Bcl-2 family of proteins [135]. They are integral elements of the mitochondria initiated “intrinsic” pathway of apoptosis. This large protein family includes both antiapoptotic (such as Bcl-2 and Bcl-Xl) and proapoptotic members (Bid, Bax, Bak and Bad). The proapoptotic subfamily is further divided into BH3 only and the multi-domain members (represented by Bax and Bak)[60].

Members of the antiapoptotic Bcl-2/Bcl-Xl subfamily contain all four conserved homology (BH) domains BH1-4. Members of the proapoptotic Bax/Bak subfamily only contain BH1-3. The antiapoptotic members Bcl-2 and Bcl-Xl appear to function, at least in part, by inhibiting the release of apoptogenic factors from mitochondrion. They are thought to recognize and interact with the
amphipathic BH3 domains of proapoptotic Bcl-2 proteins. Members of the opposing subfamilies can dimerize in aqueous solution. This ability represents a mechanism for inactivation of the proapoptotic factors, through recognition and antagonizing by antiapoptotic Bcl-2 proteins; i.e. the BH1-3 domains of Bcl-XL forms an elongated groove that can bind the BH3 domain of proapoptotic Bcl-2 proteins[31].

The BH3-only proteins are thought to act as sensors for the various apoptotic stimuli, relaying the signals to the mitochondrion. At the mitochondrion, the BH3 proteins activate multidomain proapoptotic Bcl-2 proteins directly, or antagonize the activities of the antiapoptotic members such as Bcl-XI or Bcl-2[39].

**Fig.3.5** members of the Bcl-2 family of apoptosis regulating proteins. The figure illustrates the organization of the Bcl-2 homology (BH) domains of different types of Bcl-2 proteins. Antiapoptotic Bcl-2 include a BH4 domain. Several Bcl-2’s include a transmembrane domain (TM) rich in hydrophobic residues, in their carboxyl-terminal end. The BH3 domain is thought to be critical for the apoptogenic effects of pro-apoptotic Bcl-2 proteins [31].

Members of the Bax/Bak subfamily undergo homo-oligomerisation upon activation by an apoptotic signal. The homo-oligomers have been thought to form pores in the mitochondrial outer membrane, resulting in the release of apoptogenic factors, such as Cyt-c from the inner membrane space. The exact mechanism of proapoptotic Bcl-2 mediated pore formation is still under investigation [36;86]. Bax has been proposed to be integral for the opening of the mitochondrial permeability transition pore (MPTP), either directly being a part of the pore structure, or promoting formation by its other proteins including Cyp-D, VDAC and ANT[146].

The BH3-only protein Bid is cleaved by Caspase-8 to its highly apoptogenic form, truncated Bid or t-Bid. Binding of t-Bid mediates the homo-oligomerisation of Bax with the resulting pore formation of the MK outer membrane [36].

In addition to triggering Bax and Bak oligomerisation, t-Bid initializes rearrangements of the mitochondrial cristae [36;99]. These invaginations of the inner membrane are thought to sequester a large portion of the cyt-c store, in addition to those bound to IM cardiolipin. The exact distribution of cyt-c in mitochondria is unknown. The alterations and rearrangement of the mitochondrial
morphology by t-Bid, under negative regulation by Bcl-Xl, thus represents another mechanism through which apoptogenic factors could be released. Mitochondrial morphological rearrangements during cell-death, triggered by t-Bid, may be an example of the interaction of the intrinsic and extrinsic pathways of apoptosis[129;189].

The heavy metal cadmium can induce both Bid and Bax activation in WI 38 cells. In these cells, Cd$^{2+}$ induces caspase mediated Bid cleavage and activation. Bax is then activated by Calpain, a family of Ca$^{2+}$ activated cysteine proteases [122]. The Calpain family of proteases is increasingly being associated with different forms of PCD including apoptosis, both as a mediator by controlling apoptosis signalling, as well as executioner of the cell death mechanism [175].

Fig.3.6 Bcl-2 mediated crosstalk between various apoptotic pathways. The figure shows a putative schematic diagram of Bcl-2 interaction in a selection of apoptotic pathways. BH3-only Bcl-2 proteins act as sensors and signal integrators, relaying the apoptotic stimuli to the mitochondria, by antagonizing anti-apoptotic Bcl-2's such as Bcl-XL or Bcl-2, or activating multidomain pro-apoptotic proteins. The multidomain pro-apoptotic Bcl-2 proteins Bax and Bak undergo oligomerization and facilitate the permeabilisation of the mitochondrial membrane, resulting in release of apoptogenic factors, including cytochrome c (CytC), Endonuclease G (Endo-G), apoptosis-inducing factor (AIF) and Smac/Diablo. The pro-apoptotic elements released from the mitochondria during Bcl-2 facilitated permeability transition induce apoptosis by caspase dependent or independent mechanisms. Apaf-1, apoptosis protease activating factor-1[31].

3.4. Caspase

Caspases, or cysteine aspartate-specific proteases, is a family of intracellular proteins primarily responsible for the breakdown of proteins during different forms of PCD including apoptosis and necrosis. Initiator caspases are responsible for activating downstream executioner/effecter caspases or enhancing an already existing apoptotic signal, through activation of other initiator caspases. Effector caspases cleave intracellular substrates eventually leading to cell death. Their primary
function during apoptosis is to, 1) arrest cell cycle and inactivate DNA repair, 2) inactivate the inhibitors of apoptosis and 3) dismantle the cellular cytoskeleton [117].

Caspases were first recognized as being important and sufficient for apoptosis based on the observation that the Caenorhabditis elegans death gene (CED-3) was essential for all programmed cell deaths in C.Elegans development and growth. It was later established that CED-3 showed a close homology to a human protein, interleukin-1β-converting enzyme (ICE, now called caspase-1). Caspase-1 is not thought to be involved in human apoptosis, though it has been implicated in inflammation. Other members of the large CED-3/ICE-like protease-family have been identified that are necessary for correct initiation or execution of several apoptotic pathways. The caspases can be divided into two functional subgroups based on their (often hypothetical) function in apoptosis: initiators (Caspase-2,-8,-9 and -10) or effectors/executioners (caspases -3,-6, and -7) [195].

All caspases share similarities in amino acid sequence, structure and substrate specificity. They are expressed as 30-50 kDa single chain proenzymes containing three domains: an NH2-terminal domain, a large 20 kDa subunit and a smaller 10 kDa subunit. They are synthesized as inactive zymogens (procaspases) that are cleaved at an internal proteolytic site to yield the active protease. Initiator caspases possess long N-terminal prodomains that contain protein-protein interaction motifs. Effector caspases usually contain shorter or no prodomains[80;120].

Four pathways to caspase activation have been described, that lead to apoptosis. They can be briefly categorized as: 1) the mitochondria mediated pathway; 2) the death-receptor mediated pathway; 3) granzyme-B mediated pathway; and 4) the endoplasmic reticulum mediated pathway [195].

The mitochondria mediated or the “intrinsic” pathway involves a permeabilisation of the mitochondrial membranes culminating in a release of apoptogenic factors, responsible for activation of caspase 9 (as part of the apoptosome). Caspase 9 activates downstream caspases-3,-6 and -7. Caspases -3 and -9 appear the most important for this type of apoptosis as they determine the form and progress of apoptosis[45;123;135].

The alternate apoptotic pathway, the death-receptor mediated or “extrinsic” pathway, activates a different set of caspases. After association of a deathdomain-containing ligand to the plasma membrane-bound death receptor (FAS), a member of the large tumor necrosis factor receptor superfamily (TNFR), oligomerisation or conformational changes occur that recruit an adaptor molecule, FADD, forming the death inducing signalling complex together with procaspase-8 (and probably procaspase-10). The inactive zymogen Procaspase-8 is processed and caspase-8 is activated. Caspase-8 directly activates caspase-3 leading to a cascade of caspase activations and eventually to apoptosis[79;178;183].

The intrinsic and extrinsic pathways can be linked through the proapoptotic Bcl-2, Bid. Bid is cleaved into the active form, t-Bid by caspase-8, which translocates from the cytosol to the mitochondrion where it can participate in the activation and formation of the MPTP, by activation of Bax [99].
3.5. Calpain.

Calpain or calcium-activated neutral proteases are a family of cytosolic endopeptidases. Conventional calpains are classified as m- or µ-type calpains depending on their requirement of a calcium concentration for activation, respectively activated by high or low micromolar calcium concentrations. Calpain is regulated mainly by spatio and temporal changes in cellular calcium levels and by its unique endogenous inhibitor calpastatin[58;161].

Calpains are thought to mediate a variety of intracellular signalling processes including cell proliferation, embryonic development and differentiation pathways. They are also thought to be involved in some mechanisms of apoptosis, necrosis and in several types of pathology including Cardiac ischemia/reperfusion injuries, Alzheimers disease and muscular dystrophy[101;130].

The physiological importance and function of calpain is not fully understood, but a number of potential substrate proteins have been proposed. Several substrate proteins have been associated with carcinogenesis including products of oncogenes and tumor supres sor genes. Calpains plays an important role in p53 induced apoptosis by hydrolizing the protein lowering its in vivo level. Other
substrate proteins include calmodulin binding-proteins, cytoskeletal proteins, G-proteins, enzymes involved in signal transduction including the IP$_3$-receptor and membrane receptors. Calpain obviously has a broad spectrum of target proteins, and is probably involved in a wide range of cellular signaling functions[106;144].

Toxicants may activate Calpains possibly directly or by affecting cellular calcium levels. Cadmium may invoke release of calcium from cellular stores, interpreted as a signal for Calpain activation and inducing apoptosis[96;184].

The two classes of Calpains, m- or µ-type are heterodimers typically containing an 80 kDa large catalytic subunit and a smaller 30 (28-30) kDa calcium-binding regulatory subunit. The 80 kDa subunit contains 4 (I-IV) domains, with the protease domain subunit II further divided into IIA and IIB, a feature common for cysteine proteases. Domains IIA and IIB are common for most human isotypes of calpain, with larger variations found in the other domains. In the absence of calcium the two protease subdomains, IIA and IIB are separated by structural constraints imposed by domain interaction. Ca$^{2+}$-induced structural changes that release these constraints are necessary for activation and formation of a functional catalytic site. The 30 kDa small subunit contains 2 domains (V-VI). The domains I, III, IV, V and VI are involved in regulation of enzymatic activity by dimerisation of the two subunits at domain IV and VI. Inhibition of calpain by binding of endogenous inhibitors requires the proximity of these two domains[43;58;161;182].

![Fig.3.8](image.png)  
**Fig.3.8** structure and relationship of the Calpains, Ca$^{2+}$-dependent cysteine proteases involved in regulation and execution of some types of apoptosis.[161]
4. Calcium as a signal molecule

The Ca\(^{2+}\) ion represents a ubiquitous and highly versatile intracellular signal, involved in a great number of physiological processes and cellular functions. It is involved in processes ranging from muscular contraction, neuronal signalling, glycogen metabolism, hormonal secretion as well as cellular differentiation and proliferation. It is directly involved in regulating enzyme function as a cofactor or second messenger, as well as being a potent activator of gene transcription[29;143;179]. Calcium involvement in so diverse a set of processes requires cells to initiate, interpret and react to signals with widely differing spatio-temporal properties. Consequently the cellular machinery involved in calcium signalling consists of a vast number of components, including calcium sensing receptors, ion-pumps, -exchangers and channels, transducers, buffering proteins, and calcium regulated enzyme systems[8;190]. Components of this large Ca\(^{2+}\) signalling toolkit is uniquely combined and expressed in each type of cell, resulting in cell-type specific calcium signal reaction schemes. However, the common theme for calcium signalling is almost always the generating of brief calcium pulses. The transients generated are interpreted by the signalling machinery and reacted upon[6;16;70] [8].

Signal calcium is either derived from internal stores (eg. endo- or sarcoplasmic reticulum), or external medium. Normal levels of intracellular levels of Ca\(^{2+}\)\(_i\) are at the nanomolar range (~100 nm), with extracellular calcium levels reaching concentrations often 10.000 times greater (~1mM). Mobilization of Ca\(^{2+}\) from intracellular stores or uptake from extracellular sources can elevate the cytosolic calcium levels to more than 1µM. Calcium concentrations in the bloodstream is normally at a near constant. Because of the sensitivity of cellular calcium signalling, a tight regulation of calcium homeostasis is essential, and consequently the homeostatic mechanism responsible for a constant Ca\(^{2+}\) level in the bloodstream is very complex[17;143].

4.1. Physiological role of Calcium

The following should be considered a brief introduction to a very complex subject: calcium sensing cells secrete more or less of calcitropic hormones into systemic circulation. Parathyroid hormone (PTH) is secreted from cells in the parathyroid gland. Elevated levels of Ca\(^{2+}\)\(^{2+}\) in the blood inhibits release of PTH and production of 1,25-dihydroxyvitamin D3 from the proximal tubule, and increases release of calcitonin from the thyroid gland. Changes in the secretion rate of these hormones alter the function/activity of effector organs and tissues involved in calcium homeostasis, principally intestine, kidney and bone. These tissues normalize extracellular calcium by altering the transport of ions into and out of cellular stores, bone, intestine and urine, in response to hormonal activation. The secretion rate of the calcitropic hormones is very sensitive to changes in extracellular calcium concentration, and the effector tissues response to these hormones is likewise effective, making the whole system for maintaining correct calcium homeostasis extremely efficient [137].
Fig. 4.1 A simple overview of the homeostatic system responsible for regulating and maintaining physiological calcium at a near constant. Solid arrows show the target of parathyroid hormone (PTH) and 1,25(OH)_{2}D_{3}. Dotted arrows show examples of where calcium and phosphate ions regulate the functions of target organs. ECF, extracellular fluid; 25(OH)D, 25-dihydroxyvitamin D; 1,25(OH)_{2}D_{3}, 1,25-dihydroxyvitamin-D_{3}[28].

4.2. Cellular calcium signalling

The release of calcium from internal and external sources to the cytosol, has a unique spatio-temporal pattern determined by the mechanism of activation. The apparatus interpreting this signal is designed to recognize and differentiate between localized increases in Ca^{2+}, as happens with transient openings of calcium channels in the endoplasmic reticulum, or recognize global increases of calcium[126;165]. In neurons, the ability for localized calcium sensing complexes to autonomously react to changes in calcium greatly enhances these cells computational capacity [29]. The speed with which signal components react, and the speed with which normal calcium levels are restored, is another method for utilizing the change in calcium concentration, as a means of differentiating signal output. Some components of the calcium sensing machinery react fast (e.g. synaptic transmission and cardiac contraction), with reaction times of microseconds. In other instances the Ca^{2+} transients last longer, even minutes, propagating as “waves” resulting in a different reaction scheme that is typically less localized. With continuous stimulation, the calcium signal may be repeated, resulting in series of Ca^{2+} oscillations [17;27]. Cells are able to determine changes in frequency of these oscillations in addition to the concentration of each calcium spike, resulting in a highly sophisticated mechanism of signalling. Ca^{2+}-frequency modulated signalling is important in numerous cell types, regulating processes ranging from mitochondrial redox state to differential gene transcription[27;38;124].

4.3. Calcium movement

Calcium entry from the external medium is controlled by a number of stimuli including membrane depolarization, extra- or intracellular messengers and agonists, and depletion of intracellular stores [134]. Calcium release from the internal stores is controlled by calcium itself or a group of
messengers including inositol-(1,4,5)-triphosphate (IP$_3$), cyclic ADP ribose (cADPR), nicotinic acid adenine dinucleotide phosphate (NAADP) and sphingosine-1-phosphate (S1P). Phospholipase c (PLC) isoforms, generating IP$_3$ that releases calcium from internal stores, are activated by different mechanisms, including G-coupled receptors, increases in Ca$^{2+}$ concentrations and tyrosine-kinase coupled receptors[70;137].

### 4.4. Calcium sensing receptor

The Calcium sensing receptor (CsR) belongs to the superfamily of g-coupled proteins. Binding of a ligand to the CsR activates an isoform of phospholipase c releasing IP$_3$ and DAG to the cytoplasm. Binding of IP$_3$ to the IP$_3$-Receptor on the endoplasmic reticulum (ER), induces a transient opening of the channel, releasing calcium from the store. CsR’s are sensitive to a number of signals including various divalent cations including calcium and other divalent cations. Activation of a CsR can initiate a complex series of intracellular signalling cascades. An extracellular (calcium) signal, detected by the CsR and interpreted by the associated cellular signalling machinery, can propagate to the nucleus and alter gene transcription [70]. The CsR isoform expressed in A6 cells is sensitive to cadmium [51].

![Signal transduction mediated by a Calcium sensing Receptors (CsR) expressed in A6 cells. CsR's belong to the superfamily of g-coupled proteins. Binding of a ligand to the CsR activates an isoform of phospholipase c releasing Inositol(1,4,5)P$_3$ (IP$_3$) to the cytoplasm. Ins(1,4,5)P$_3$ binds to the Ins(1,4,5)P$_3$-Receptor (IP$_3$-R) on the endoplasmic reticulum. Activation of IP$_3$-R induces a transient opening of the channel, releasing calcium from the intracellular calcium stores [128]. The CsR isoform expressed in A6 cells is sensitive to a number of divalent cations including calcium, cadmium and zink. Activation of a CsR can initiate a complex series of intracellular signalling cascades. CsR mediated release of calcium from the endoplasmic reticulum, can be detected by mitochondria due to the close proximity of the two organelles.](image-url)
proximity of the two organelles [70]. This can affect mitochondrial functions controlled by calcium, including metabolic pathways and initiation of cell death. Mitochondrial Calcium Uniporter (MCU).

ER replenish calcium lost through the IP3-R, through the action of the Sarco- and Endoplasmic Reticulum Calcium ATPase (SERCA)[157;169]. The release of calcium from the ER stores is not necessarily of a significant size to elicit a response from the cellular calcium sensing machinery, and especially the mitochondrion that are integral in calcium regulation. Resting levels and stimulated levels of Ca^{2+}_c are well below the affinity of the MK calcium transporters. With the rapid and transient changes in normal cellular calcium levels, the MK was originally not thought to be able, to accumulate a significant amount of Ca^{2+} to activate any signals. The last few years have seen a change in this view, with the work of several groups demonstrating a MK Ca^{2+} increase parallel to increases in Ca^{2+}_c. This is possible with the close proximity of MK to calcium channels in intracellular stores, or as is the case in neurons, strategically placed to sense Ca^{2+} changes near the plasma-membrane in synapses [168]. The release of calcium from cellular stores produces micro-domains of high calcium concentrations. It has been shown that these transient localized micro-domains of calcium largely exceed bulk cytosol and meet the low affinity of MK Ca^{2+} transporters. The consequence of the close proximity of MK and ER is the capacity of MK to “sense” the microenvironment at the mouth of the IP3-R and the high Ca^{2+} micro-domains generated by their opening upon cell stimulation [163].

4.5. Store operated calcium channels

Release of Ca^{2+} from ER by a phospholipase C generated IP3 mediated signal, initiated by stimulation of a surface receptor, is often followed by influx of calcium across the plasma membrane. This is the case in numerous and widely different cell- and tissue types. The uptake of extracellular calcium as a response to the depletion of intracellular stores (i.e. ER), was first described by Putney [134]. It was originally proposed to be a mechanism responsible for replenishing Ca^{2+} of intracellular stores. It is now termed Store-operated Calcium entry, because of its dependence on and regulation by the level of free Ca^{2+} in the intracellular calcium stores of the ER lumen. It provides a direct means for Ca^{2+} to enter the cell across the plasma membrane via store-operated channels (SOC)[132;157]. In addition to its store-replenishing function, SOC’s can contribute to a general elevation of cytosolic calcium levels and may thus be important in intracellular calcium signalling [168]. Store-replenishing allows calcium stores to quickly regenerate after calcium release and maintain the repetitive calcium oscillations that underlie many intracellular calcium signals. It has been accordingly been proposed to be involved in certain types of apoptosis [121;126].

Not all calcium currents across the plasma membrane initiated by IP3 signals are store-operated, but have instead been termed signal-driven calcium entry. The specific molecular mechanism responsible for the opening of SOC’s is unknown. It is probable that a number of homologous SOC exist, as their distribution and needed function presumably is dependent on tissue type. SOC’s have been identified by patch-clamp techniques in numerous cell types including hematopoietic- and endothelial cells, hepatocytes and β-cells. The best described is the CRAC channel, a prototypic SOC, found in a few cell types including T-cells, mast cells and related cell lines. A hallmark of CRAC is its lack of voltage dependence and its high selectivity of calcium over other cations [132]. While the SOC is relative specific to calcium ions, cadmium may also pass through, presumably due to the similar size in ionic radii of the two metal-ions [108].
Fig. 4.3 Four models for store-operated calcium entry. The exact mechanism for store-operated calcium entry is not known. Four theories of capacitative or store-operated calcium entry are shown here. All models are similar in that an agonist activates an IP$_3$ signal releasing calcium from ER. a) Discharge of calcium releases a diffusible component - calcium influx factor (CIF) activating store-operated calcium channels of the plasma-membrane. b) In the exocytosis model, depletion of stores causes fusion of vesicles containing CRAC channels, with the plasma membrane. c) Calcium discharged from a sated calcium pool inhibits opening of the store-operated channels. With emptying of stores, this inhibition is removed. d) In the conformational coupling theory, emptying of calcium stores incurs conformational structure changes in the IP3-receptor acting as a signal to the plasma membrane by direct protein-protein interaction. R, agonist receptor; Ag, agonist; G, heterotrimeric G protein; polyphosphoinositide phospholipase C; CRAC, Ca$^{2+}$ release-activated Ca$^{2+}$ (channel); ER, endoplasmic reticulum; Ins(1,4,5)P$_3$, inositol 1,4,5-trisphosphate; Ins(1,4,5)P$_3$R, Ins(1,4,5)P$_3$ receptor; CIF, Ca$^{2+}$-influx factor; SP, scaffolding protein [134].
4.6. Mitochondria and Calcium

![Mitochondrial Calcium Transporters and Channels](image)

Fig. 4.4 Mitochondrial Ca\(^{2+}\) transporters and channels. The outer membrane has been omitted for clarity. Complex I, III and IV of the electron transfer chain (ETC) is shown with the associated ATP-synthase (complex V). Known 3D structures obtained from the Protein Data Bank ([HTTP://www.rcsb.org/pdb](http://www.rcsb.org/pdb)) are shown. UP, Ca\(^{2+}\)-uniporter; RaM, Rapidmode Ca\(^{2+}\)-uptake; RyR, ryanodine receptor; PtP, Permeability Transition Pore; \(\Delta \Psi_m\), membrane potential [27].

In addition to its role as the cell’s “powerhouse”, the site of oxidative phosphorylation, mitochondria is integral for many other cellular processes and metabolic reactions. Mitochondria are the site of steroid and porphyrin synthesis, urea-cycle and amino acid metabolism (interconversion). It is also central for many cellular functions, integrating and regulating signal pathways, for example through the actions of glucose sensing/insulin regulation and the regulation of cellular calcium homeostasis. The mitochondrial regulation of these metabolic functions affects numerous other signalling pathways [12].

Mitochondria are tubular organelles, defined by a set of differentiated membranes, the inner and outer MK membranes. The inner membrane space is located between the two membranes, and the mitochondrial matrix lies at the centre inside the inner membrane. The outer membrane (OM) is permeable to small proteins and some ions. Proteins (MW<10 kDa) enter through the mitochondrial porins, a large conductance channel. The inner membrane (IM) is ion-impermeable with numerous extrusions or invaginations into the matrix termed cristae. The respiratory chain complexes and ATP-synthase complex responsible for oxidative phosphorylation are all located on the IM. The ion-impermeability of the IM allows the respiratory chain complexes to establish a large electrochemical gradient (\(\Delta \mu_H\)), through the translocation of H\(^+\) from the matrix to the inner membrane space. The proton gradient established by the ETC is utilized by the ATP-synthase complex to generate ATP [22;63].

The electrochemical gradient is composed of an electrical (\(\Delta \Psi_m\)) and chemical component (\(\Delta p\text{H}\)) as per the Nernst equation:

\[
\Delta \mu_H = z F \Delta \Psi_m + RT \ln \left[ \frac{[H^+]_m}{[H^+]_{out}} \right]
\]
Calcium may enter the mitochondria in several different ways, with unique channels handling transport of calcium to the inner membrane space and from there to the matrix.

The primary mechanism responsible for calcium uptake into the inner membrane space is through a voltage dependent anion channel (VDAC). This ruthenium red-sensitive Ca$^{2+}$ channel is integral to the regulation of outer membrane permeability to ions and metabolites. The location and number of VDAC at places with close MK-ER proximity or contact sites, may be a factor in the regulation of MK sensitivity to ER calcium release [27].

The electrical component is thought to be primarily in the form of $\Delta \psi_m$ creating a huge driving force for Ca$^{2+}$ entry into the organelle. The Mitochondrial Calcium Uniporter (MCU) is responsible for the majority of Ca$^{2+}$ uptake and transport into the matrix. Because of the net movement of charge from Ca$^{2+}$ uptake, it comes at the expenditure of the electrochemical potential ($\Delta \psi_m$) generated by the electron transport chain. Reverse MCU (Ca$^{2+}$ export) is also possible and linked to binding of Ca$^{2+}$ to the outer surface of the inner membrane, as well as a soluble inter-membrane component [27;63].

Two other mechanisms for calcium uptake exist, that are thought to be the basis for the phenomenon of excitation-metabolism coupling, i.e. a Ca$^{2+}$ induced contraction, coupled with stimulation of the mitochondrial oxidative phosphorylation (ox-phos) [12].

“Rapid mode” uptake or RaM occurs at mili-scale timeframe and allows a rapid minute regulation of matrix calcium concentration. Matrix calcium changes by RaM are coupled to changes in cytosol. A ryanodine receptor isoform has been located in the inner membrane of excitable cells (mRyR) [22].

The primary mechanisms for Ca$^{2+}$ efflux is achieved through the exchange of Na$^+$ via the Na$^+$/Ca$^{2+}$ exchanger. Na$^+$ is pumped out of the matrix in exchange for protons. Both Ca$^{2+}$ uptake and efflux is therefore linked to consumption of the electrochemical gradient established by the electron transport chain [27].

The primary role of MK calcium uptake is to stimulate Ox-Phos, including enzymes of TCA cycle, pyruvate dehydrogenase and isocitrate dehydrogenase as well as ATP-synthase complex V of the Electron Transport Chain (ETC) pathway. Overall it appears that Ca$^{2+}$ is a positive effector of the ATP-producing machinery, upregulating enzymatic activities, increasing ATP-output so as to meet changing cellular demands. Many other enzymatic processes in the MK are likewise affected by calcium, giving the impression of Ca$^{2+}$ being a global positive effector of MK function. This, however, is only true at “normal” calcium concentrations, as the Ca$^{2+}$ levels observed during certain pathologies, indeed have a negative impact on MK function [63].

As mentioned earlier, the Mitochondria Permeability Transition Pore can be triggered by pathologically high levels of mitochondrial calcium. The release of apoptogenic factors from the MK is the defining hallmark of intrinsic pathway of apoptosis, and the permeabilisation of the MK membrane is a requirement, though it is possible it may be accomplished by means other than the action of the MPTP [10;12;60;64;197].

Calcium has been proposed to directly affect MK membrane structure, both as part of normal MK function and regulation and at high levels inducing dysfunction. It has been suggested that calcium may be able to release membrane bound Cyt-c without activating MPT, by affecting MK fission.
events; ectopic expression of the p20 fragment of BAP31 (a protein that binds to Bcl-2 and Bcl-xl) causes MK translocation of DLP1, resulting in fragmentation and Cyt-c release. The signal for MK recruitment of DLP1 is likely Ca$^{2+}$, since depletion or inhibition of calcium uptake attenuates the fragmentation [57].

Fig.4.5 Overview of mitochondria and ER, structure and interaction. Three specific micro-domains are shown (dotted boxes). 1) Ca$^{2+}$ signalling micro-domain. ER and mitochondria calcium signalling is accomplished due to the close proximity of the two organelles 2) "intrinsic" apoptotic mechanism. The boxed area shows some of the components of the apoptotic pathway associated with mitochondria. VDAC, ANT and Cyp-D assemble into the MPT-pore after an apoptotic signal, releasing Cyt-c and other apoptogenic factors into the cytoplasm. These are activated and assemble into the apoptosome. 3) Metabolic micro-domain and path of ATP. This micro-domain shows an overview of and location of metabolic components, including the electron transport chain, TCA cycle in the mitochondria and glycolysis in the cytosol [22].

5. Modulators of mitochondrial function

Oxidative phosphorylation (OxPhos) comprises the main source for generating ATP in aerobic organisms. OxPhos is comprised by an Electron Transport Chain, comprised of a series of linked redox enzyme complexes. The ETC consists of NADH coenzyme Q reductase, cytochrome bc$_1$ complex, cytochrome c oxidase (respectively Complex I, III and IV), Succinate dehydrogenase (complex II) and the ETC associated ATP-syntase (complex V). According to Mitchell’s chemiosmotic theory, the transport of electrons along the ETC intermediaries generates a proton gradient across the inner mitochondrial membrane, which is utilized by the ATP synthase complex to generate ATP. As the primary means of cellular energy production, the function and regulation of the OxPhos is extremely complex, with a number of intrinsic and extrinsic mechanisms controlling the system[22;27;76].
Inhibition of OxPhos can in principle be achieved by one of two primary methods: a) inhibition of the enzyme complexes and other constituents of the electron transport chain or the ATP synthethase, or b) by elimination of the proton gradient required by complex V to generate ATP [85].

Classical uncouplers of the electron gradient include carbonylcyanide-\textit{m}-chlorophenylhydrazone (CCCP) and carbonylcyanide-\textit{p}-trifluoromethoxyphenylhydrazone (FCCCP) [76]. An example of an inhibitor of the electron transport chain is sodium azide (NaN\textsubscript{3}), an inhibitor that binds to the heme group of cytochrome \textit{c} oxidase[88].

![Mitochondrial Electron Transport Chain](image)

**fig.5.1** An overview of the mitochondria and the enzyme complexes of the mitochondrial electron transport chain (ETC) and the associated ATP-synthase. The ETC is located on the inner mitochondrial membrane translocating protons from the matrix to the inner membrane space, establishing the proton gradient utilized by ATP-synthase to generate ATP.

5.1. Rotenone

Rotenone is a member of the flavonoid family of compounds and the common name for a broad spectrum insectizide. The correct IUPAC name of rotenone is (2R,6aS,12aS)-1,2,6,6a,12,12a-hexahydro-2-isopropenyl-8,9-dimethoxychromeno[3,4-b]- furo(2,3-h)chromen-6-one. It is a very
effective inhibitor of electron transfer from the Fe-S center of complex I of the ETC to Ubiquinone, acting at a domain common for hydrophobic complex I inhibitors, a large membrane located pocket of the complex. Rotenone inhibition of complex I enhances electron leakage from the ETC and increase formation of SO and hydrogen peroxide, and can therefore negatively affect both ATP production and the oxidative status of the cell [41].

5.2. **Antimycin A**

Antimycin a is a powerful fungicide produced by the *Streptomyces* bacteria. It binds between Cyt b and Cyt c of the ETC, possibly near the Qi site of complex III, disrupting the transfer of electrons from semiquionone to ubiquinone during the Q cycle. When electron transfer is hindered due to binding of antimycin a, complex III or cytochrome c oxidoreductase, may leak electrons increasing the generation of ROS [9;32;82;180].

In addition to its ETC inhibitory function, antimycin a can function as an inhibitor of the anti-apoptotic Bcl-2 family members, Bcl-2 and Bcl-XL. Antimycin is thought to suppress Bcl-XL by mimicking the pro-apoptotic Bcl-2 family member, Bak. By targeting the same binding-regions of anti-apoptotic Bcl-2’s normally targeted by Bak, antimycin may disrupt anti-apoptotic effects of these proteins. Inhibition of anti-apoptotic Bcl-2 proteins has been shown to enhance apoptosis in tumor cells overexpressing these[30;82].

Incubation with antimycin inhibits ATP-production and has been used as a model for chemically induced anoxia. Harriman et al used antimycin to study the effects of ATP-depletion on ER calcium release [68]. Pre-incubation with thapsigargin, an inhibitor of SERCA, inducing ER calcium release prevented antimycin a induced cytosolic increase in calcium. Pre-incubation with antimycin a prevented the Thapsigargin induced ER calcium release. This shows that antimycin a dependent ATP-depletion can cause ER calcium release. This increase in cytosolic calcium was large enough to activate calpains and promote cell-death [68].

5.3. **Sodium Azide**

Sodium azide or NaN₃ is a highly toxic compound synthesised from the reaction of sodium amide with nitrous oxide. It is comparable in toxic effect to cyanide salts, binding to heme groups of various proteins. Sodium azides toxic effect on respiratory enzymes is thought to be limited to cytochrome oxidase. Its primary method of toxicity in mitochondria is due to its rapid binding to and acting as an inhibitor of the cytochrome oxidase complex, complex IV of the ETC. NaN₃ is known to bind reversibly to the binuclear ETC complexes heme-cofactors, preventing transferral of electrons from reduced cytochrome c to molecular oxygen[187].
Sodium azide has been shown to have different effects on the cytochrome oxidase complex, depending on whether exposure is chronic or acute. While these differences are still being investigated, chronic sodium azide exposure is thought to lead to an irreversible inhibition of cytochrome oxidase function, possibly due to a chelating effect of the toxicant. Acute exposure is usually reversible. Sodium azide has been in use for 50 years as a mitochondria inhibitor and often used to mimic the effects of oxygen depletion of mitochondria [88].

5.4. Oligomycin

Oligomycin belong to a group of compounds called macrolides, synthesized by *streptomyces* bacteria and used as an antibiotic. Oligomycin is a well-known inhibitor of the F0F1 ATP-synthase complex of the oxidative phosphorylation pathway. It is presumed to effect its toxic function by binding to the F0 subunits 9 and 6 that contribute to the proton channel of the ATP-synthase complex. The ATP-synthase utilizes the proton gradient established by the action of the ETC to phosphorylate ADP generating ATP. Binding of oligomycin inhibits proton translocation through the complex, that otherwise would drive ATP formation. ATP degradation by reverse action of the ATP-synthase is likewise inhibited [180].

Disruption of the normal proton release through the ATP-synthase complex, may hyperpolarize the mitochondria, which would favour an increased generation of ROS. Generation of ROS by oligomycin may not be ubiquitous in all model-systems, as there have been observations where an increase in ROS has not been observed [42].

Inhibitors of the ETC (such as rotenone) and inhibitors of the ATP-synthase complex can both reduce ATP levels. A significant difference in toxicity may be from the difference in ROS generation.
5.5. CCCP

![Chemical structure of CCCP](image)

Carbonyl cyanide m-chlorophenylhydrazone, or CCCP is often cited as a prototypical uncoupler of ETC and inhibitor of oxidative phosphorylation. It functions by eliminating the proton gradient established by the normal activity of the ETC, through translocation of the negatively charged uncoupler out of the mitochondrion and diffusion of the protonated molecule back into the matrix. CCCP carries a negative charge delocalized over a spread of ten atoms in its ionized form, resulting in a weak electrical field surrounding the anion form. This unusual property is what allows CCCP to shuttle protons across the non-polar environment of phospholipid membranes [76].

The activity of the ATP-synthase, ETC complex V, is effectively inhibited due to its requirement of the proton gradient normally established by complex I-IV. Paradoxically, the activity of the ETC components upstream in oxidative phosphorylation of the ATP synthase complex may be enhanced by addition of CCCP. Accordingly it has seen use in studies of uncoupler stimulated mitochondrial respiration [76;85].

CCCP has been linked to production of ROS and induce superoxide dismutase. Incubation with CCCP may affect mitochondria a number of ways, including disrupting ultrastructure and functions other than oxidative phosphorylation, in addition to promoting cell death via apoptosis [76].

5.6. Ionomycin

Ionomycin is a calcium ionophore derived from the bacteria *Streptomyces conglobatus*. It enhances calcium transport across cell membranes, raising cytosolic calcium levels, thereby affecting and disrupting normal calcium-regulated functions. It has been used in clinical studies of the immune system because of its ability to stimulate intracellular cytokine production.[77]

Ionomycin affects all cellular membranes and has been shown to enhance the permeability of Ca$^{2+}$ in cellular organelles including sarcoplasmic reticulum, liposomes and mitochondria. It is commonly used in studies of Ca$^{2+}$ transport over biological membranes and calcium signalling mechanisms, due to its high specificity for divalent over monovalent cations. Though Ionomycin is not entirely specific to Calcium it shows a higher affinity for this ion than for other cations such as Mg$^{2+}$ [56].

Ionomycin has been known to cause cell death by necrotic and possibly apoptotic pathways, through calcium mediated mechanisms[56].
5.7. Thapsigargin

Thapsigargin was originally isolated from the Mediterranean plant *Thapsia garganica* L. (*Linnaeus*). It belongs to a group of naturally occurring guaianolides also found in other members of the genus *Thapsia*. The high lipid solubility accounts for its excellent penetration of cell membranes. Thapsigargin is a commonly used inhibitor of the sarco-endoplasmic reticulum Ca\(^{2+}\)-ATPases (SERCA) in mammalian cells. Its specificity and high potency makes it an important tool in studies of cellular calcium signalling and the function of calcium stores [166].

Agonist induced release of calcium from endoplasmic reticulum stores is considered central in maintaining cellular calcium homeostasis and in a number of signalling pathways. This signalling mechanism is dependent on the replenishing of ER stores by SERCA. Inhibition of SERCA prevents the pumps from counterbalancing the loss of calcium through active release or passive leaking, leading to a rise in cytosolic levels of calcium. Thapsigargin mediated calcium release is therefore indirect, relying upon inhibition of thapsigargin-sensitive uptake pumps and another source for the release of calcium from the stores[165;190].

Thapsigargin inhibition of SERCA generally occurs in the nanomolar range, but a wide range of effective concentrations have been reported for different isotypes. It has a very high affinity for SERCA1 isoform of the calcium pump, binding stoichometrically allowing for titration of the enzyme. The high affinity of thapsigargin for SERCA, results in a practically irreversible binding, persisting after dilution or removal of excess inhibitor[165;166].

5.8. Ruthenium Red

Ruthenium Red (RR) is a ruthenium containing polycationic dye. It is known to interact with and inhibit a range of calcium binding proteins, including Ryanodine Receptors, Sarcoplasmic reticulum Ca\(^{2+}\)-ATPase and calcium binding to calsequestrin [151]. Ruthenium Red is a specific non-competitive inhibitor of the mitochondrial calcium uniporter, making it a useful tool in investigating calcium dynamics of mitochondria [10]. The active component of ruthenium Red is presumed to be Ru 360 [12].

Exposure to RR may cause mitochondrial calcium levels to fall as the MCU is the main source of uptake and replenishing of calcium in the mitochondrial matrix. Calcium will be lost from mitochondria through leaking and during the normal functions. As the dynamic mitochondria are...
very reliant on calcium as a mediator and signalling molecule, inhibition of the MCU may indirectly affect numerous functions including that of the OxPhos machinery[10;90;91;97].

In addition to this complication, in whole cell model systems RR may have a dual or graded effect. Griffiths showed in rat cardiomyocytes that 5µM RR affects the sarcoplasmic reticulum releasing calcium from the stores, increasing cytosolic calcium levels sufficient to affect contraction. At higher levels (10-25µM), RR also targeted the MCU [61]. Care must therefore be taken when interpreting experiments using RR.

5.9. Cyclosporin A

Cyclosporin A (CsA) is an 11 amino acid cyclic non-ribosomal peptide produced by the fungus Tolypocladium inflatum Gams. Its primary medical use is as an immunosuppressant during organ transplant.

It is commonly used in studies of mitochondria mediated apoptosis, due to its ability to inhibit MPT pore opening. The specific mechanism responsible for CsA inhibition of MPT is not known, partially due to the uncertainty regarding MPT pore composition [150]. Mounting evidence suggests that the MPT pore may consist of elements including hexokinase, voltage dependent anion channel (VDAC), creatine kinase, the adenine nucleotide translocator (ANT) and cyclophilin-D (CyP-D)[69;81]. The two most commonly used inhibitors of MPT pore opening are Bongkrekic Acid and CsA. Bongkrekic acid directly lock ANT in its m-state and thereby preventing the MPT pore from forming. CsA enters the mitochondrial matrix and binds to the active site of CyP-D, presumably forming a CyP-D-CsA complex preventing pore opening. CsA blocking of the MPT-pore has been shown to increase the calcium capacity of mitochondria, presumably by blocking of a normal transient opening of the pore in its low-conductance state[52;60;193].
5.10. Calpain Inhibitor I and II

The search for an inhibitor of the calcium regulated apoptotic protease-family of Calpains, have been ongoing since their initial discovery more than 30 years ago. General protease inhibitors including EDTA and EGTA were among the first to be identified as inhibitors. A number of calpain inhibitors were identified and isolated from *streptomyces* species, including Leupeptin, Strepin P-1 and Antipain. These compounds inhibit calpain by reacting with the active-site cysteines thiol group. This is a feature common for many of the Calpain inhibitors described in the literature, as they are often active-site directed peptide inhibitors. Most are not specific for Calpains, but show inhibition of other cysteine proteases such as papain and cathepsin B or L[43].

Research into calpain inhibitors led to the discovery of calpeptin (Z-Leu-Nle-H). The compound is cell permeable and inhibits human m-class calpains, but also inhibits other cysteine proteases. Attempts to improve on calpeptin through structure activity analyses, led to the discovery of calpain inhibitors I, II and III. The common feature of these peptide-aldehyde inhibitors are the “capping” of their N-terminal with a lipophilic substituent[43]. Commonly used calpain inhibitors, Calpain inhibitor I (N-Acetyl-Leu-Leu-Norleucine-Aldehyde) and II (N-Acetyl-Leu-Leu-Normethionine-aldehyde) are both cell permeable active-site directed peptide-aldehyde inhibitors [43].

A wide range of other inhibitors have been discovered, some active-site targeted, others targeted at the calcium binding site [144].
6. Cadmium

Cadmium is an industrial and environmental pollutant, with no known normal biological function. The major sources of cadmium exposure are through industrial pollution and waste, from production of batteries, paints and mining, or through inhalation of tobacco smoke. It is recognized as an environmental toxicant, with genotoxic effects and has been the subject of numerous studies[26;138;192].

Cadmium is a multi-target toxicant for a number of cell types and tissues. The etiology and pathology of cadmium toxicity depends on and varies highly at different concentrations and tissue types[7;49;133;139]. Acute and chronic exposures to cadmium have been implicated in several pathologies, including cardiac, renal and hepatic toxicity. Cadmium accumulates in the liver and kidneys, with resulting lesions leading to organ dysfunction.

A review by Il’yasova and Schwartz specifically discusses cadmium’s role in renal cancer, referencing several large studies finding significant correlation between cadmium exposure and renal dysfunctions [72].

In vivo renal damage is primarily due to reabsorption defects in the proximal tubuli, leading to proteinuria, and later to aminoaciduria, glucosiduria and phosphatiduria. The proximal tubuli have the largest cadmium uptake of renal tissues and research has traditionally been focused on describing cadmium toxicity in this part of the kidney. The distal tubuli however, is of significance in the regulation of calcium homeostasis. Cadmium induced defects in these tissues may therefore have a large secondary toxic effect[53;100;109;160].

Cadmium is thought to induce cell death both through necrosis and/or apoptotic pathways, two forms of programmed cell death (PCD)[9;62;91;97]. It is however not uncommon for toxicants to be able to induce cell death by both mechanisms [26;133]. Apoptosis is normally observed at low levels of exposure, whereas necrosis sets in at higher concentrations. It has therefore been hypothesized that the two mechanisms, an ordered ATP-dependent breakdown versus the disordered cell destruction of necrosis, represents the two extremes of cadmium induced cell death. The term “necrapoptosis” has sometimes been used to describe this type of cell death with a common origin, but different outcome [94;95]. Apoptosis is dependent on the maintenance of an adequate metabolism, due to its ATP-requirement. High levels of cadmium might induce cellular damage in excess of what can be tolerated, to allow an adequate ATP-production, and a disordered cellular breakdown occurs. The concentrations determining the mechanism of cell death is tissue and celltype specific[62;122;191]. There may also be differences in the toxic mechanism of in vivo and in vitro cadmium exposure; cadmium in vivo is usually bound to metallothionins, rather than found free as the divalent metal ion. The exposure pathways affect the pathophysiology of cadmium toxicology[7;100].

6.1. Cadmium Toxicity

Cadmium asserts multiple toxic effects and has been classified as a human carcinogen. Cellular and molecular mechanisms known to be involved in cadmium’s genotoxicity include, activation of proto-oncogenes, inactivation of tumor-suppressor genes, disruption of cell adhesion in addition to DNA damage and disruption of DNA-repair pathways[20;48;73;173;191].
Despite the heavy metals ability to generate reactive oxygen species (ROS), cadmium isn’t directly involved in any redox reactions and direct generation of ROS, as it does not catalyze any Fenton-type reactions. Indirect mechanisms are therefore proposed as the means of at least part of its toxic effects, and specifically any genotoxic effects it may have. The major component of its genotoxicity is presumed to be the ability to affect cellular signalling transduction pathways and gene expression[54;139;177].

Cadmium has been shown to affect cell cycle in a cadmium sensitized CHO cell line (CHO K1). The cell cycle was arrested in the G2/M phase after 8 hours when exposed to 1µM Cd, whereas cells exposed to 4µM stopped at the S phase after 8 hours. Experiments done by Yang et al. Showed that cell cycle arrest was global and not directly linked to any specific phase (G1,S,G2,M)[185]. Other genotoxic effects are caused by interference with enzymes in the cellular antioxidant system, by replacing essential metals, such as Cu from prosthetic groups, or binding to sulfhydryl groups of GSH. Cadmium has been known to affect gene induction by indirect means both through ROS generation, but primarily through modulation of cellular signal transduction pathways by enhancing protein phosphorylation and activation of transcription and translation factors. Inhibition of components of the DNA repair mechanisms is a mechanism whereby cadmium can enhance the toxicity of other toxic compounds[55;173].

Part of cadmiums toxicity is due to its ability to act as a dithiol chelating reagent, having a high affinity for the free electronpair of SH groups of aminoacids such as Cysteine, but also binding to Histidine, Aspartate and Glutamate. Another other important trait of Cd²⁺ is the ability to act as a calcium agonist. The ability of Cd²⁺ for ionic “mimicry” of Ca²⁺ is probably due to the closeness of the crystal radii, and similar charge of the two metal ions. Cadmium has thus been implicated in apoptosis signalling by disruption of calcium signalling and cellular calcium homeostasis [26].

In the kidney cell line A6 isolated from the distal tubuli of xenopus laevis, Cd²⁺ may initiate release of calcium from the intracellular stores of the endoplasmic reticulum, by binding to a plasma membrane located calcium sensitive receptor (CSR)[51]. The extracellular calcium sensing receptor belongs to the family of G-protein receptors, activation of which first results in generation of an IP₃ mediated release of calcium from ER and as a consequence of this, an activation and opening of Store Operated calcium channels in the plasma membrane, resulting in uptake of calcium and Cd²⁺ from the external medium. The resultant rise in cytosolic calcium levels can activate apoptotic and/or necrotic pathways[128;154;168]. A6 cells are highly polarized when at confluent growth and react differently to cadmium when exposed at the basolateral or apical sides [51].

The exact etiology of cadmium induced apoptosis is often dependent on the heavy metals ability to affect and activate a number of proteases involved in regulation and execution of PCD, including various caspases and calpains. Activation of these apoptogenic proteases may be under direct regulation of calcium, such as the calpains, with ionic mimicry or cadmium induced cellular increases in calcium, as potential explanations for apoptosis induction[96;122]. Other proteases may be indirectly activated by other proteins affected by cadmium, such as Bcl-2 members. Activation of proapoptotic Bcl-2 by cadmium could potentiate other effects of the heavy metal, while inhibition of antiapoptotic Bc-2 could activate proteases downstream in the apoptosis pathways. Elucidation of a mechanism for cadmium mediated toxicity may become difficult as it appears cadmium can sometimes activate apoptotic pathways dependent as well as independent of caspases and Calpains [89;109;191].
As an additional complication, cadmium has at times been suggested to activate apoptosis through both of the two major mechanisms, mitochondria- or deathreceptor pathways. The two pathways may be linked by the activity of Bcl-2 members, calpains and caspases, suggesting why cadmium toxicity is a complex affair.

Cadmium induced apoptosis has been associated with the production of reactive oxygen species (ROS) in several studies. ROS include the superoxide, hydroxyl radical, hydrogen peroxide and other very reactive species, by-products of MK oxidative phosphorylation. Cadmium has been seen to increase the cellular level of ROS and its cytotoxicity has been associated with this phenomenon. Increase in ROS may lead to a reduction in MK membrane potential $\Delta \psi_m$, thereby increasing membrane permeability, releasing apoptogenic factors into the cytosol. ROS may also act as signal molecules of the death receptor pathway of apoptosis[9;122;139].

Cadmium has often shown to be implicated in disruption of electron flow through the electron transfer chain of the mitochondrion. The exact mechanism is still unclear, but it seems to be at locations on the ETC, at or near complexes I and III. Wang et al report that Cd$^{2+}$ only increases ROS production at complex III, binding between semiubiquinone and cytochrome Q$_0$[177]. Belyaeva et al. report that Cd$^{2+}$ toxicity of isolated mitochondria, shows a substantial dependence on mitochondrial energization and function, indicating that this organel may have a large role to play in cadmium toxicity of whole cells. They report that Cd$^{2+}$ chelation of thiol s of ETC components is the primary mechanism responsible for inhibition of ETC activity, acting at complex III, close to the Q$_0$ site and near heme b$_1$, presumably by oxidation of cytochrome b[10].

Cadmium has been implicated in the disruption of the mitochondrial membrane potential, shown to be able to induce the opening of the permeability transition pore of mitochondria, possibly directly by interaction with MPT components, thus inhibiting substrate oxidation and having a direct stimulatory effect on proton leak from mitochondria[90;91]. Prolonged opening of this pore results in activation of an apoptotic signal, the mitochondrial permeability transition mechanism, with release of cytochrome c and activation of caspase 9. Cd$^{2+}$ inhibition of ETC components, acting at complexes I and III, or indirectly by disrupting redox status by depletion of antioxidants such as GSH, generate ROS, which can potentiate the opening of the MPT pore [9]. While the composition of the MPT pore is still unresolved, it has been shown that cadmium can enter the mitochondrial matrix through the Ca$^{2+}$ uniporter, binding to thiol residues of the ANT, a putative member of the MPT-pore complex [97].

Belyaeva et al propose a slightly different scenario responsible for cadmium induction of MPT. They and others have suggested that the MPT-pore contains two cation sensitive (Me$^{2+}$) sites, one externally where binding of a cation including Ca$^{2+}$, decreases the probability of pore opening. Binding of Ca$^{2+}$ to the second internal site increases the probability of pore opening. They suggest that Complex I and/or complex III of the ETC may contain these Me$^{2+}$-binding loci[10].

Cadmium is thought to bind to both Me$^{2+}$ sites, resulting in an increased probability of an MPT event, even with binding to the external site. Binding to the internal Me$^{2+}$ site results in a CsA sensitive MPT, where binding to the external site is presumably CsA insensitive. Cadmium induction of the MPT pore is also strongly affected by modulators of ETC function. Rotenone may inhibit pore formation, presumably due to the sensitivity of the pore to electron-flow through
complex I. In certain model systems, rotenone has been demonstrated to be an even stronger inhibitor of MPT than CsA [11].

MPT pore opening and disruption of ETC strongly affect the respiration rate of mitochondria. The apoptotic cell death pathways activated by cadmium are dependent on continued ATP production, with loss of ATP potentially leading to necrosis. This may partly explain how cadmium toxicity is heavily determined by respiratory status and mitochondrial function, with low cadmium concentrations potentially causing cell death by apoptotic pathways, and at high levels by necrosis.

Inhibition of caspase-8 has been shown to reduce the cell death from cadmium exposure, suggesting that cadmium mediated apoptosis may occur by both of the two primary mechanisms, the extrinsic (or death receptor mediated) and intrinsic pathways. Cadmium binding to proteins involved in regulating apoptosis, such as calpains and members of the Bcl-2 family, can presumably determine whether it continues in an orderly fashion or by disruption of normal cell death pathways, ends in necrosis [83;96;122].

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**Fig.6.1** A general scheme for cellular Cadmium toxicity. Cadmium is a multi-target toxicant affecting various cellular subsystems including DNA repair mechanisms or antioxidant defences by depleting stores of antioxidants and increasing ROS production. Cadmium has direct and indirect effects that can initiate different cell-death pathways, either apoptosis or necrosis. It may affect gene expression, resulting in cell cycle arrest, inhibiting proliferation and is also a potent carcinogen causing DNA damage by direct or indirect means [20].

The above scheme was proposed by Bertin and Averbeck as a generalized overview of cellular consequences of cadmium toxicity. The scheme summarizes most of the relevant targets as well indirect effects of cadmium [20].
7. The biochemical basis of the MTT viability assay

Tetrazolium salts are a group of heterocyclic compounds often forming highly coloured formazans upon reduction. The first tetrazolium salts were synthesized in 1894. The prototypical tetrazolium salt Triphenyl Tetrazolium Chloride (TTC), also commonly used as a redox indicator, has been modified over the years by addition of nitro-, iodo- and methoxygroups to the phenylrings. A review by Altman gives a good overview of the chemical properties of different classes of tetrazolium salts, as well as the historical basis for research of and with this class of chemicals [3].

\[
\text{TTC}
\]

The modifications to the parent compound affect solubility and numerous other parameters, resulting in a group of compounds with a range of biochemical properties, usable in a diverse set of biological measurement systems. The bioreduction of tetrazolium into a coloured product with wavelength absorption maxima different to the parent compound has been utilized in both histological and cell-based assays [18].

\[
\text{Measuring of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium }
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Bromide (MTT), is a commonly used method in determining cell proliferation and in evaluating toxicity of a wide variety of compounds. MTT has routinely been used in assaying cell growth/inhibition and cell viability since Mossmans detailing of its application in a rapid colorimetric assay in 1983[116]. It was found that, under certain conditions, the amount of formazan formed by living cells correlated with the cell density: an increase in cell density from growing cells, relative to a starting density, would result in a proportionate increase in MTT reduction. Likewise it was thought that a decrease in activity could be ascribed to a fall in cell density from dying cells. This hypothesis would show to be true in some experimental models, but that many parameters were able to alter the activity of the assay without proportionately affecting cell count [15;74;75;125].

Originally developed as an alternative to other techniques such as those based on radioactive labelling and uptake of H<sup>3</sup>-Thymidine. The assay was developed for use with 96-well microculture plates, one of a number of microculture tetrazolium assay (MTA). The solubilised formazan crystals
formed after MTT reduction is read using a standard spectrophotometric microplate reader, allowing a high output of data[2]. It was considered for use at the USA National Cancer Institute in screening programs of anti-cancer drugs. The programme opted to test more than 10,000 new compounds per year, in terms of cytotoxic and/or cell growth inhibitory effects. Advantages of the assay were the high output, the speed of measurements and relatively low costs and, important compared to radioactive assays, it was very safe to use and easy to dispose of waste[115;147;172]. Other MTA’s were since considered for use in screening for AIDS-antiviral activity. Unfortunately the use of the assay has had to be reconsidered as it has become clear that changes in activity may not always directly be correlated to the number of cells, and despite its continuing application, the actual mechanism of MTT reduction is poorly understood[19;102;110;167].

Since measurements of viability and cell death is vital to a wide range of fields including research in toxicology, viability and cell death assays have received a lot of attention. The MTT assay, because of its many advantages has been assessed and subject of a lot of work. Because of its problems and possible flaws, a lot of consideration is required when using the assay in its common form, measuring viability[84;171;181;196].

Early studies of the mechanism using respiratory chain inhibitors suggested that Succinate-dependent MTT reduction in Rat liver homogenates occurred at two primary sites of the mitochondrial electron transport chain; at Coenzyme Q, and cytochrome c [102]. This was originally taken as evidence that MTT is reduced in the mitochondria of living viable cells, even though definite proof of an association of MTT reduction with mitochondria was lacking. The observation that other non-mitochondrial dehydrogenases and flavin oxidases can reduce MTT has also put to question the exclusive association of MTT reduction with the organelle. The exact cellular site, enzymes and substrates involved in MTT reduction has thus not been satisfactorily determined[13;14;113].

Originally thought to be a direct measure of cell growth, a review by Marshall et al in 1995 examining the assay, suggested its use as a measure of pyridine nucleotide redox status. The current understanding of the assay suggests its use only as a measure of cell ‘viability’. This term is of course vague and may be to restrictive a term in certain experimental models. With continual changes in definitions because of the accumulating knowledge and better understanding of the molecular mechanism of MTT-reduction, interpretations of earlier experiments utilizing the assay, may have to be re-evaluated [110].

Berridge et al. argue in their review of tetrazolium assays that the integrated metabolic signal read by MTT-reduction with a particular cell type, under defined growth conditions has consistently shown to be a robust measure of cell viability. Changes in growth conditions and exposure to cytotoxic or cytostatic compounds may however alter the metabolic signal, and give information about these compounds as regards their cellular effects. Continued study of this commonly used assay is therefore still very important [18].

The reaction has been studied in numerous different species tissues and cell lines, including CHO, human T-lymphoblastic Jurkat cells, nb2 rat lymphoma, b12, rat brain cells and kidney cell lines[71;115;147;186]. The assay is usually applied as a cell based assay, but the reaction has also been studied in cellular fractions and in isolated mitochondria[35;102]. Discrepancies in the results and difficulties in finding a unifying theory may thus be partly due to species or tissue specific
differences in the handling of MTT, as well as artefacts of the experimental methods used in examining the reaction.

The parameters relevant for understanding MTT bio-reduction include; the site of reduction (intra- or extracellular, membrane located, in the plasma or tied to a specific organelle), enzyme systems responsible for the conversion, relevant cofactors and substrates involved, deposition and exocytose of the formazan crystals. Understanding these parameters will hopefully clarify what the assay actually measures [110;172].

7.1. Cellular uptake of MTT

Berridge et al. examines several different tetrazolium dyes were for their ability to be reduced at intra or extracellular sites. The reduction of four tetrazolium dyes MTT, XTT (Sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt), MTS (5-[3-(carboxymethoxy)phenyl]-3-(4,5-dimethyl-2thiazolyl)-2-(4-solfophenyl)-2H-tetrazolium inner salt) and WST-1 (Sodium 5-(2,4-disulfophenyl)-2-(4-iodophenyl)-3-(4-nitrophenyl)-2H-tetrazolium inner salt), was compared to cytochrome c reduction in the presence or absence of superoxide dismutase (SOD) and an electron carrier mPMS. In the absence of mPMS cellular reduction of WST-1, XTT and cyt-c was minimal, while MTS gave a weak signal and MTT was strongly reduced. With the addition of mPMS, WST-1, MTS and XTT were strongly reduced. These results suggested an extracellular reduction of WST-1 and XTT, these dyes being unable to enter the cell due to their net negative charge, and MTT and to a lesser degree MTS being reduced at intracellular locations [19].

MTT is known to be impermeable to synthetic lipid membranes, but is able to rapidly cross the plasma membrane of living cells. The plasma membrane potential has thus been proposed to be the major cellular determinant in MTT uptake, or conversely uptake could be through active transport through endocytosis, as suggested by liu et al [102].

7.2. Substrate dependency of MTT reduction

The MTT assay was initially studied using succinate as a substrate, and the correlation of activity with changes in succinate concentrations was taken as evidence for a connection to the mitochondria and electron transport chain activity. It has since been established that NAD(P)H may be an equal or better substrate for MTT reduction [19].

In their review from 1997, Liu et al compared the activity of the MTT assay when incubated with a combination of Succinate, Malate, Glutamate or all three substances in the presence or absence of ADP. They found that while succinate was an adequate substrate for MTT reduction, the NADH linked substrates, malate and glutamate where capable of enhancing MTT reduction. The presence of ADP enhanced MTT reduction by >60% (state 3 respiration) compared to its absence (state 4 respiration). The observation that MTT reduction can be linked to NADH suggests that enzymes separate from the electron transport chain could be involved [102].
7.3. Cellular localization of MTT reduction

One important reason that the exact cellular localisation of MTT reduction is still unclear has been the long absence of good methods for determining penetration and distribution of tetrazolium salts in the cell interior of living whole cell systems. Accordingly many experiments examining the reaction have been done with cell free solutions of a reducing species and tetrazolium salts. It is unclear to what degree these experiments can be extrapolated to intact cells [102].

Bernas et al. described a series of experiments using fluorescence and backscattered light confocal spectroscopy to determine formazan deposition in or around living cells in vitro. The experiments showed that MTT is readily taken up by HepG2 cells, a human hepatoma cell line, and that the plasma membrane does not pose any significant hindrance for MTT uptake. MTT-formazan was accumulated as oval deposits around the plasma membrane and at several intracellular sites. The experiments showed that MTT reduction and accumulation of formazan crystals, did not cause any observable damage to the plasma membrane or compromise the cells ability to perform exocytosis (of the formazan crystals), indicating that, at least short-term, MTT reduction did not severely compromise normal cell functions. It was concluded that mitochondria probably did not present the primary site of MTT reduction in vitro and presumably not in vivo, which is still a controversial view [13].

Liu et al described in 1997 a putative mechanism for the cellular reduction of MTT in B12 cells, a cell line made from nitrosoethyurea-induced rat brain tumours similar to neural precursor cells. MTT was shown to be deposited in vesicles at perinuclear sites in the cells. Some of these vesicles were determined to be lysosomes/endosomes, reduction was determined to be confined to these sites by a N-ethylmaleimide (NEM)-sensitive flavin oxidase. MTT formazan would then be transported to the cell surface through exocytosis. With these observations and other data, Liu et al. proposed the above mechanism for MTT reduction in B12 cells and suggested that mitochondria are an unlikely candidate as the primary site for this [102].

7.4. Cellular MTT-reduction and Mitochondria

Contrasting these findings are the observations of many others that mitochondrial function directly affects MTT-reduction. If the reduction of MTT is largely dependent on dehydrogenase or reducing enzymes of the electron transport chain of the mitochondria, as it has been supposited, the reaction could reasonably be expected to be affected by inhibitors of OxPhos and effectors of mitochondrial function [148].

Jabbar et al. Using COR-L23 cells observed a reduction in cell count with interferon treatment, but a size increase in individual surviving cells and a 40% increase in mitochondrial activity assessed by uptake of the fluorescent dye 123Rhodamine. They observed that the theoretical reduction in MTT-formazan production from the reduced cell count, was partially compensated for, presumably by the mitochondrial activation of the surviving cells [74].

Paggliacci et al found that formazan production increased, despite a decrease in cell count of tumour cell lines exposed to the anti-carcinogenic isoflavone genistein. They attributed this effect to an increased mitochondrial activity of cells which had been blocked at the G2/M transition. It was of possible concern that the genistein-dependent changes in MK function and/or number, and its
derived effect on MTT reduction could represent a serious bias when analyzing the effects of this or similar drugs[125].

Sobbotka and Berger analyzed a modified MTT assay, for use in assessment of anti-proliferative effects of neoplastic agents [155]. They compared the efficacy of several anti-proliferative drugs as determined by an MTT assay, with conventional cell counting using a Coulter Counter and determining cell death with a Tryphan Blue Exclusion assay. They found a high correlation between cell count and MTT-formazan production, in drug-free experiments. After continuous 72 hour incubation with the antineoplastic drugs, vinblastine, HECNU and HPC, they found that the MTT assay slightly or considerably in the case of HECNU, underestimated the anti-proliferative properties of the drugs. Sobotka and Berger suggested that these differences could partly be attributed to changes in mitochondrial function. A 2.4 fold change in cell volume observed at the MTT IC50 was also proposed as a possible explanation for part of the change in correlation. Sobbotka and Berger also expressed a concern that a part of the deficiency in correlation could be due to the MTT assays sensitivity to changes in glucose concentration of the incubation medium, a potential problem caused by the experimental setup [155].

Bernas and Dobrucki argued that mitochondria were at least partly involved in MTT reduction [13]. In a series of experiments utilizing backscattered light confocal microscopy to image the formation of MTT-formazan deposits in the human hepatoma cell line HepG2. Mitochondria were identified through the binding of fluorescent dies dependent (JC-1 and TMRE) or independent (NAO) of MK electric potential. 25-45% of the MTT-formazan formed was observed to be associated with mitochondria. The fluorescence of the mitochondrial electric potential probes, TMRE and NAO decreased rapidly, suggesting a change in electric potential upon MTT exposure of the mitochondria. Other experiments however dismissed this possibility. The monomeric form of JC-1 decreased similarly, but its aggregated form decreased by less than 30% at the onset of MTT incubation and remained at this level throughout the experiment. Bernas and Dobrucki, based on the evidence of their experiments, concluded that MTT interacted with and displaced the monomeric form of JC-1 binding to the outer surface of the inner mitochondrial membrane. The aggregate forms of JC-1 are presumably displaced from their monomeric form, in the intra-mitochondrial space, similar to other cyanine dyes in energized mitochondria. MTT does not penetrate as readily to the inner mitochondrial space, explaining the difference in MTT’s ability in rendering the two forms of the probe non-fluorescent. Backscattered light confocal microscopy revealed that MTT was not deposited at all near the nucleus. The co-localization of MTT deposits and mitochondria decreased somewhat during the exposure time of 25 minutes [13].

In a comprehensive review and detailed study of the MTT assay, Liu et al examined MTT reduction in B12 cells and compared it experiments on mitochondria isolated from purified rat brain[102]. MTT activity was compared in the presence or various inhibitors of the mitochondrial electron transport chain, including rotenone, antimycin a and oligomycin. It was found that, with exposure to ETC inhibitors MTT activity was reduced in both systems, but generally more in isolated mitochondria, when compared with the activity of whole cells. Exposure to the uncoupler FCCP increased activity in whole cells, and curiously decreased activity in isolated mitochondria. Liu et al was unable to explain this difference. These and other observations led Liu et al to several important conclusions[102]:

1) MTT reduction in whole cells was different from MTT reduction in isolated mitochondria. 2) Intracellular deposits of formazan didn’t correspond to the location of mitochondria. 3) Mitochondrial marker enzyme activity was not increased in formazan containing vesicles. 4) human
ρο cells contain no mitochondrial DNA and no functional ETC are still able to convert MTT to its formazan product. 5) referencing a former study by Nikkhah et al from 1992 where electron microscopy imaging found that MTT formazan was accumulated in non-mitochondrial vesicles[118;119]. These observations led Liu et al to caution thinking of the MTT assay as an expression of mitochondrial activity, that it is possibly reduced by NEM-sensitive flavin oxidases and therefore linked to glycolysis and NAD(P)H generated from this mechanism rather than enzymes of the electron transport chain. Liu et al considered the assay to be a better model of cellular exocytosis of formazan containing vesicles, a process that is fortunately also linked to cellular viability, and therefore not requiring all previous work with the assay to be reconsidered[102].

Despite a very comprehensive review and investigation into the assay, Liu et al was unable to unequivocally establish the mechanism of MTT uptake, reduction, localisation and exocytosis of the product. Some of the conclusions in this work may have to be considered, since observations seem to differ slightly depending on cell type, and experimental setup, suggesting that MTT reduction can have a cell specific manner of execution [102].

7.5. Reduction of MTT and intermediate electron acceptors.

In a comparison of several different classes of Tetrazolium salts, Bray et al. report that MTT reduction is unaffected by oxygen, suggesting that they do not form radical intermediates, that can react with oxygen[3].

Some Tetrazolium salts require an intermediate electron acceptor (IEA) to achieve any significant conversion to its product, and while it has been proven that the IEA menadione, may enhance MTT-formazan production, it is clearly not necessary for it to occur. Goodwin et al demonstrates that different IEAs have markedly different effects on the MTT assay. Co-enzyme Q₀ is highlighted as an IEA that added to the MTT assay allows the detection of much lower cell densities [59]. A worry in using IEA may be instabilities of the charge-transfer complex of IEA-tetrazolium salt, a problem in the case of XTT and the IEA PMS. It is not clear which intracellular pathways are responsible for the differences in IEA effects on the MTT assay [59]. Marshall et al reviews possible explanations, suggesting the facilitated non-enzymatic transfer of electrons from a reduced compound to MTT via an IEA with an appropriate redox potential. An alternative possibility could be through an enhanced transfer of electrons across membranes [110].

In summary: The current most dominant hypothesis is that MTT reduction is a complex mechanism, highly cell-type specific, dependent on experimental setup and therefore with a lesser or stronger connection to mitochondrial activity. NADH seems the best substrate, but a number of enzymes, some mitochondrial, and some non-mitochondrial are able to reduce it. MTT-reducing activity is affected by a number of substrates involved in energy metabolism including glucose and pyruvate. It can be affected by inhibitors of the mitochondrial electron transport chain, as well as inhibitors of N-ethylmaleimide (NEM)-sensitive flavin oxidases.
7.6. Alternative uses for the classic MTT assay

The MTT assay has predominantly been used as a measure of the vague quantity “viability”, often but not always correlating with the number of living cells. With the current lack of understanding of the assay, most users of the assay consider this its best application. However, other possible uses have been demonstrated.

Janjic and Wollheim demonstrated that the assay could reflect islet cell metabolism, they observed a good correlation between MTT reduction and glucose concentration of the medium. Janjic and Wollheims aim of their study was to determine its use as a rapid assay for assessing beta cell function in diabetes mellitus. Normal beta cells are able to respond rapidly and modulate glycolytic and metabolic rates as a function of extracellular glucose concentration. They found that MTT formazan production correlated with extracellular glucose concentrations and that formazan production was abolished with exposure to beta cell cytotoxic compounds, and they therefore concluded it was of possible use in assessing beta cell function [75].

Takahashi et al examined the MTT assay for use in evaluating oxidative metabolism of in vitro systems [164]. They compared the activity of MTT in astroglial and neuronal cells, when exposed to different energy sources, including glucose, malate, acetate and pyruvate. There was a marked difference in specific activity between the two cell types; while glucose was a suitable substrate in both cell types, it increased activity, relative to the control of no substrate, markedly more in astroglial cells than neurons. Conversely, lactate and pyruvate was a poor substrate for astroglial MTT reduction, unlike neurons where lactate was as good a substrate as glucose. This clear difference in substrate preference in MTT-reduction seemingly suggests the difference in substrate preference in energy production of the two neural cell types. This conclusion might not be entirely correct however, as Takahashi et al pointed out one curious observation. Lactate produces one molecule of NADH and one molecule pyruvate, through the action of LDH, and knowing the MTT assays affinity for NADH, it would presumably be a better substrate than pyruvate. In neuronal cells pyruvate showed approximately two-fold increases in MTT reducing activity as glucose at the same concentrations, a higher specific activity than lactate. So while the assay demonstrably can be used to describe aspects of redox metabolism in different cell types, the mechanism of MTT reduction and substrate dependence must be better understood before it can properly be utilized in this manner [164].

Abe and Matsuki combined the MTT assay with another common viability assay, the LDH release assay. They argued that the MTT assay could be used to measure redox status and estimation of LDH activity would measure damage to the plasma-membrane. The method devised was successful in estimating t-butyl hydroperoxide toxicity in cultured rat cortical astrocytes, with formation of MTT-formazan shown to be directly proportional to LDH activity [1]

Molinari et al. used confocal microscopy and digital analysis to estimate MTT bio-reduction of single cells. Total optical density, cell area and specific activity of MTT reduction was measured for V79 cells and cultured macrophages. They established that the production of formazan of single cells varied and was proportional to cell size. Individual cells in non-synchronous cell cultures are relatively heterogeneous in terms of cell volume. The contribution of formazan from a single cell in the culture will thus vary highly, dependent on cell size, and may result in the production of formazan not being proportional to the number of cells [114].
8. Experimental Methods

The following chapter describes the experimental procedures used in the project, with primary focus on the MTT assay and discussion of the technical implications and potential problems relevant when optimizing the assay for other experiments.


A6 is a continuous cell line derived from the kidney of the aquatic toad *Xenopus laevis*. In culture these cells form a homogenous, confluent polarized cell layer. When grown on solid support (e.g. In a NUNC-flask) they orient with the basal surface against the supporting substrate. The formation of dome- or hemicystlike growths is due to the transepithelial transport of water and ions from the apical to the basolateral side. The A6 epithelium cell line is thought to be a suitable model for the study of mammalian cells, as it shows morphological and physiological similarities with these cell types. A6 cells have additional experimental-technical benefits, such as a lower temperature optimum for growth, which allows experiments to be performed at room temperature and minimizes risks of infections and contaminations by microorganisms.

Materials:
- Cell culture: A6 cells american type culture collection (Rockville, MD. USA) Series-67
- Growth medium: 63% DMEM medium (Invitrogen), 25% MiliQ H₂O, 10% FBS (Biochrom AG), 2% Penicillin-Streptomycin (InVitrogen Corporation).
- Subculture: 0.5% Trypsin-EDTA (InVitrogen Corporation)

Procedure:
Cells are grown in 50 ml NUNC culture flasks containing 10 ml growth medium and incubated at 26°C, 5% CO₂, 100% RH. Medium is changed twice weekly, old replaced by 10 ml fresh medium. Subcultivation is done approximately once a week when cells are confluent and differentiated, determined by observation of dome or hemicyst formation. 2ml subculture medium (Trypsin-EDTA) is added to the NUNC flask and incubated at 37°C for 3-5 min. The subculture medium is removed and cells are gently loosened from the flasks by shaking. 5 ml fresh growth medium is added to wash cells of the sides of the flask. The trypsinated cells are distributed among new NUNC-flasks with 1 ml cell culture (approximately 10⁸ cells) and 9 ml fresh medium per flask.
8.2. Cell count and visual identification of cell death by tryphan blue exclusion assay.

Trypan blue is an electrically charged dye and therefore unable to pass the membranes of living viable cells. Living, intact cells will show as bright spots surrounded by the blue dye. Dead cells will lose membrane integrity and be coloured by the dye. Cell survival and the total cell count can be estimated with a microscope, by visual identification and counting of coloured (dead) and uncoloured (living) cells. The cell count is calculated as cells/ml. The cell count is first established for the known volume deposited in, and counted with the aid of the Bürker-Türke counting chamber. This cell-count is then corrected for the dilution factor of the added Trypan Dye, and multiplied to find the total number of cells pr. ml. Cell-death is calculated as the number of dead cells as a percentage of the total number of cells counted.

Equipment:

- Microscope: Leica 300
- Counting chamber: Bürker-Türke counting chamber.
- CR412 Benchtop centrifuge.

Materials:

- Cell culture: A6 cell line trypsinated from confluent cells.
- Growth medium: 63% DMEM medium (InVitrogen), 25% MiliQ H2O, 10% FBS (Biochrom-AG), 2% Penicilin-streptomycin (InVitrogen).
- Dyes: 0,5% Trypan Blue (Sigma).
- Resuspension medium: 5 mM Glucose - NaCl-Ringer (dissolved in MiliQ H2O:112,5mM NaCl; 2,5mM NaCO3; 2,5mM KCl; 5mM Glucos; 1,0mM CaCl2 • 2H2O)
- CdCl2 (Sigma) 1M Stock diluted with H2O as appropriate for individual experiments.
- Test-reagents: Thapsigargin (Sigma), Oligomycin - mixture of A, B & C (Aldrich), Antimycin A - mixture of A(1), A(2), A(3) & A(4) (Fluka), Ionomycin (Sigma), rotenone (Serva), CCCP (Carbonyl-Cyanide-m-Chlorophenylhydrazone) (Sigma), NaN3 (Fluka), Ruthenium Red (Aldrich), Cyclosporin a (Fluka/Biochemika).

Procedure:

Cells are trypsinated when at confluent growth (after 5-10 days), as described in the cell cultivation procedure. The contents of one growth flask is resuspended in 5ml of standard growth medium to de-activate any remaining Trypsin and transferred to a 10 ml Nunc-tube. The suspension of single cells is washed in 5mM Glucose - NaCl-Ringer and centrifuged 3 minutes at 2000 RPM using a CR412 Benchtop centrifuge. The cells are resuspended in 5 ml of 5 mM Glucose - NaCL-Ringer. 495 µl of the cell-suspension is transferred to eppendorf test-tubes and incubated with 5µl dilution of CdCl2 for a final test concentration of 0-400µM and incubated for 0-3 hours. After drug exposure, 1% Trypan Blue is added 1:1, for a final concentration of 0,5%. 10 µl of the sample is transferred to the Bürker-Türke counting chamber and living (white) and dead (dyed blue) cells are identified in a light-microscope and counted.
8.3. The classic MTT assay.

The assay is based on the metabolic reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). In principle the viable cell number/well is directly proportionate to the production of coloured formazan crystals, from the tetrazolium substrate, which following solubilisation can be measured spectrofotometrically ($\lambda \approx 595\text{nm}$). The MTT formazan has an absorbance maximum between 590-620 nm. The wavelength used in these experiments, was chosen due to the technical specifications of the Synergy HT microplate reader.

The assay used here was modified slightly from the assay by Mosmann, and other similar assays [116]. The original assays were designed for maximum ease of use, aiming for a high output using 96-well microtiter plates. In the original assays, cells are plated in each well of a 96-well microtiter plate and grown to a suitable cell density. The test reagents are then added to each well and usually incubated for 24 or 48 hours before the MTT is added. After a further 3 hour incubation period with MTT and test reagents, the incubation medium is removed by gentle aspiration. The formazan crystals formed upon MTT reduction is dissolved in a suitable organic solvent. The most common solvent is isopropanol acidified with HCl or with added DMSO. Isopropanol ensures that all formazan is dissolved and can be measured with a microplate-reader at 595 nm (590-620nm). Viability is then given as a percentage of MTT reducing activity of control samples[18;67;116;119;196].

The common MTT assay was modified for several reasons. The MTT assay was to be used in experiments determining effects of cadmium induced toxicity in A6 cells. A6 cells show a large transepithelial resistance and are highly polarized when grown to confluence, because of asymmetrical distribution of channels and receptors. These cells have been shown to be more sensitive to cadmium on the than basolateral their apical side [23;51]. As cadmium induced effects would be a primary focus for the experiments, it was therefore important to change the incubation method of cells from cells settled to cells free in suspension. This is convenient as it removed the preparatory step of seeding and growing cells in the microtiter plate before use, but effectively lowers the number of samples that can be measured. It furthermore allowed the inclusion of centrifugation steps, making it easier to change incubation medium from standard growth medium to glucose-NaCl Ringer and making it possible to remove undisolved cell material after the adding of solvent. The choice of solvent is usually what constitutes the major difference between MTT assays, as it must be effective in dissolving all formazan and cell material without affecting the measurements[102;114;196]. The centrifugation step of the modified assay allowed a mechanical removal of undisolved cell material after addition of pure isopropanol that could otherwise affect accurate spectrofotometric measurements. This is also in accordance with observations by Sobottka and Berger, that the removal of medium after MTT incubation distinctly increased solubility of formazans in isopropanol[155].

Incubation of A6 cells free in suspension, here incubated in standard eppendorf test-tubes does not readily allow for 24 hour exposure periods, but was adequate for the short-term and acute measurements of toxicity (0-3 hours drug exposure periods), that was the focus of this project. A6 cells are able to function normally at room temperature for several hours, allowing the incubation to be done on a standard plate shaker.
8.4. The Modified MTT-assay.

The method described here is a modified version of the rapid colorimetric MTT-assay originally described by Mossman[116]. The method is described in three versions: the first presents a generic overview of the assay. Second, a version presents it for use in determining cell densities or the optimum cell densities at which the MTT assay method here is suitable for a given cell line. A possible concern with the assay is that it may not show a linear correlation between MTT production and specific cell densities, at all densities. The third iteration of the assay method present it for use in measuring toxicity of compounds, that either affect cell viability or activity of MTT reducing enzymes.

Equipment:

- Spectrofotometric microtiter platereader (Synergy HT).
- CR412 Benchtop Centrifuge
- Standard Tabletop Centrifuge.

Materials:

- Cell-culture: A6 cells trypsinated from confluent growth. Resuspended in 5mM Glucose-NaCl Ringer.
- Growth media: 63% DMEM medie (InVitrogen), 25% miliQ vand, 10% FBS (Biochrom AG), 2% Penicilin/streptomycin (InVitrogen).
- 5 mM Glucose - NaCl-Ringer (dissolved in MiliQ H₂O:112,5mM NaCl; 2,5mM NaCO₃; 2,5mM KCl; 5mM Glucos; 1,0mM CaCl₂ • 2H₂O)
- Incubation time: 3 hours with the MTT reagent.
- Wavelength: 595 nm read using spectrofotometric microplate-reader (synergy HT)
- MTT-Stock solution: MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Aldrich), 2,5 mg/ml dissolved in sterile MiliQ H₂O; stored at 4°C until use

Method: All samples are mixed and incubated in appendorf test tubes at room temperature. Measurement of absorbance values is done by transferring the samples to wells on a 96-well Microtiterplate. Absorbance values measured w. synergy HT microplate-reader at 595 nm.

- Cells are harvested from the cultureflasks at confluent growth, by Trypsination and resuspension in fresh growth medium.
- Cell densities are determined by Tryphan Blue Exclusion assay, and diluted to an appropriate cell concentration (10⁶ cells pr. ml).
- 500µl of cell suspension is added to each appendorf test tube.
- 25 µl stock MTT reagent is added to each testtube.
- Cells are incubated in eppendorf-testtubes, for 3 hours at room temperature on a plate shaker.
- The incubation medium is removed after incubation, by centrifugation (2 min. at 8000 rpm using a standard tabletop centrifuge) and pipetting off most of the supernatant. A small amount (25-50µl) is left to ensure no cells are removed.
- 500 µl isopropanol is added to each test-tube to solubilize the formazan crystals and ensure a homogenous solution.
• After a 5-min. incubation period at room temperature, the test-tubes are spun in a centrifuge (1 min. 8000 rpm using a standard tabletop centrifuge) to remove any undisolved cell material.
• 250µl supernatant from each sample is transferred to the wells of a 96x250µl test plate.
• The plate is read on an ELISA reader using a test wavelength of 595.
• Blinds are prepared as per the standard procedure, but samples are incubated at 90°C for 10 minutes before MTT is added, to ensure no living cells. There is usually no significant difference in absorbance values between the control sample and the reference containing pure isopropanol.

8.5. Determination of optimum cell density

• Cells are harvested from exponential growth phase of the maintenance cultures.
• Cell count is determined by a Tryphan exclusion assay after harvesting.
• Cells from the maintenance culture are diluted with standard growth medium to the following concentrations: 0, 2*10⁵, 4*10⁵, 6*10⁵, 8*10⁵ and 1*10⁶ cells per ml.
• 500 µl of the diluted cell suspensions are transferred to eppendorf testtubes.
• 25 µl stock MTT reagent is added to each test tube.
• Cells are incubated in eppendorf-testtubes, for 2 hours at room temperature on a plate shaker.
• The incubation medium is removed after incubation, by centrifugation (2 min. at 8000 rpm using a standard eppendorf tabletop centrifuge) and pipetting off most of the supernatant. A small amount (25-50µl) is left to ensure no cells are removed.
• 500 µl isopropanol is added to each test-tube to solubilize the formazan crystals and ensure a homogenous solution.
• After a 5-min. incubation period at room temperature, the testtubes are spun in a centrifuge (1 min. 8000 rpm using a standard eppendorf tabletop centrifuge) to remove any undissolved cell material.
• 250µl supernatant is transferred to the wells of a 96x250µl testplate.
• A testplate holds blanks (containing no cells), control (testubes heated 10 min. at 90°C before incubation w. MTT), and the series of cell dilutions (N=4).
• The optical densities are read on a spectrophotometric platereader at a single wavelength of 595 nm.
• Viability of test samples is calculated as a percentage of the control, after subtraction of blind samples, ie. \((\text{Abs}_{\text{test}} - \text{Abs}_{\text{blind}})/(\text{Abs}_{\text{control}} - \text{Abs}_{\text{blind}})\) * 100% 
• A standard curve should give a linear relationship between absorbance values and cellcount.
8.6. Incubation with reagents:

Materials:
- Stock solution: MTT (Aldrich), 2,5 mg/ml, in deionized H2O; store at 4°C until use.
- Test reagents: CdCl2 (Sigma), Antimycin A - mixture of A(1), A(2), A(3) & A(4) (Fluka/Biochemika), rotenone (Serva), NaN3 (Sigma), Oligomycin - mixture of A,B & C (Aldrich), CCCP (Sigma), Thapsigargin (Sigma), Ionomycin (Sigma), Ruthenium Red (Aldrich), Cyclosporin A (Fluka/Biochemika), Calpain Inhibitor I (Sigma), Calpain inhibitor II (Sigma).
- Solvent: Isopropanol (AppliChemLab).

Method:
- Cells are harvested from exponential growth phase from the growth flasks.
- The cell solution is washed and resuspended in 5 mM Glucose-NaCl-Ringer, if used in shorter experiments.
- Cell densities are determined by trypan blue exclusion assay, and diluted to appropriate cell densities (~1*10^6 cells per ml).
- 490 µl of the cell suspension is added to each testtube.
- 10µl solution containing appropriate test reagents and/or G-ringer is added to a total volume of 500 µl.
- Incubation of the test tubes containing test reagents, at room temperature on a platenshaker.
- After drug incubation, if needed the test tubes can be centrifuged, the cells washed and resuspended in fresh 5 mM Glucose-NaCl ringer before incubation w. MTT.
- 25 µl stock MTT reagent is added to each testtube.
- Cells are incubated in eppendorf-testtubes, for 2 hours at room temperature on a plateshaker.
- The incubation medium is removed after incubation, by centrifugation (2 min. at 8000 rpm using a standard eppendorf tabletop centrifuge) and pipetting off most of the supernatant. A small amount (25-50µl) is left to ensure no cells are removed.
- 500 µl isopropanol is added to each test-tube to solubilize the formazan crystals and ensure a homogenous solution.
- After a 5-min. incubation period at room temperature, the test tubes are spun in a centrifuge (1 min. 8000 rpm using a standard eppendorf tabletop centrifuge) to remove any undisolved cell material.
- 250µl supernatant is transferred to the wells of a 96x250µl testplate.
- A testplate holds blanks (cell free reagent control), control and different test reagent concentrations.

8.7. Statistics:
The results of MTT assays are given as mean ±SE and analyzed for difference in means, with the Student's t-test. Experiments with all sample replicates from the same day were analyzed by a paired t-test. Experiments with replicates from different days were analyzed with a non-paired t-test. P values less than 0.05 were taken as showing significant difference between samples. The Windows program Microsoft Office: Excell, including the expansions Analysis Toolpack (I+II), was used to calculate P-values.
9. Results

The following chapter presents data from the experiments performed in this project with the modified MTT assays, the Trypan Blue Exclusion assay and Visual Identification of cell death, all assays described in the methods chapter.

Briefly described, using the modified MTT assay, samples were incubated with the MTT reagent and the various inhibitors or other compounds being evaluated, absorbance values were measured determining the proportion of reduced MTT (λ=595nm) between different exposure types and controls. Absorbance values were transformed to relative activities of MTT-reducing enzymes, exposure sample activities given as a percentage of the absorbance values of control samples (mean ±SE%). The data from the experiments is presented in a series of graphs with the exposure type and concentration plotted on the x-axis and the relative activity of MTT-reducing enzymes plotted on the y-axis (ie. controls having 100% activity of controls).

Experiments where all sample replicates where obtained on the same day, used a minimum of 4 replicates (ie. n=4+). Several experiments where conducted over a period of days, with repetition of the entire experimental methodology on each separate day (as described in the methods chapter). Each of the replicates obtained from different days where averages of two samples using the same experimental methodology. The relative activities of MTT assays was then calculated as the means of activities of replicates from different days and having a minimum of 3 replicates (n=3+). Each replicate in this case being the average of two samples using the same experimental method. The following data presentation chapter details the number of replicates of a specific experiment using the MTT assay and in the case where experiments were performed several times over a series of days, if each replicate represents the average of two samples.

The MTT-assay was examined for two primary purposes; 1. to estimate any dependence of MTT-reduction on mitochondrial electron transport chain reductases and dehydrogenase enzymes (experiments 9.3-9.11), 2. to describe aspects of cadmium toxicity of A6 cells evaluated by changes in mitochondrial enzyme activities (experiments 9.12-9.24).

Several experiments using the MTT-assay to analyze the effects of CdCl₂ in combination with other reagents, have their data presented using two different methods of calculating relative activities, for example experiment 9.12(1) and exp.9.13(2). In exp.9.12 (1) activity of MTT-reducing systems is calculated and expressed relative to absorbance values of the controls as described in the methods chapter. Exp.1.13 (2) calculates and presents the data (absorbance values) of the original experiment (i.e. exp.9.12(1)) slightly differently:

The effect of cadmium on MTT-reduction has activity expressed as a percentage of the activity of controls. In order to make an estimation of the effect of an additional agent on cadmium toxicity, the activity of co-incubation is expressed as a percentage of the activity of the single treatment. In the case of Experiment 9.13(2) antimycin a+cadmium is expressed as a percentage of the MTT-reducing activity of A6 cells exposed to antimycin a. This makes the direct comparison of single treatment with combination treatments easier and enables an estimation of how the additional toxicants mediate an increase or decrease in the relative effects and potency of cadmium.
9.1. MTT-formazan production in A6 cells at different cell densities

**Experiment 9.1** examines the cell-density optimum for the modified assay method. The figure presents the data from a series of experiments using the modified MTT assay. A6 cells were trypsinated from confluent growth and resuspended in 5 mM Glucose-NaCl ringer. Cells were counted using a light microscope and Bürker-Türke counting chamber, and diluted to cell densities of 0.2, 0.4, 0.6, 0.8 and 1*10^6 cells per ml. MTT reagent was added to each sample and incubated 3 hours at room temperature. The MTT–formazan crystals produced with bioreduction of MTT, was collected and dissolved using the method described earlier. The dissolved MTT-formazan was measured using the Synergy HT microplate reader at a wavelength of 595 nm.

![Graph showing MTT formazan production](image)

**Fig. 9.1** MTT assay: A6 Dilution series of experiments (n=3). A trendline was fitted, forced through X,Y (0,0): y = 0.1046x, R^2 = 0.9934.

The absorbance of blinds (λ<sub>595</sub>≈0.034) was deducted from each test-sample (ie. λ<sub>595</sub>≈0.792-0.034) and activity was given as a percentage of maximum (ie. 1*10^6 cells per ml). There is a linear relationship between cell-densities and absorbance (R^2=0.9934), showing that the modified assay is capable of giving accurate measurements at different A6 cell densities.
9.2. Differences in cell death estimates of the MTT- and Trypan Blue Exclusion assay

Experiment 9.2 compared the estimated "viability" determined with the MTT assay with cell-death determined by the Trypan Blue Exclusion assay. The vital dye Trypan Blue Exclusion assay was used to estimate CdCl₂ induced cell death. This was compared to results obtained by using the modified MTT assay.

![Graph](image)

**Fig.9.2** Comparison of the MTT-assay (n=3 each replicate is the average of two samples) and Trypan Blue exclusion (n=4) assays used in measuring Cadmium Chloride induced cell death. Results of a 3 hour exposure to varying concentrations of CdCl₂

The MTT assay overestimates cell death when compared with results of the Trypan Blue assay measuring loss of plasma membrane integrity. The Trypan blue assay estimates living cells to be 93,25 ±1,01% at the highest concentration of CdCl₂ (200µM), with the MTT assay showing a decrease in MTT reducing activity to 24,4 ±0,84% compared to controls at the same concentration. The MTT assay reveals a negative correlation between increasing CdCl₂ concentration and activity of MTT reducing enzymes.

The discrepancy between the results of the two methods of determining cell death and viability can possibly be explained by cadmium mediated changes in enzyme activities of MTT reducing enzymes. The change in enzyme activity is effective before, and not proportionate to the degree of disruption in plasma-membrane integrity.

The dosis-response curve of cadmium indicates that its effect as an inhibitor of MTT reduction is close to the maximum at a concentration of 200µM.
9.3. ETC complex I inhibitor Rotenone decreases MTT reduction

If MTT reduction is dependent on mitochondrial ETC enzymes, it should reasonably be expected to be affected by ETC inhibitors. An inhibitor acting directly at the site of action of MTT reducing enzymes would have a greater effect on modifying activity than enzymes up or down-stream of the site(s) of action.

The experiment was used to examine the effects of the ETC complex I inhibitor rotenone on reduction of MTT.

![Graph showing MTT assay results](image)

**Fig.9.3** MTT assay: 3 hour incubation with complex I inhibitor Rotenone (5 and 10µM) (n=4, each replicate is the average of two samples)

Incubation with rotenone, decreases the activity of the MTT assay to 81,0 ±4,8\% (5µM) (p<0,01) and 81,6 ±4,4\% (10µM) relative to the control. There is no significant difference in the MTT reducing activity from exposure to 5µM and 10µM rotenone (p>0,05).
9.4. ETC complex IV inhibitor Sodium Azide decreases MTT reduction

NaN₃ or sodium azide has several mechanisms of toxicity. It inhibits cellular energy metabolism, both at the level of glycolysis and mitochondrial oxidative phosphorylation. OxPhos is inhibited primarily by NaN₃ binding to cytochrome reductase, complex IV of the ETC.

The experiment was conducted in order to examine the effect on reduction of MTT, from inhibiting the ETC at complex IV.

Exposure to 5mM Na azide decreases MTT reducing activity to 84.6 ±4.8% relative to controls (p<0.05).

![MTT assay: 3 hour incubation with Sodium Azide (5mM) (n=4)](image-url)
9.5. ETC complex III inhibitor Antimycin A increases MTT reduction

Antimycin a is an inhibitor of complex III of the mitochondrial electron transport chain. The experiment was conducted to examine the effects on reduction of MTT, with inhibition of ETC complex III.

Incubation with antimycin a increases production of formazan relative to the control. Antimycin a (1µM) showed an activity of 113,18 ±3,22% and antimycin a (2µM) 116,71 ±2,72%. With exposure of 5µM antimycin a, activity increases to a maximum of 123,8 ±2,9% relative to controls (p<0,01). The results show a concentration dependent increase in activity.
9.6. ATP-synthase inhibitor Oligomycin decreases MTT reduction

Mitochondrial ATP-synthase is associated with the mitochondrial ETC, utilizing the proton gradient established by the ETC complexes to generate ATP. This experiment used the ATP-synthase inhibitor oligomycin to investigate the effect on reduction of MTT, with the derived indirect inhibition of the entire electron transport chain.

![Graph showing comparison between Control and Oligomycin (5µg/ml) treatments](image)

**Fig.9.6** MTT assay: 3 hour incubation with ATP-synthase inhibitor oligomycin (5µg/ml) (n=3, each replicate is the average of two samples)

The ATP-synthase inhibitor oligomycin decreases the activity of the MTT-assay to $34.1 \pm 6.9\%$ relative to control values ($p<0.01$). Oligomycin is more effective in inhibiting MTT reduction than the ETC inhibitor rotenone (exp.9.3).
9.7. CCCP increases activity of MTT-reducing enzymes

This experiment examined the effect on reduction of MTT with exposure to CCCP. CCCP acts to increase the activity of all ETC enzymes, through removal of the proton gradient across the MK inner membrane. Uncoupler stimulation by CCCP could therefore be used to assess the maximum activity of MTT reducing enzymes associated with the ETC.

![Fig.9.7 MTT assay: 3 hour incubation with ETC uncoupler CCCP (2,5µM) (n=3, each replicate is the average of two samples)](image)

Incubation with the ETC uncoupler CCCP increases MTT reduction to $300.2 \pm 15.6\%$ relative to the control ($p<0.01$). CCCP eliminates the proton gradient established by the ETC, thus preventing ATP-synthase from generating ATP from ADP. CCCP is commonly used to establish the maximum capacity of the ETC, as uncoupling will increase activity of all enzyme complexes involved[76;85]. The increase in MTT reduction to 300.2% of controls, can be attributed to an uncoupler stimulated increase in ETC activity.
9.8. Thapsigargin dependent calcium release from ER increases reduction of MTT

This experiment was done to investigate the effects on reduction of MTT, after calcium release from ER stores and with increased cytosolic and mitochondrial calcium levels.

Thapsigargin inhibits SERCA of the endoplasmic reticulum. Calcium ions are lost from the store, through leaking or as part of the normal function of ER during calcium signalling\cite{165, 166}. Inhibition of SERCA prevents reuptake of calcium ions and restocking of the ER calcium stores. Thapsigargin may therefore be seen as indirectly emptying the ER calcium stores. Calcium ions released from the ER will increase cytosolic and mitochondrial calcium levels, affecting calcium dependent processes.

![Fig.9.8 MTT assay: 3 hour incubation with Thapsigargin (1,6µM) (n=4, each replicate is the average of two samples)](image)

Incubation with Thapsigargin (1,6µM) increases the activity of the MTT assay by 22.3 ±3.4% relative to controls (p<0.05).
9.9. Ionomycin dependent increase in calcium levels increases reduction of MTT

This experiment was done to investigate the effect on reduction of MTT with a global cellular increase in calcium.

Ionomycin is a calcium ionophore. Incubation with Ionomycin increases the permeability of calcium of all membranes, including plasma-, ER- and mitochondrial membranes.

![Graph showing MTT assay results](image)

**Fig.9.9** MTT assay: 3 hour incubation with Ionomycin (5µM) (n=4).

Ionomycin causes a cytosolic increase in calcium levels. MTT activity is 16.9 ±5.6% greater than controls (p<0.05), when cells are exposed to 5µM ionomycin.
9.10. **Ruthenium Red decreases MTT reduction**

This experiment was conducted in order to investigate the effects on reduction of MTT with a decrease in mitochondrial calcium.

Ruthenium Red is an inhibitor of the mitochondrial transporter VDAC, and a specific inhibitor of mitochondrial calcium uniporter (MCU). Blocking of the MCU decreases mitochondrial calcium levels, as calcium will be lost from the mitochondria through leaking or during calcium signalling. Ruthenium Red induced loss of calcium can affect all calcium dependent mitochondrial enzymes.

![Bar chart showing MTT assay results](image)

**Fig.9.10** MTT assay: 3 hour incubation with Ruthenium Red (5 and 25µM) (n=4)

Incubation with ruthenium red (5µM) decreases the reduction of MTT to 85.8 ±4.6% relative to controls (p<0.05). Incubation with ruthenium red (25µM) decreases the reduction of MTT to 70.4 ±3.6% relative to controls, showing a concentration dependent inhibition of MTT reducing activity (p<0.01).
9.11. Co-Incubation of ETC inhibitors and uncouplers.

This experiment was done to investigate the effects on reduction of MTT with a combination of the mitochondrial inhibitors used in earlier experiments.

![Graph](image)

**Fig.9.11(1)** MTT assay: 3 hour Co-incubation of rotenone (10µM), Oligomycin (µg/ml) and CCCP (2,5µM) (n=4, each replicate is the average of two samples).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% activity of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
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<tr>
<td>rotenone (10µM)</td>
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<tr>
<td>oligomycin (5µg/ml)</td>
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</tr>
<tr>
<td>CCCP (2,5µM)</td>
<td>473,7±64</td>
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<tr>
<td>rotenone+oligomycin</td>
<td>17,2±3,1</td>
</tr>
<tr>
<td>rotenone+oligomycin+CCCP</td>
<td>444,9±48,2</td>
</tr>
</tbody>
</table>

**Fig.9.11 (2)** The specific activities of the experiment shown in **Fig.9.11(1)**

The absolute activities of the single exposures determined with this experiment differ slightly from the earlier described experiments. Most noticeable is the experiment with CCCP giving an increase in MTT reduction of 473,7 ±64% relative to controls, with the experiment depicted in **Fig. 9.7** giving a value of 300,2 ±15,6%. When grown over several cycles of trypsination and subcultivation, A6 cells may accumulate defects and change slightly in their response to toxicants such as CCCP.

Whatever the explanation is for this discrepancy, the experiments confirmed the reaction scheme of the ETC inhibitor rotenone and ATP-synthase inhibitor oligomycin as decreasing activity and CCCP increasing it. Co-incubation of rotenone and oligomycin with CCCP (444,90 ±48,18%) did not significantly decrease activity when compared with CCCP alone (p>0,05).

The earlier experiments established that inhibition of different complexes on the ETC could have different effects on reduction of MTT. This experiment was performed to investigate the effect on
reduction of MTT from combined exposure to the complex I inhibitor rotenone and complex III inhibitor antimycin a.

The experiment shows the expected pattern of antimycin a increasing activity to 117,82 ±1,79% of controls and that incubation with rotenone decreases activity 75,03 ±2,24%. Co-incubation of the complex I and III inhibitor shows an activity of 87,78 ±1,33%. The two inhibitors partially cancel each others effect on MTT reduction, resulting in an intermediate activity. Formazan production is higher than that observed with rotenone exposure, but significantly lower than controls and samples exposed to antimycin a (p<0,05).

This experiment examined the effect on reduction of MTT with co-exposure to cadmium and complex III inhibitor antimycin a.

Antimycin a increases the activity of MTT reducing enzymes, and is able to partially negate the decrease in activity from incubation with CdCl2. The experiment shows a decrease in activity to 30.2 ±3.6% with cadmium exposure and an increase to 117.3 ±1.9% with antimycin a. Co-incubation reduces activity to 44.8 ±3.1% when expressed as a percentage of the controls, showing a significant increase in activity compared to CdCl2 alone (p<0.01).
9.13. **Co-incubation of Antimycin A and CdCl₂ (2)**

The original data obtained in **Exp.9.12 (1)** are reiterated here but with activities calculated differently, with values for CdCl₂+antimycin a given as a percentage of CdCl₂. Plotting the data this way makes it easier to see any synergistic or antagonistic effects of combining cadmium and antimycin a.

![Fig.9.13(2) MTT assay: 3 hour Co-incubation of antimycin a (2µM) and CdCl₂ (100µM) (n=4, each replicate is the average of two samples). Values of CdCl₂ alone are plotted relative to the control. Samples of CdCl₂ co-incubated with antimycin a are plotted relative to the activity of CdCl₂.](image)

The results show that the antimycin increases MTT reduction to 117,27 ±1,94% relative to its control. Co-incubation of antimycin with CdCl₂ has an activity of 151,49 ±11,22% relative to the activity of samples with CdCl₂. The increase in activity of co-incubation of antimycin with CdCl₂, relative to CdCl₂ alone, is greater than the observed antimycin induced increase relative to the controls (p<0,01).

This experiment examined the effect on reduction of MTT with co-exposure to cadmium and complex I inhibitor rotenone.

![Graph showing MTT assay results](image.jpg)

**Fig.9.14** MTT assay: 3 hour Co-incubation of rotenone (10µM) and CdCl₂ (200µM) (n=4).

This experiment gave a greater decrease in MTT reducing activity from cadmium exposure (19,2 ±0,6%) compared to experiments described earlier (eg. exp.9.2), but show the same tendencies as these, with a large cadmium mediated decrease in MTT-formazan production.

The experiment shows that rotenone (10µM) reduces MTT-formazan production to 67,9 ±1,0% of controls, but is not synergistic with CdCl₂. Rotenone co-incubated with cadmium (23,1 ±0,45%) shows a slightly higher formazan production than the experiment with cadmium alone (p<0,05).
9.15. Co-incubation of Oligomycin and CdCl₂

This experiment examined the effect on reduction of MTT with combined treatment with cadmium and ATP-synthase inhibitor oligomycin. Oligomycin inhibits ATP-synthase and thus indirectly inhibits the entire ETC. Cadmium is a multi-target toxicant that may affect ETC complexes as well as the associated ATP-synthase.

![Figure 9.15](image)

**Fig.9.15** MTT assay: 3 hour Co-incubation of Oligomycin (5µg/ml) and CdCl₂ (100µM) (n=4).

Oligomycin reduces activity of the MTT assay to 40,8 ±2,3% of control. Cadmium decreases activity to 24,0 ±0,5% and co-incubation decreases the activity significantly from this to 11,7 ±0,15% (p<0,01). Oligomycin and cadmium assert an additive or synergistic effect in inhibiting MTT-formazan production.

Cadmium alone shows a slightly higher effectiveness as an inhibitor, than in some earlier experiments (ie. CdCl₂ (100M) 36,34 ±0,47% in exp. 9.2). This is possibly due to the age of the cells used in this experiment, or potentially a problem with the cadmium stock solution. Toxicity seems closer to the results obtained for those for higher concentrations of cadmium (ie. CdCl₂ (200µM) 24,44 ±0,84% in (exp.9.2)).
9.16. Co-incubation of CCCP and CdCl₂(1)

CCCP was seen in experiment 9.7 to increase reduction of MTT, presumably by uncoupler stimulation of the ETC. This experiment examines the effect on cadmium inhibited MTT reduction with co-treatment of CCCP.

![Fig.9.16(1) MTT assay: 3 hour Co-incubation of CCCP (2,5µM) and CdCl₂ (100µM) (n=4, each replicate is the average of two samples).](image)

The ETC uncoupler CCCP increases MTT production to 300,2 ±15,6%. Co-incubation of CCCP with CdCl₂ increases MTT-formazan production to 216,7 ±11,9, with a significant difference between the two treatments (p<0.05). Treatment with CCCP prevents the observed CdCl₂ mediated decrease in activity of 40,7 ±7,2% relative to the controls.
9.17. Co-incubation of CCCP and CdCl$_2$(2)

The original data presented in Exp.9.16 (1) is reiterated here but with the relative activities calculated differently. Values for CdCl$_2$+CCCP are given as a percentage of CCCP. This method of calculating the relative activities makes it easier to give an estimate of synergistic or antagonistic effects.

[Graph showing MTT assay: 3 hour Co-incubation of CCCP (2.5µM) and CdCl$_2$ (100µM) (n=4, each replicate is the average of two samples). Values of CdCl$_2$ alone are plotted relative to the control. Samples of CdCl$_2$ co-incubated with CCCP are plotted relative to the activity of CCCP.]

The results show that the CdCl$_2$ decreases MTT reduction to 40.73 ±7.20% relative to its control. Co-incubation of CCCP with CdCl$_2$ has an activity of 72.76 ±6.63% relative to the activity of samples with CCCP (p<0.05).

Samples with CdCl$_2$ give a greater relative decrease in activity when compared to its control, than CCCP+CdCl$_2$ compared to CCCP (p<0.01).
9.18. Co-incubation of Thapsigargin and CdCl2

Thapsigargin releases calcium from the intracellular stores of the ER, seen in exp.9.8 to increase reduction of MTT. Emptying the intracellular stores of calcium may affect cadmium toxicity by opening of store operated calcium channels in the plasma-membrane, thus increasing cellular uptake of cadmium.

This experiment was done to observe the effect on cadmium inhibition of MTT reduction, with co-treatment of Thapsigargin.

![MTT assay graph](image)

**Fig.9.18(1)** MTT assay: 3 hour Co-incubation of Thapsigargin (1,6µM) and CdCl2 (5 and10µM) (n=4, each replicate is the average of two samples).

Low concentrations of cadmium (5 and 10µM) diminish or negate the Thapsigargin induced increase in MTT reduction. CdCl2 (10 µM) had an activity of 85,56 ±2,02%, Thapsigargin (1,6µM) an activity of 122,31 ±3,42%. Co-incubation of CdCl2 (10µM) and Thapsigargin had an activity of 85,03±2,72, not significantly different from CdCl2 alone (p>0,05). Co-incubation of CdCl2 (5µM) and Thapsigargin had an activity of 105,52 ±1,42% compared to control samples, significantly greater than the 94,58 ±1,99% activity of CdCl2 (5 µM ) exposure (p<0,05).
9.19. Co-incubation of Thapsigargin and CdCl\textsubscript{2} (2)

**Fig.9.19(2)** presents the original data from **Exp.9.18(1)** reiterated but calculated differently. The activity of samples with CdCl\textsubscript{2} (10µM) is plotted relative to the reagent-free controls. Samples of CdCl\textsubscript{2} co-incubated with Thapsigargin (1,6µM) are plotted relative to the activity of Thapsigargin. This method of calculation and plotting the data, makes observations of synergistic or antagonistic effects easier.

![Graph showing the activity of CdCl\textsubscript{2} and Thapsigargin in different combinations.](image)

**Fig.9.19(2)** MTT assay: 3 hour Co-incubation of Thapsigargin (1,6µM) and CdCl\textsubscript{2} (5 and10µM) (n=4, each replicate is the average of two samples). Values of CdCl\textsubscript{2} alone are plotted relative to the control. Samples of CdCl\textsubscript{2} co-incubated with Thapsigargin are plotted relative to the activity of Thapsigargin.

The experiment showed that co-incubation of Thapsigargin and CdCl\textsubscript{2} increased the relative cadmium induced reduction in activity (66,93 ±1,58%), when compared with the CdCl\textsubscript{2} induced decrease relative to its controls (85,56 ±2,02%) (p<0,01).
9.20. Co-incubation of Ruthenium Red and CdCl2(1)

Ruthenium Red was observed in exp.9.10 to decrease reduction of MTT. Ruthenium Red inhibits the MCU and prevents uptake of calcium into the mitochondria. Cadmium may enter into the mitochondria through calcium transporters such as the MCU. This experiment examined the effect on cadmium impairment of MTT reduction with co-treatment of Ruthenium Red.

![Fig.9.20(1) MTT assay: Results of experiments with 3 hour Co-incubation of Ruthenium Red (25µM) with CdCl2 (40µM) (n=4). Samples were pre-incubated with Ruthenium Red for 30 minutes before addition of CdCl2 and MTT reagent.](image)

The results of the experiments with co-incubation of cadmium with ruthenium red show that ruthenium red can have a synergistic effect with cadmium. Ruthenium Red (25µM) had an activity of $70.35 \pm 3.57$, CdCl2 (40µM) $66.75 \pm 3.24$ and when co-incubated an activity of $35.93 \pm 2.53$. The combination treatment was significantly different from both of the single series of exposures ($p<0.01$).
9.21. **Co-incubation of Ruthenium Red and CdCl\_2(2)**

The original data from **Exp.9.20(1)** is reiterated here but calculated and plotted differently, with values for CdCl\_2 (40\(\mu\)M)+ruthenium red (25\(\mu\)M) given as a percentage of the activity of ruthenium red. This method of plotting the data makes it easier to observe any synergistic or antagonistic effects of co-incubating cadmium and ruthenium red.

![Chart](chart.png)

**Fig.9.21(2)** MTT assay: Results of experiments with 3 hour Co-incubation of Ruthenium Red (25\(\mu\)M) with CdCl\_2 (40\(\mu\)M) (n=4). Samples were pre-incubated with Ruthenium Red for 30 minutes before addition of CdCl\_2 and MTT reagent. Values of CdCl\_2 alone are plotted relative to the control. Samples of CdCl\_2 co-incubated with Ruthenium Red are plotted relative to the activity of Ruthenium Red.

The results show that the CdCl\_2 decreases MTT reduction to 66.75 ±3.24% relative to its control. Co-incubation of ruthenium red with CdCl\_2 has an activity of 51.29 ±3.93% relative to the activity of samples with ruthenium red. This indicates that ruthenium red may act synergistically in enhancing the negative effects of CdCl\_2 on MTT reduction. The observed CdCl\_2 induced decrease in activity from co-exposure is greater than the combined decrease in activity of single treatments, showing a synergistic effect of co-incubating cadmium and ruthenium red (p<0.05).
9.22. Co-incubation of Cyclosporin A and CdCl2(1)

Cyclosporin A is an inhibitor of the mitochondrial permeability transition pore. Cadmium has been shown to sometimes initiate apoptosis by targeting the MPT-pore. The experiments done earlier with the trypan blue assay (exp.9.2) did not suggest a large frequency of cadmium induced apoptotic cells.

This experiment was performed to examine the effect of CsA on the basal MTT reduction, as well as determining if inhibition of the MPT-pore could ameliorate the effects of cadmium.

![Graph](attachment:image.png)

**Fig.9.22(1)** MTT assay: 3 hour Co-incubation of CsA (5µM) and CdCl2 (40µM) (n=4). Samples were pre-incubated with CsA for 30 minutes before addition of CdCl2 and MTT reagent.

Incubation with CsA alone increases the activity of the MTT assay to 130,5 ±3,7% compared to controls. Co-incubation of CsA with CdCl2 (40µM) negates the cadmium induced decrease in MTT reduction otherwise observed, a significant increase from 52,8 ±1,9% to 69,4 ±4,8% (p<0,05).
9.23. Co-incubation of Cyclosporin A and CdCl₂(2)

The original data presented in Exp.9.22(1) are reiterated here but calculated and plotted slightly differently, with values for CdCl₂ (40μM)+CsA (5μM) given as a percentage of the activity of CsA. This method of plotting the data makes the estimation of synergistic or antagonistic effects easier.

![Graph showing MTT assay results](image)

**Fig.9.23(2)** MTT assay: 3 hour Co-incubation of CsA (5μM) and CdCl₂ (40μM) (n=4). Samples were pre-incubated with CsA for 30 minutes before addition of CdCl₂ and MTT reagent. Values of CdCl₂ alone are plotted relative to the control. Samples of CdCl₂ co-incubated with CsA are plotted relative to the activity of CsA.

The results show that the CdCl₂ decreases MTT reduction to 52.82 ±1.92% relative to its control. Co-incubation of CsA with CdCl₂ has an activity of 53.38 ±4.15% relative to the activity of samples with CsA. There is no significant difference when compared to the relative decrease in activity of CdCl₂ only treatments (p>0.05), indicating there is no additive or synergistic effects from cadmium and CsA co-exposure.
9.24. **Co-incubation of Calpain inhibitor I+II and CdCl₂**

Oh et al. reported that Calpains was involved in cadmium induced cell-death of WI38 cells, initiating apoptosis, by mediating Bcl-2 members, Bid and Bax activation of mitochondrial permeability transition [122]. Similarly, Yang et al. report from observations of CHO cells that cadmium stimulates an increase in intracellular calcium levels, thereby activating the calcium-dependent Calpains, resulting in a Calpain mediated activation of MPT.

Because of Calpains broad range of targets, cadmium activation of Calpains could be expected to impair mitochondrial function in several ways[39;80;159]. If Calpains are involved in cadmium mediated mitochondrial dysfunction of A6 cells, the experiments done in this project will not show their exact effects. The experiments done will only show if they can be associated with observations of cadmium induced decreases in mitochondrial function; if Calpain inhibition ameliorates the Cadmium dependent inhibition of MTT reducing enzymes.

![Fig.9.24(1) MTT assay: 3 hour Co-incubation of Calpain Inhibitor I (100µM) and CdCl₂ (200µM) (n=4). A6 cells were preincubated with Calpain Inhibitor I for 30 minutes before addition of CdCl₂ and the MTT reagent.](image)
Fig.9.24(2) MTT assay: 3 hour Co-incubation of Calpain Inhibitor II (100µM) and CdCl₂ (200µM) (n=4). A6 cells were preincubated with Calpain Inhibitor II for 30 minutes before addition of CdCl₂ and the MTT reagent.

The above experiments show that incubation with Calpain inhibitors I (100µM) and II (100µM) have a small or no effect compared to controls, respectively with activities of 93,97 ±3,22% and 98,70 ±0,76%. Calpain Inhibitor I show a lesser activity than controls. The Calpain inhibitors used in this project are not entirely specific to Calpains, but may inhibit other serine proteases, thus possibly having a very indirect on MTT-reduction and cellular metabolism[43;111;130;188]. Calpain inhibitor dependent decrease in MTT-reduction can also possibly be explained by their induction of apoptosis [105].

The experiment with Calpain Inhibitor I shows cadmium (200µM) inhibiting MTT-reduction to 25,39 ±0,45%. Co-incubation with the Calpain inhibitor I shows an activity of 23,21 ±2,25%, not significantly different from CdCl₂ (p>0,05).

The experiment with Calpain Inhibitor II shows cadmium (200µM) inhibiting MTT-reduction to 37,68 ±2,07%. Co-incubation with Calpain Inhibitor II decreases activity to 30,44 ±1,73%, significantly smaller than CdCl₂ (p<0,05).
9.25. **Time-dependent variant MTT assay with CdCl$_2$ exposure.**

**Fig.9.25 (I-III)** gives results for a series of experiments using a slight variation of the MTT assay otherwise used in the project. A6 cells were incubated in 5mM Glucose-NaCl Ringer with CdCl$_2$ (5, 10, 50, 100 and 200µM) for respectively 1, 2 or 3 hours. The samples were then centrifugated, incubation medium was removed and cells washed before resuspension in 5mM Glucose-NaCl Ringer. MTT was added for an additional incubation period of 3 Hours and samples were analyzed as normal for the variant MTT assay. The sample number for these experiments is only n=2, making it impractical to use any statistics in analyzing the data.

![Diagram](image1)

**Fig.9.25(I)** MTT assay: A6 cells were incubated with CdCl$_2$ for 1 **hour** before washing and resuspension in 5mM Glucose-NaCl Ringer and MTT reagent was added. Samples were then incubated 3 hours with MTT before measuring (n=2).

![Diagram](image2)

**Fig.9.25(II)** MTT assay: A6 cells were incubated with CdCl$_2$ for 2 **hours** before washing and resuspension in 5mM Glucose-NaCl Ringer and MTT reagent was added. Samples were then incubated 3 hours with MTT before measuring (n=2).
Fig.9.25 (III) MTT assay: A6 cells were incubated with CdCl₂ for **3 hours** before washing and resuspension in 5mM Glucose-NaCl Ringer and MTT reagent was added. Samples were then incubated 3 hours with MTT before measuring (n=2).

The result of the above experiments after **1 hour** of CdCl₂ exposure indicates a positive correlation between concentration and inhibition of MTT-reduction. There is no difference between results obtained for **2 and 3 hour** exposure periods, indicating that a maximum of MTT inhibition is obtained after 2 hours, and is independent on increasing CdCl₂ concentrations from 50μM to 200μM. This series of experiments show that low concentrations of CdCl₂ (50μM ) may inhibit MTT reduction as much high concentrations (100-200μM), when cells are washed after exposure and before incubation with the MTT reagent.

Fig.9.25 (IV) summarizes data from these experiments, plotting the activity of CdCl₂ (200μM) after 0-3 hours of incubation. Activity decreases to 61.7% after a one hour exposure period, falling to 57% after three hours.
9.26. Overview of MTT-assay experiments

An overview of MTT reducing activity of each experiment, expressed as a percentage of controls. Notes in the right-hand column indicate experiments were one reagent shoved slightly different activities from those previously observed. An example is the experiment with co-incubation of rotenone, oligomycin and CCCP were CCCP alone shoved a significant higher activity than it did in other experiments.

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<th>Relative activity %</th>
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<td>CdCl₂ (100µM) + Antimycin a (2µM)</td>
<td>44,78</td>
<td>3,13</td>
<td></td>
</tr>
<tr>
<td>CdCl₂ (200µM)+ Rotenone (10µM)</td>
<td>23,11</td>
<td>0,46</td>
<td></td>
</tr>
<tr>
<td>Oligomycin (5µg/ml) + CdCl₂ (100µM)</td>
<td>11,70</td>
<td>0,15</td>
<td>B</td>
</tr>
<tr>
<td>CdCl₂ (100µM)</td>
<td>23,97</td>
<td>0,51</td>
<td>B</td>
</tr>
<tr>
<td>CCCP (2,5µM) + CdCl₂ (100µM)</td>
<td>216,66</td>
<td>11,88</td>
<td>C</td>
</tr>
<tr>
<td>CCCP (2,5µM)</td>
<td>300,20</td>
<td>15,63</td>
<td>C</td>
</tr>
<tr>
<td>Thapsigargin (1,6µM) + CdCl₂ (5µM)</td>
<td>105,53</td>
<td>1,42</td>
<td></td>
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<tr>
<td>Thapsigargin (1,6µM) + CdCl₂ (10µM)</td>
<td>85,03</td>
<td>2,72</td>
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</tr>
<tr>
<td>Ruthenium Red (25µM) +CdCl₂ (40µM)</td>
<td>35,93</td>
<td>2,53</td>
<td></td>
</tr>
<tr>
<td>CsA (5µM) + CdCl₂ (40µM)</td>
<td>69,41</td>
<td>4,76</td>
<td>D</td>
</tr>
<tr>
<td>CdCl₂ (40µM)</td>
<td>52,82</td>
<td>1,92</td>
<td>D</td>
</tr>
</tbody>
</table>

Fig.9.26 Overview of results of the experiments. The values are given as % activity of controls from each separate experiment.
9.27. **Visual identification of A6 Cell-death.**

Images were taken using the method described in the methods chapter, using a Leica Light-microscope (x100) and IM50 imaging software. A6 cells were harvested from confluent growth, using the standard method of trypsination. 20µl of cell-suspension was seeded in each of 24 wells of 24x1ml microplate. 1 ml standard growth medium was added and cells were allowed to grow overnight. With experiments, the growth medium was replaced with 5mM Glucose-NaCl Ringer including the various test reagents. Images was taken before the change of medium, after (at time=0) and at regular intervals (30 min.) until end of the experiments after 3 hours. Images were taken of the effects of the various reagents used in the MTT assays described earlier. This chapter will only present the result of a selection of the images taken. A comprehensive collection of the images taken is presented in an appendix.

The reported observation of changes in cell morphology of single cells, as well as the extent of changes to the number of cells over time, will be estimations and approximations.

9.28. **A6 cell confluent mono-layer.**

![Fig.9.28(1) Dome-like growth of A6 cells at confluent growth. Cells were grown in 25 ml culture flasks 10-14 days after subcultivation, before domes were prominent. Images were taken at x100 magnification.](image)

A6 cells at confluent growth transport Cl⁻ ions from the apical to the basolateral side, with a concurrent net movement of water to the space between the cell monolayer and the supporting surface. Tight junctions binding the A6 cells tightly together, prevent water leaking back to the apical side. Water pressure can then push cells of the monolayer away from the growth surface into
“dome”-like formations seen on the above image as rounded shapes, under the microscope seen as 3D domes of A6 cells protruding up into the growth medium.

Fig.9.28(2) A6 at confluent growth without dome formation. images taken after ~7 days at x200 magnification.

The above image shows the A6 cells before transport of water across the confluent cell-layer has created the domes observed in Fig.9.28(1).

Images are of (a) controls after 3 hour of incubation, (b) CCCP (2.5µM) at time $t=0$, (c) CCCP (2.5µM) after 3 hours (d) CdCl$_2$ (200µM) after 3 hours of incubation and (e) CdCl$_2$ co-incubated with CCCP after 3 hours.

(a) Control: A6 cells after 3 hours incubation ($t=3$).

(b) CCCP (2.5µM): A6 cells at the beginning of experiments ($t=0$).
(c) CCCP (2.5 µM): A6 cells after 3 hours incubation (t=3).

(d) CdCl$_2$ 200 µM: A6 cells after three hours of incubation (t=3).
(e) CdCl₂ 200µM + CCCP 2,5 µM: A6 cells after 3 hours of incubation (t=3).

Image (a) and (b) are good representations of how A6 cells look in this experimental setup. The cells are seeded at a low cell-density and allowed to grow overnight. The shape of the wells results in an accumulation and highest cell-density in the middle of the well. The beginning stages of a confluent A6 monolayer are visible in the upper portions of image (b). Single cells and smaller collections of cells are found gathered around the central mass. Images were primarily taken of low-density areas, as this eases the tracking of changes in cell morphology over time. Image (c) shows A6 cells after 3 hours incubating with CCCP (2.5 µM). The cells appear smaller and rounded compared to controls. Image (d) shows the effects on A6 cells after three hours of cadmium (200µM) exposure. Many cells show changes in morphological features, including swelling and disruption of the normal shape of the plasma membrane. Several cells shrink in size, possibly indications of apoptotic cell-death. Image (e) shows the effect of co-incubating A6 cells with cadmium and CCCP. The degree and severity of changes in plasma-membrane structure is markedly less than that observed in image (d), with cells exposed to cadmium. Several of the cells shrink in size compared to the observations in image (a) or (b). The number of A6 cells with marked features of cell-death is also considerably smaller than what can be estimated from image (d).
In the following chapter the experiments performed in this project will be discussed thoroughly and results compared with existing literature, principally to establish if: a) the MTT assay can assess the effects of various mitochondrial mediators and inhibitors, b) the reduction of MTT is associated with specific mitochondrial reductases and c) which mitochondrial targets the MTT assay predicts for cadmium toxicity.

Modifications were made to the original MTT assay to allow for incubation of free cells, allowing for inclusion of centrifugation steps. This variant assay was then tested on the A6 model system, first with a series of experiments incubating MTT at varying cell-densities (exp. 9.1).

It has been of some concern that the MTT assay may be imprecise when assaying very high or very low cell densities [147]. Finding the optimum cell densities where there is a direct linear relationship between cell densities and measured absorbance is therefore of prime concern. The experiment indicates that a cell density of around $1 \times 10^6$ per ml, is acceptable, as absorbance values will typically lie in the range of ~0.0 to ~1.0. Higher cell-densities result in high absorbance values that could be measured by dilution of the MTT-formazans formed with more isopropanol. However, a too high formazan production from a high cell density may have some procedural problems, as formazan crystals formed from MTT-reduction, can be deposited outside cells and mistakenly removed with the supernatant after MTT incubation, before isopropanol is added. A lower level of formazan production minimises the risks of this occurring. Large deposits of formazan could conceivably mechanically affect the cells.

The MTT-assay was used to measure cadmium induced changes in viability of A6 cells, the results then compared with cell-death measurements by the Trypan-blue Exclusion assay (exp. 9.2). The experiment shows that there is a large discrepancy in what the Trypan Blue assay measures and what is observed with the MTT assay. This apparent decrease in viability can not entirely be ascribed to cell death or loss of plasma-membrane integrity, as confirmed by the Trypan Blue assay. The MTT assay is now mostly used as a means of determining viability, as it has many advantages compared to other more tedious methods, but was originally used as a cell-death assay; experiment 9.2 illustrates the potential problems with the assay as it may sometimes overestimate cell death[74;125;155]. In short-term experiments as done in this project, the MTT assay does not measure cell-death. The difference between the values obtained by the two assays must instead be ascribed to changes in the activity of MTT reducing enzyme systems.

The reduction of MTT has often been ascribed to the activity of dehydrogenases or reducing enzymes of the mitochondria[1;19;35;114]. While other mechanisms for its bio-reduction have been presented in the literature[13;19], it was decided to focus on and explore a possible mitochondrial link.

A series of experiments (exp.9.3-exp.9.11) were conducted to determine any effect of mitochondrial modulators and inhibitors on the reduction of MTT. The effects of the mitochondrial inhibitors and other toxic agents used in the experiments were also estimated by visual identification with the light-microscopy imaging technique (exp.9.29). The method used here for visual identification of morphological changes or disruption of the plasma membranes of A6 cells, with exposure to the toxic agents used in the MTT experiments, gives a qualitative estimate of cell-
death. If the visual identification experiments indicate negligible toxicant induced differences in cell-death when compared to controls, changes in MTT-formazan production can be ascribed to changes in the activity of MTT-reducing enzymes.

The specific concentrations of the compounds being examined were chosen after consulting the literature to optimize it for inhibition of MTT reduction, without causing cell-death within the 3 hour timeframe of the MTT assays.

The experiments using visual identification of morphological alterations of the cells, was adequate in establishing that the degree of cell-death caused by exposure to the toxic agents, was less than what was needed to account for the reduction of MTT-formazan production.

The images of A6 cells exposed to the various toxic substances used in the MTT assays, are collected and presented in the appendix, with the exception of images of cells exposed to CCCP in combination with CdCl₂. The images from this experiment were considered unusual and presented in the results chapter, to receive a more extensive examination and discussion.

Several of the MTT-assays also showed an increase in activity of MTT-reducing enzymes that due to the short exposure periods could not be explained as cell growth (eg. exp.9.5 and exp.9.7).

10.1. The reduction of MTT is linked to mitochondrial function

Experiments 9.3-9.5 examine the effects of mitochondrial inhibitors on MTT reduction.

Inhibitors targeting a specific enzyme complex on the mitochondrial electron transport chain may decrease the activity of MTT reducing enzymes, if the ETC complex is directly responsible for MTT conversion [103].

MTT reduction can take place downstream on the ETC from the site of action of the inhibitor. This could also decrease the observed relative activity, when compared to the controls, since no electrons would be available to reduce the MTT-salt to its formazan product[3;59]. Conversely, if an inhibitor has no immediate effect, the site of MTT-reduction could reasonably be located upstream of the site of action of the inhibitor or not be associated with the ETC at all. With a series of inhibitors targeting various parts of the ETC, it may be possible to pinpoint the location or coupling point of mitochondrial MTT reduction [19].

Of course the situation may be more complicated than the scheme described above indicates. MTT reduction may occur at more than one cellular location, and so may be only partially dependent on ETC enzymes [3]. The ETC has a number of regulatory mechanisms and feedback systems. Inhibition at one site on the ETC could possibly increase activity at other locations.

Another possible complication is the fact that few of the inhibitors of mitochondrial function used in this project are entirely specific for mitochondria. Several of the compounds tested have effects beyond the mitochondria that could significantly affect the results. An example is cyclosporin a, a compound that binds to cyclophilins. In mitochondria CsA may bind to Cyp-D and prevent the opening of the mitochondrial permeability transition pore, thus protecting against depolarization from an MPT event[69;149]. However CsA is not specific to Cyp-D but can bind to other Cyp-family enzymes, such as Cyp-A. Cyp-A is known in some cell systems, to regulate the activity of calcineurin, which in turn regulates activity of nNOS. nNOS can generate NO that is required for
glutamate-dependent mitochondrial depolarization[46]. There are therefore several ways CsA can prevent depolarisation of mitochondria.

Many of the tested compounds have a similar broad range of cellular targets, and it is vital to be careful before advocating a single simple solution to an experimental observation.

The use of inhibitors of OxPhos will influence energy metabolism in the A6 cells, and this could potentially be problematic when interpreting the data from the experiments examining MTT conversion. Depletion of cellular ATP-pools is sometimes implicated in activation of cell-death pathways, in addition to affecting activity of enzymes in the energy metabolism machinery[27;180]. Exposure of A6 cells to an inhibitor such as antimycin a might result in changes at several levels affecting the production of MTT-formazan. Changes in formazan production may well be a combination of reduced activity of reductases/dehydrogenases responsible for MTT conversion, in additional to cell-death. If reduction of MTT should prove not to be entirely limited to mitochondria, it might add another layer of complexity in interpreting data.

Changes in ATP-pool sizes of A6 cells, after exposure to inhibitors of ETC enzymes, was not monitored in this project but has been described in the literature. Lynch and Balaban examined the energy metabolism of A6 cells under normal conditions, compared to when either OxPhos or Glycolysis was inhibited [107]. It was found that ATP production was significantly lower with only active Oxphos or Glycolysis compared to controls. Glycolysis was unable to fully compensate for the reduction in ATP production after inhibition of the ETC, a feature sometimes observed in immortalized cell-lines [107].

**Rotenone:**
Rotenone is an inhibitor of electron flow through Complex I of the ETC [41]. Experiment 9.3 shows that MTT reduction in the presence rotenone is lower than in the control samples. This decrease in bioreduction of MTT could be ascribed to at least a partial dependence on ETC dehydrogenases or reductases being inhibited by rotenone [19]. Rotenone does not give a total inhibition of formazan production. Possible explanations for this observation could be that MTT reduction is related to the activity of more than one enzyme of the ETC, activity of which is not shut down entirely by rotenone or bioreduction takes place at other locations in the cell.

There is no significant difference in the activity of MTT-reducing enzymes with exposure to 5µM or 10µM rotenone. This indicates a possible maximum for rotenone inhibition, small compared to the maximum for cadmium inhibition observed in experiment 9.2. Cadmium is known to be a multi-target toxicant, possibly targeting both ETC complex I and Complex III[162], indicating that MTT reduction happens at more than one site or downstream from rotenone.

The visual identification experiments show that 10µM rotenone may induce some degree of cell death within the 3 hour exposure period. The images (appendix VII) show that the cells decrease in volume and some groups of cells dissapear. The morphological changes show that the cells retain the ability to regulate their volume. Volume regulation has an ATP requirement, showing that some ATP production continues after exposure to rotenone, primarily through glycolytic reactions. Cells shrinking could also indicate the onset of apoptotic cell-death as it is a common morphological hallmark in the early stages of apoptosis.
**Sodium Azide:**
Sodium azide targets ETC cytochrome reductase, inhibiting OxPhos[187]. MTT production when A6 cells are exposed to NaN₃ (5mM) is lesser than in the control samples (exp.9.4). NaN₃ inhibition occurs downstream from the site of action of rotenone [19;26]. Inhibition downstream of enzymes responsible for MTT reduction could be expected to have a lesser effect on MTT reduction than inhibition directly at the site of conversion, as upstream components of the ETC may increase in activity to compensate for this [85]. However, NaN₃ seems to be as effective an inhibitor as rotenone.

The experiments in visual identification (appendix VII) show that exposure to 5mM azide affects the cells morphology after 3 hours of exposure. The cells decrease slightly in volume. There is not any significant degree of cell-death induced by azide, within the three hour timeframe of the experiments.

**Antimycin A:**
**Experiment 9.5** shows the result of incubation with 1, 2 and 5µM antimycin a[32]. Antimycin a, an inhibitor of the ETC complex III, increases activity of MTT reducing enzymes. The antimycin a mediated increase in the activity of enzymes upstream of complex III, could be a consequence of a positive feedback mechanism responsible for regulating ETC activity, increasing the reduction of MTT.

Another possible explanation is that antimycin a derived ATP-depletion affects an ER release of calcium, as the organelles calcium-uptake pump, SERCA, requires ATP to function [38]. Antimycin a depletion of ATP causing an indirect inhibition of SERCA can increase cytosolic Calcium levels [141]. This could eventually affect an increase in mitochondrial calcium and in the activity of calcium dependent enzymes involved in reduction of MTT.

The ETC inhibitor antimycin a displays an unusual effect on reduction of MTT compared to the experiments using other ETC inhibitors, in that it increases formation of MTT-formazan. It makes it unlikely that MTT reduction takes place directly at complex III, but there might be an explanation for this observation. Antimycin a targets ETC complex III and inhibits the transfer of electrons to ubiquinone. AA inhibition may shift equilibrium of Q⁻ resulting in reverse action of the complex and accumulate QH₂ [177]. If QH₂ is a substrate for reduction of MTT, inhibition of complex III by antimycin could potentially aid this process. The theory is not in conflict with the observation of cadmium decreasing production of MTT-formazan despite the fact that it also may be targeting complex III, as it presumably also acts at other sites of the ETC[10;177]. Needless to say, this theory is merely speculation, an intriguing idea that requires additional experiments to verify.

A6 cells undergo few morphological changes after 3 hours with antimycin a (2µM), as observed with the visual identification experiments (appendix VII). The cells decrease only slightly in size and there is a very small increase in cell deaths compared to the controls.

**Oligomycin:**
Oligomycin inhibits the mitochondrial ATP-synthase complex [85]. Not directly a part of the ETC, ATP-synthase utilizes the proton gradient established by the action of complexes I-IV. Inhibition of ATP-synthase rapidly depletes ATP-levels, negatively affecting all ATP-dependent processes.
The ETC is not directly dependent on ATP, but activity is controlled by ATP-levels [85]. Oligomycin induced ATP-depletion may disrupt all normal cellular functions, affecting generation of NADH needed in the electron transport chain. It may affect the activity of the citric acid cycle enzymes depleting succinate, indirectly inhibiting ETC complex II, normally using Succinate as a substrate [87]. Experiment 9.6 shows that oligomycin is a more potent inhibitor of MTT-reduction more than rotenone, NaN₃ or antimycin. The inhibition of ATP-synthase can result in an accumulation of electrons in the ETC and an increase in the proton gradient that may hyperpolarize the mitochondria. This eventually results in an inhibition of the ETC itself, from working against the proton gradient [42].

Part of the reduction in activity could be due to ATP-depletion affecting viability. While ATP-depletion would normally cause enzymes of the ETC to increase in activity through feedback mechanisms, it will induce cell-death through apoptosis or necrosis if ATP levels decrease enough [27].

The experiments using visual identification (appendix VII) show that oligomycin (5µg/ml) exposed cells decrease in size. The regulation of cell volume is dependent on ATP. The experiments therefore show that ATP production is continued after exposure to oligomycin. Decrease in cell volume is a hallmark of apoptosis, and continued exposure to oligomycin may lead to cell-death using the apoptotic pathway.

Carbonyl cyanide m-chlorophenylhydrazone:
CCCP is an uncoupler of Oxidative Phosphorylation [85]. Used in experiments to establish the maximum respiration rate of mitochondria, the depletion of the proton gradient increases the activity of all ETC enzymes [76]. Experiment 9.7 shows a great increase in formazan production after exposure to CCCP. This increase would be consistent with a theory of MTT-reduction dependent on the action of ETC reductases, when the activities of these are increased by uncoupler stimulation.

This theory is in accordance with observations from the literature: Berridge et al report that the uncoupler FCCP, a compound related to CCCP, increases MTT-formazan production [18]. Collier and Pritsos have investigated effects of the mitochondrial uncoupler dicumarol on MTT reduction, following the reaction for up to 24 hours. They observed a marked increase in activity, primarily in the first few hours, potentially resulting in an overestimation of cell viability. They ascribed this effect to uncoupler generated ROS, and thus an indirect effect [35]. Interestingly it has been observed that CCCP in experiments with isolated mitochondria may decrease activity rather than enhance it as is the case in whole cell models. This difference has not yet been explained [103].

The experiments in this project using the uncoupler CCCP had some experimental-technical difficulties. There was a large production of formazan crystals in all experiments with CCCP, possibly large enough to affect cell functions and viability as growth of large amounts of formazan crystals could possibly harm or even disrupt plasma-membrane integrity. Removal of supernatant after MTT-incubation before addition of the solvent isopropanol was problematic, as the large amount of formazans hindered disposal of the incubation medium without removing any formazan from the samples. Cell-densities could have been decreased for these experiments, but directly comparing the results with other experiments could then be somewhat problematic.
Goodwin et al. describes in their review a use for intermediate electron acceptors in increasing the reduction of MTT, so as to detect smaller cell-densities. CCCP could possibly act as an IEA, but would then be expected to increase formazan production in blinds, as reported for other IEAs[59]. CCCP does not increase activity of MTT reduction in the blind samples of the experiments ($\lambda_{595} = \sim 0.04$, similar to other blinds). The observed increase is therefore likely enzymatic, not independent of cell functions and not an auto-oxidation reaction enhanced by the presence of CCCP.


The experiments described using ETC inhibitors (antimycin a, rotenone and azide), ATP synthase inhibitor (oligomycin) and an uncoupler of oxidative phosphorylation (CCCP), leads to the proposal of a simple model for what is measured by the MTT assay. None of the single ETC inhibitors proved as effective in preventing MTT reduction as oligomycin. Oligomycin presumably inhibits the entirety of ETC reductases involved in reduction of MTT. CCCP increased reduction of MTT, by stimulating the activity of ETC reductases involved in reduction of MTT.

The MTT assay measures the combined activity of reductases involved in reduction of MTT. ETC reductases are the likely candidates for much of this activity. Inhibition of the MK ATP-synthase is the most effective method of inhibiting MTT reduction.

![Diagram of electron transport chain](image)

**Fig.10.1** A schematic representation of the electron transport chain of A6 cells, from NADH to O$_2$. The positions of ETC inhibitors are indicated with the compound name followed by (-) if displaying an inhibitory effect on MTT reduction, or (+) if showing a stimulatory effect. The ETC complexes likely to be associated with reduction of MTT are also shown. CCCP, an uncoupler of OxPhos increases MTT reduction by increasing activity of ETC reductases. Oligomycin, an inhibitor of ATP-synthase indirectly inhibits all ETC reductases responsible for MTT reduction.
10.3. The activities of MTT reducing enzymes are regulated by calcium

Based on Experiments 9.3-9.7 a model for MTT reduction was proposed arguing that ETC enzymes are likely responsible for reduction of MTT. The activity of ETC enzymes is regulated by calcium. Three different compounds were used to affect cellular and mitochondrial calcium levels; thapsigargin, ionomycin and ruthenium red.

**Thapsigargin:**
Thapsigargin inhibits SERCA of the endoplasmic reticulum, reducing the ATP-dependent reuptake of Calcium [166]. Calcium released from the intracellular stores of the ER increases both cytosolic and mitochondrial calcium levels, affecting all calcium dependent processes [17;131]. While a continual, global increase in cellular calcium levels may cause cell death through activation of calpains [159], it can also positively affect the activity of calcium dependent enzymes of the ETC [22;63].

**Experiment 9.8** shows an increase in MTT-reduction, relative to the controls. This observation can be explained as a Thapsigargin mediated increase in mitochondrial calcium resulting in increased activity of calcium-dependent MTT-reducing enzymes, consistent with the theory of MTT reduction by ETC enzymes [19;103].

The visual identification experiments (appendix VII) show that thapsigargin (1.6µM) decrease the volume and morphology of the cells similar to exposure to ETC inhibitors rotenone and azide. These morphological changes are not the result of ATP depletion, but are indicators of the advent of calcium induced apoptotic cell-death.

**Ionomycin:**
Calcium concentrations vary greatly between different cellular compartments, with low resting levels in cytosol compared to mitochondria and especially the calcium stores of the endoplasmic reticulum. The enhanced calcium permeabilization of the plasma membrane by ionomycin, results in an increased influx and cytosolic rise in calcium. This may increase the activity of all calcium-dependent processes, including mitochondrial functions such as the electron transport chain, increasing mitochondrial respiration [174].

The result of **Experiment 9.9** show that exposure to Ionomycin (5µM) increases the activity of MTT-reducing enzymes. Depending on the experimental setup, Ionomycin can also increase mitochondrial loss of Calcium, which would normally have a negative impact on mitochondrial calcium dependent functions. Ionomycin has however been shown, in some cases to enhance mitochondrial respiration [77]. Replenishing of mitochondrial calcium is an energy dependent process, requiring the expenditure of the electrochemical potential established by the action of the ETC, by the mitochondrial calcium uniporter. While loss of mitochondrial calcium can negatively affect the activity of the respiratory components, the loss of the proton gradient due to MCU activity and subsequent re-establishing of it has been shown to sometimes enhance ETC activity and increase oxygen consumption [77].

While Ionomycin may eventually cause cell death through calcium dependent mechanisms, the increase in MTT-reduction observed here illustrates that the generic MTT assay may be inadequate in determining changes in viability and establishing cell death when induced by toxicants associated with calcium mobilization, such as Ionomycin and Thapsigargin.
**Ruthenium Red:**

Ruthenium Red is an inhibitor of the mitochondrial uniporter responsible for maintaining high mitochondrial calcium levels [151]. Inhibition of the MCU can empty the mitochondria for calcium negatively affecting activity of MK calcium dependent enzymes and processes [27;63]. In **Experiment 9.10** A6 cells were incubated with 5µM or 25µM ruthenium red and the MTT reagent for 3 hours. There was a concentration dependent decrease in activity, which would be explained by mitochondrial MTT-reducing enzymes being regulated by calcium.

The hypothesis of ruthenium red reducing uptake of calcium into the mitochondria indirectly having a negative effect on MTT-reduction, is in accord with the observations of Thapsigargin and Ionomysin (**exp. 9.8 and exp. 9.9**) increasing cytosolic and mitochondrial calcium levels and increasing MTT-reducing activity.

### 10.4. Co-incubation of ETC inhibitors and uncouplers

**exp.9.11(1)** presents the results of combination treatment of A6 cells with the ETC inhibitor rotenone, the ATP-synthase inhibitor oligomycin and the ETC uncoupler CCCP.

CCCP could be expected to act against the oligomycin dependent inhibition of MTT reducing enzymes. Oligomycin inhibits the mitochondrial ATP-synthase complex that normally utilizes the proton gradient established by the ETC enzymes [42]. The uncoupler CCCP removes the proton gradient and electron build-up in the electron transport chain, that would normally be created by oligomycin[76;180]. This allows ETC enzymes, that otherwise would act against a proton gradient, to operate at an increased pace.

The experiment shows that MTT-reduction may occur locations distinct from complex I, as this would otherwise be inhibited by rotenone [41].

**Exp.9.11 (3)** shows the result on MTT-reduction by A6 cells, from combined exposure to the ETC inhibitors rotenone (10µM) and antimycin a (2µM). When incubated alone the two agents have the effects expected from earlier experiments (**exp. 9.3 and exp.9.5**); rotenone decreases activity and antimycin a increases the activity of MTT reducing enzymes, relative to the controls. The result of co-incubation shows that the antimycin a dependent increase in MTT reduction partially negates the effects of rotenone, that the two ETC inhibitors have a mutually exclusive effect on the MTT-reduction by A6 cells. The exposure to two different ETC inhibitors could possibly be expected to increase the overall inhibition of the ETC components; however the effect on MTT reduction is instead an intermediate activity [19]. This is an additional indication of MTT reduction taking place at a location downstream of the site of rotenone inhibition of complex I, but upstream from the site of antimycin a at complex III.

Antimycin a and rotenone may have additional effects on A6 cells other than their direct ETC inhibitory abilities would reveal. Antimycin a may interact with apoptotic regulatory proteins from the Bcl-2 family [82] and rotenone may act against the opening of the mitochondrial permeability transition pore[180], a notable event in the activation of the intrinsic pathway of apoptosis[81;82].
The two compounds have some very different cellular effects in addition to the inhibition of ETC activity, which may partly account for the observation of them being antagonistic to each other in this experiment.

Berridge and Tan in a review of the MTT assay, described a series of experiments examining effects of inhibitors of ETC complexes I-IV on the production of formazan from MTT [19]. Isolated mitochondria from 32D cells derived from the bone marrow of mice, was subjected to rotenone (2µM or 1mM), the complex II inhibitor TTFA (500µM), antimycin a (1mM) or azide (10mM), and incubated with MTT and either Succinate or NADH as substrate. MTT reducing activity was measured spectrofotometrically similar to the method described for a standard MTT assay.

Some concentrations are much higher than those used in this project (antimycin a as an example), and the use of isolated mitochondria also complicates a direct comparison of results. The experiments with succinate as substrate showed that rotenone (1mM) was ineffective in inhibiting MTT reduction, reducing it to 20-35%. The complex II inhibitor TTFA decreased activity by 85-90% compared to controls. Antimycin a decreased activity by 70% and azide by <10%. With NADH as a substrate inhibition of MTT reduction was much less severe for all inhibitors, being >95% of control activity, except for rotenone decreasing activity by 10-15%.

The reduction in activity described for succinate fuelled mitochondria show a reaction scheme dissimilar to the experiments presented in this project (Exp.9.3-9.5). In the results presented here, rotenone decreases activity as expected when compared to the above experiment. Azide decreases activity as much as rotenone in this project, an effect not observed by Berridge and Tan. Antimycin a actually increased activity in whole A6 cells, compared to the efficient inhibitory effect observed in isolated mitochondria.

Berridge an Tan proposed on the basis of these and other observations, that MTT reduction was dependent on the NADH pool and only indirectly on the activity of ETC complexes. This would support the contention that cellular MTT reduction is not primarily involved with the mitochondrial inner membrane. While isolated mitochondria obviously can reduce MTT, cellular MTT reduction was proposed as being primarily based at microsomes, the plasma-membrane or the mitochondrial outer membrane [19].

Slater et al examined the effects of antimycin and azide on a Succinate-tetrazolium reductase system [152]. The assay used in these experiments was a non-colorimetric variant, instead measuring µg MTT-formazan produced at various tissue levels. The experiments used homogenated rat liver extracts, and incubation time (10 minutes) was significantly shorter than the incubation periods of the assays in this project.

Slater et al found that antimycin decreased MTT-formazan production, but only affected approximately 40% of the total MTT reduction. They argued that this percentage of MTT reduction occurs via an interaction with elements of the respiratory chain prior to the antimycin-sensitive site. They found that azide slightly stimulated the Succinate-reductase system, indicating that the main coupling-site between the respiratory chain and MTT reduction did not involve cytochrome oxidase. It was concluded that Reduction of MTT was associated with two distinct sites on the ETC, the latter site in the region of complex III [152].
The findings of Slater et al are the opposite of what was shown in the experiments of this project. In this project antimycin a had a slight stimulatory effect and azide showed a slight inhibitory effect on reduction of MTT.

A primary difference was the use of Succinate as the main energy source in Slaters experiments and Glucose in this project. The metabolic fuel has been shown to be important for reduction of MTT[75;164;172]. This is because the MTT assay measures the total output of MTT reductases; glucose may enhance the activity of a different subset of MTT reducing enzymes than Succinate.

The experiments with visual identification of morphological changes in the A6 with exposure to cadmium or the various ETC inhibitors (appendix VII) reveal an interesting difference. Generally the ETC inhibitors tend to incur controlled changes and decreases in cell volume, whereas with cadmium the cells increase in size. This suggests that, depending on concentrations cadmium may induce cell-death by necrotic means, whereas the ETC inhibitors will activate apoptotic cell-death pathways.

10.5. ETC targets of Cadmium toxicity examined by the MTT assay

Dorta et al [44]have proposed a reaction scheme for the events of cadmium induced mitochondrial toxicity. Isolated rat liver mitochondria were monitored for up to 25 minutes after a cadmium exposure of 5-25µM. The succinate energized mitochondria were examined for several parameters including mitochondrial swelling, transmembrane resistance (Δψ), mitochondrial respiration, ATP levels, protein thiols, GSH, iron release, lipid peroxidation and cytochrome c release. Cadmium changes to these parameters were examined with or without N-ethylmaleimide (NEM), ruthenium red (RR), CsA+ADP or Butylhydroxytoluene (BHT).

Co-incubation with RR delayed the onset of the MPT event and mitochondrial swelling. CsA+ADP prevented mitochondrial swelling nearly in entirety. RR prevented the uncoupling and inhibition of respiration otherwise observed. RR also prevented cadmium induced mobilization of iron, an effect not inhibited by CSA+ADP, NEM or BHT. Lipid peroxidation was partially prevented by RR and NEM but not CSA+ADP or BHT [44].

The experiments led to Dorta et al proposing the following as the sequence of events for cadmium induced mitochondrial toxicity:

1. Cadmium interacts with protein thiols on the mitochondrial membrane stimulating its uptake via the MCU.
2. MPT is initiated, dissipating Δψ in conjunction with the action of the MCU, causing uncoupling of the electron transport chain and depressing ATP levels. These preliminary events take place within a few minutes of cadmium exposure.
3. The electron transport chain undergoes inhibition, generating ROS together with cadmium-mobilized iron, causing gradual membrane lipid peroxidation. These events occur later in the reaction scheme after the initial events of points 1 and 2. Cadmium induced a mobilization of iron from the mitochondria, presumably by direct competition, displacing iron of ETC cytochromes.

The above reaction scheme is proposed for isolated rat liver mitochondria and will likely vary when compared with that of A6 whole cells, but could be indicative of the mechanism likely to be found in those systems.
A primary difference is the exposure pathway. In A6 cells free cadmium is thought to activate the extracellular cation sensing receptor initiating an IP₃ signal propagating to the ER [51]. IP₃ activates the IP₃-receptor of the ER, releasing calcium from the organelle. Store-operated calcium channels in the plasma-membrane sense the emptying of the ER store, opening and increasing entry of calcium and cadmium.

After IP₃-R mediated, SOC-dependent cadmium entry into the A6 cell, the mitochondrial reaction scheme may be similar to that described above by Dorta et al [44].

The following experiments with the MTT assay were done to observe the effect of modulators of mitochondrial function (experiments 9.1-9.11) on cadmium impairment of MTT reduction. Comparing the data obtained from cadmium experiments, with experiments with cadmium co-incubated with the modulators of mitochondrial and cellular functions described earlier, can reveal aspects of cadmium's relation to mitochondrial function. These experiments can then be compared with the hypothetical reaction scheme described by Dorta et al above [44], to estimate its accuracy in A6 cells [44].

A synergistic effect of cadmium and a specific inhibitors of ETC enzyme complexes on reduction of MTT, indicate that the two act at similar locations.

Antimycin and Cadmium:

Experiment 9.12(1) is the results of an experiment with co-incubation of cadmium (100µM) and antimycin a (2µM). The experiment showed that antimycin a increases MTT-reduction and is able to compensate somewhat for the cadmium dependent decrease in activity.

Experiment 9.13(2) presents the data from Exp.9.12(1) expressed differently; antimycin a (2µM) is plotted relative to controls, and CdCl₂+antimycin a is plotted relative to the activity of CdCl₂. This method of plotting the data makes it easier to give an estimation of the contribution of antimycin a to MTT-reducing activities, and specifically its effect in combination with CdCl₂.

This method of calculating the data reveals that the antimycin a derived difference in activity between samples exposed to cadmium and cadmium+antimycin a, is greater than the increase in activity observed with antimycin a activity compared to toxicant-free samples. This indicates that antimycin a directly mitigates the effects of the cadmium dependent disruption of MTT-reduction.

Wang et al. have examined the effects of cadmium on the relative enzyme activities of ETC complexes I-IV in isolated mitochondria of liver, heart and brain of guinea pigs. They found that while all cadmium concentrations tested (ranging from 5-40µM) affected the activity of all ETC complexes, complex II and III were the most susceptible. There was an apparent tissue specific concentration-activity relationship: cadmium (40µM) decreasing activity of complex III of heart mitochondria to ~55% activity, and to ~30% in liver, cadmium here being effective at lower concentrations [177].

Another experiment presented in this paper was an examination of cadmium's effect on antimycin a-insensitive pre-steady state reduction kinetics of of three complex III components, cytochromes b₅₆₆, b₅₆₂ and c₁. The isolated mitochondria were pre-incubated with antimycin a (5µM) or antimycin a+cadmium (10µM). In all three experiments, antimycin+cadmium failed to completely inhibit reduction of the cytochromes, but was more effective than antimycin a alone. Incubating
with myxothiazol (5µM), a specific inhibitor of Q₀, in combination with antimycin a, entirely prevented the reduction of MTT. When myxothiazol was used instead of antimycin, cadmium had no effect on the reduction rates of the three cytochromes. This result and other data examined in the paper suggested that cadmium inhibits non-competitively in the vicinity of Q₀ at complex III [177]. This has been confirmed by other authors [11].

The notable difference from the observations made by Wang et al., is that in my experiments antimycin a actually increased activity of MTT-reducing enzymes, implying that MTT reduction does not take place at Q₀ of complex III but at another location, activated by antimycin a. Antimycin a inhibits respiration by blocking transfer of electrons from cytochrome b₅₆₆ to ubiquinone, entailing an accumulation of semiubiquinone anions (Q⁻) at the Q₀ site.

![Diagram](image)

**Fig.10.2** The scheme proposed for cadmium and antimycin a inhibition of Complex III. Antimycin a (AA) prevents transfer of electrons to ubiquinone and cadmium (Cd) is proposed to non-competitively inhibit transfer of electrons to cytochrome b₅₆₆[177].

Accumulation of Q⁻ can rapidly reduce O₂, forming the superoxide anion radical, O₂⁻ and regenerating the quinone. The redox cycling of QH₂ can generate high amounts of ROS that may be able to reduce MTT, either directly or through an intermediate electron acceptor such as GSH[59]. ROS has been suggested as a possible source for tetrazolium salt formation. The observation in this project of cadmium decreasing reduction of MTT (exp.9.2), despite it itself being a potential source for ROS[9;20;177], decreases the likelihood of ROS being the only explanation for the observed Antimycin a increased MTT reduction (exp.9.5 and 9.12).

**Rotenone and cadmium:**

**Experiment 9.14** shows the effect on bioreduction of MTT when A6 cells are co-incubated with rotenone (10µM) and CdCl₂ (200µM). Both rotenone and cadmium inhibits production of MTT-formazan. Co-incubation of cadmium and rotenone shows a slightly higher activity than cadmium alone, in this experiment. This difference is very small and while MTT reducing activity is significantly different from samples with cadmium alone, it is likely an experimental artefact. The lack of a synergistic effect with co-incubation of cadmium and rotenone in this experiment indicates that cadmium instead acts at an ETC target distinct from that inhibited by rotenone.
**Oligomycin and Cadmium:**

**Experiment 9.15** shows the effects on reduction of MTT of co-incubation of cadmium (100µM) and oligomycin (5µg/ml). Oligomycin inhibits the ATP-synthase [180]. Oligomycin is an effective inhibitor of MTT-reduction even though it presumably does not directly inhibit the enzymes responsible for this. The inhibition of ATP-synthase may result in a hyperpolarisation of the mitochondrial inner membrane, as the proton gradient established by ETC enzymes is not utilized to drive ATP production [42]. The activity of ETC enzymes working against a high proton gradient and accumulation of electrons in the ETC, may indirectly be inhibited by oligomycin. Cadmium has a number of cellular effects, but may directly target complex III of the ETC [177], acting in concert with oligomycin to prevent the action of MTT-reducing enzymes of the ETC. Co-incubation would then result in an very effective limiting of MTT-formazan production, as evidenced by this experiment. The synergistic effects of cadmium and oligomycin could also indicate that cadmium may target the ATP-synthase.

**CCCP and Cadmium:**

**Experiment 9.16(1)** shows that A6 cells exposed to CdCl₂ (100µM) and co-incubated with CCCP (2.5µM) maintain a high formazan production. Cadmium is unable to counteract the CCCP induced increase in reduction of MTT.

The CCCP dependent increase in activity of MTT reducing enzymes can be explained as an uncoupler stimulated increase in the activity of ETC enzymes [76]. Activity of the MCU, responsible for mitochondrial uptake of cadmium (and calcium), depends on the proton gradient established by a normal functioning ETC [63]. Uncoupling removes the proton gradient, preventing MCU from moving cadmium from cytosol into the mitochondrion. ETC enzymes continue their high uncoupler stimulated activity without being inhibited by cadmium.

The results of this experiment should be compared with those of experiment 9.11 (co-incubation of rotenone, oligomycin and CCCP). Here the combined action of an ETC inhibitor and an ATP-synthase inhibitor, likewise failed to affect CCCP stimulated MTT-formazan production. This indicate that cadmium targets ATPsynthase and inhibits MTT reduction in a manner similar to oligomycin.

CCCP may affect bioreduction of MTT of isolated mitochondria, differently than in whole cell systems. It has been reported that CCCP can decrease MTT reducing activity of isolated mitochondria [103]. While the cause for this difference has not yet been explained, it needs to be adressed in order to fully understand the effect of CCCP on reduction of MTT.

**Experiment 9.17(2)** shows the data from exp. 9.16(1) calculated and plotted differently; the relative activity of MTT reducing enzymes when A6 cells were exposed to CdCl₂, was plotted relative to the activity of control samples. MTT-reduction activity of A6 cells exposed to CdCl₂ and CCCP was expressed as a percentage of the activity of samples incubated with CCCP.
The difference in activities between samples with CCCP and those of CCCP+cadmium, is smaller than cadmium relative to the pure controls. The experiments show that cadmium cannot counteract the CCCP induced increase in MTT-formazan production. It also has a relatively lesser effect as an inhibitor of MTT-reduction when co-incubated with CCCP.

The visual identification of morphological changes (exp.9.29) with co-incubation of CCCP and cadmium showed that CCCP was able to prevent most of the membrane deformation observed with cadmium exposure. This confirms that CCCP prevents or ameliorates the toxic effects of cadmium. CCCP eliminates the proton gradient that may be utilized by the MCU to transport cadmium into mitochondria [44]. This indicates that cadmium mediated changes to plasma-membrane structure is connected to mitochondrial dysfunction [10;122;133;138;177].

**Fig.10.3** A schematic representation of the electron transport chain of A6 cells, from NADH to O₂. Hypothetical coupling points of cadmium with the ETC are indicated by (-). The ETC complexes likely to be associated with reduction of MTT are also shown.

**Figure 10.3** shows the ETC with the proposed sites for cadmium inhibition. The MTT assay measures the combined output of several mitochondrial reductases, however MTT reduction is likely to be associated with complex I or III. The MTT assay measures cadmium's effect as an inhibitor of the ATP-synthase, and possibly complexes I and III.

10.6. **Cadmium toxicity affected by compounds affecting cellular calcium levels**

Calcium has often been associated with cadmium toxicity[10;21;108;184]. There are several mechanisms whereby cadmium can affect calcium homeostasis, for example through ionic mimicry or inhibition of calcium ATPases. Calcium being a universal cellular messenger, it is not surprising that cadmium affecting calcium homeostasis may have severe consequences for cells. A series of experiments were done to investigate the effect of other compounds affecting calcium levels, on cadmium inhibition of MTT reduction.

**Thapsigargin and Cadmium:**

**Experiment 9.18(1)** show the relative MTT-reducing activities of the A6 cells, resulting from co-incubation of CdCl₂ (5µM and 10µM) with Thapsigargin (1.6µM). **Experiment 9.19(2)** shows the data of the experiment calculated and presented in a slightly different manner from the method used in exp. 9.18(1). The first representation of the data follows the standard of giving activities as a
percentage of the relative activity of controls. The second plots the results of incubation of CdCl₂ (5µM and 10µM) as a percentage of the pure control samples and Thapsigargin+CdCl₂ (5µM and 10µM) as a percentage of Thapsigargin (1.6µM).

Thapsigargin increases cytosolic and mitochondrial calcium levels by release of calcium from ER, due to inhibition of the latter organelles calcium pump, SERCA [165]. Emptying of ER calcium stores, can result in opening of store-operated calcium channels, increasing the cytosolic uptake of calcium and cadmium [51]. Release of calcium from ER can increase mitochondrial calcium levels due to the close proximity of the two organelles, creating localized high concentrations of calcium, increasing its uptake [33]. Calcium act as a regulator and activator of a number of mitochondrial enzymes [46], seen here as a thapsigargin mediated increase in MTT-formazan production.

Experiment 9.19(2) shows that co-incubation with thapsigargin increases the relative effect of cadmium (10µM) toxicity. This can be explained by thapsigargin mediated emptying of ER calcium stores, opening SOC's on the plasma-membrane increasing cadmium entry into the cells.

Ruthenium Red and Cadmium:

Experiment 9.20(1) shows the results on MTT-reduction from co-incubation of ruthenium red (25µM) with CdCl₂ (40µM). The decrease in relative activity with incubation of ruthenium red is consistent with a theory of calcium regulation of activity of MTT-reducing mitochondrial enzymes [46]. Ruthenium Red is a non-competitive inhibitor of the MCU, responsible for the majority of calcium uptake in mitochondria. Incubation with RR can decrease mitochondrial calcium levels affecting activity of all calcium dependent processes of the organelle[151].

Experiment 9.21(2) presents the data from exp.9.20(1) calculated and plotted with MTT-reducing activities of samples exposed to Cd expressed as a percentage of controls, and ruthenium red+cadmium expressed as a percentage of activities of ruthenium red containing samples. The difference between the activities of control and CdCl₂ exposed samples is lesser than the difference between ruthenium red and samples exposed to ruthenium red+cadmium. This indicates that ruthenium red exacerbates the effects of cadmium on MTT-reducing systems.

Cadmium exposure decreases activity as expected (eg. exp.9.2). Co-incubation of the two toxicants decreases activity, acting synergistically. This is possibly a little contrary to what one could have expected as a primary means of cadmium entry into the mitochondrion is by the MCU, a primary target of ruthenium red[61]. It may indicate that the MCU does not represent the only path of entry for cadmium into the mitochondrion or cadmium does not need to enter the matrix of the mitochondrion to assert its toxic effects.
Fig. 10.4 An overview of the pathway for cadmium entry into the mitochondria of A6 cells. Cadmium may enter through SOC's after binding to and activating the plasmamembrane cation sensitive receptor (CSR). The CSR releases IP₃ which activates the IP₃-Receptor IP₃-R on the endoplasmic reticulum. Emptying of cellular stores of calcium, signals the SOC to open and cadmium enters the cell. Cadmium can then enter the mitochondria through the mitochondrial calcium uniporter (MCU). The MCU is inhibited by Ruthenium Red, preventing uptake of calcium and cadmium into the mitochondria. Thapsigargin inhibits the endoplasmic calcium ATPase (SERCA), preventing reuptake of calcium into the ER. Ionomycin is a calcium ionophore that increase permeability of calcium and cadmium across all membranes[51].

10.7. Cadmium, the MPT-pore and calpains

The following experiments were done to examine the MTT-assays ability to describe other aspects often associated with cadmium toxicity, namely the induction of calpains and activation of the MPT-pore.

Cyclosporin A and Cadmium:
Experiment 9.22(1) shows the results of incubation with cyclosporin a (5µM) and CdCl₂ (40µM). CsA is an inhibitor of the MPT-pore. Binding of CsA to the pore prevents its opening and onset of the MPT event integral to the intrinsic pathway of apoptosis. Cadmium mediated mitochondrial toxicity has often been linked to opening of the MPT-pore, and CsA has demonstrably prevented its opening after cadmium exposure[97;149].
The results of this experiment show that CsA increases reduction of MTT compared to the controls. The chances of MPT-pore opening and an MPT event increases with the age of cell-lines and continual subcultivations [10]. While the cells used in this experiment were seemingly fine and the measured absorbance values indicated normally functioning A6 cells, the result may indicate that the MPT-pore can be effectively blocked in otherwise normally functioning cells. The MPT-pore has been subject of extensive research as it is an integral part of the intrinsic pathway of apoptosis [63;69;81;94]. It has been suggested to operate in several different ways, possibly having a mechanism for transient and reversible opening as well as the non-reversible opening observed with apoptosis induction [10]. The need for transient opening or “flickering” of the MPT-pore is not clear, but can possibly explain the observation of an increase in activity observed with addition of CsA to the incubation medium.

Another potential explanation that was examined was the possibility that the increase was an artefact of the CsA agent ethanol. Ethanol can stimulate the activity of mitochondrial enzymes, and it could be expected to increase formazan production. The results of experiments with incubation of MTT with an amount of ethanol equal to the experiments with CsA showed no significant effect on the bioreduction of MTT (data not shown).

Co-incubation of cadmium with CsA had a higher activity of MTT-reducing enzymes than samples incubated with CdCl₂ alone, but still lower than that of the controls. The simplest explanation is that CsA increases activity independent of cadmium; a CsA increase opposing the cadmium dependent decrease.

**Experiment 9.23(2)** presents the data of exp. 9.22(1) with the MTT-reducing activities of cadmium containing samples exposed relative to activities of the toxicant-free controls. Cadmium+CsA was expressed relative to the activity of CsA exposed samples. There was no significant difference in the relative size of cadmium inhibition. This could indicate that the two effects, a CsA mediated increase in activity and a cadmium induced decrease in MTT-reduction, are independent in origin. CsA is not specific to the MPT-pore constituent Cyp-D, but may bind to other cyclophilins [149]. Binding of CsA to Cyp-A has been shown in some cell models to decrease calcium entry in mitochondria [46]. Such an effect could also decrease cadmium entry in mitochondria, as it has similar points of entry into mitochondria as calcium. If this was the case in these experiments, assuming a mitochondrial location for reduction of MTT, CsA could reasonably be expected to decrease the relative toxicity of cadmium when co-incubated. Instead these experiments indicate that CsA does not change cadmium toxicity.

*Calpain inhibitors I+II and Cadmium:*

Calpains have been proposed as being involved in cadmium mediated cell-death, notably in the apoptotic pathways [89;96]. Activated either directly by cadmium or by cadmium mediated increases in calcium levels [93]. Calpains having a broad range of targets could be expected to have several direct or indirect effects that would impact mitochondrial MTT-reducing enzyme activity. They have in some instances of cadmium toxicity been shown to indirectly initiate a mitochondrial permeability transition event, through opening of the MPT-pore, by activating apoptotic Bcl-2
family proteins[122;184]. Experiment 9.24 shows the MTT reducing activities of A6 cells exposed to cadmium and either of the Calpain Inhibitors I and II. The results of co-exposure of Calpain Inhibitor I (100µM) with CdCl₂ (200µM) showed no significant difference in activity from samples only exposed to cadmium (p>0.05). Calpain Inhibitor I alone had a small negative effect on MTT reducing activity compared to controls. The inhibitors itself may be somewhat toxic and can potentially stimulate cell-death [105;176]. A6 cells exposed to the Calpain inhibitor II (100µM) showed no significant difference in MTT-reducing activity when compared with controls. Co-incubation of Calpain Inhibitor II with cadmium (200µM) actually decreased MTT-reduction, compared to samples exposed to cadmium only. This observation can possibly be explained as cytopathic effects of Calpain Inhibitor II that affect the ability of A6 cells to respond to and compensate for cadmium toxicity.

The experiments show that Calpain activation is not responsible directly or indirectly for the decrease in MTT-reducing activity observed in A6 cells exposed to cadmium. Activation of calpains by cadmium has been described in the literature, but possibly occurs after a longer exposure period than used in this project (3h)[96;122;184].

10.8. Time-dependent variant MTT-assay:

Experiment 9.25 shows the results of an experiment using the MTT assay to measure the time dependence of cadmium toxicity. Exp.9.25 (I) shows a concentration curve slightly different from the 3 hour exposure period of experiment 9.2. CdCl₂ (10µM) seems to increase activity compared to CdCl₂ (5µM), presumably this is a mistake, an error in the experimental procedure. Activity decreases gradually along the concentration gradient until a maximum at CdCl₂ (200µM).

The experiment shows little difference in the concentration/activity curves between exposure periods of 2 and 3 hours. Fig.9.25 (II) is remarkably similar in appearance to Fig.9.25 (III). Activity drops sharply with even low concentrations of cadmium (10µM), but generally maintaining MTT-reduction at this level despite increasing concentrations Cd.

The similarity of Fig.9.25 (II) and Fig.9.25 (III), is indicative of cadmium in these particular experiments asserting a maximum effect after two hours, not increased by one additional hour of exposure. This is in accordance with earlier observations of cadmium toxicity in A6 cells, showing that cadmium enters cells and assert effects within 15 minutes [51].

Fig.9.25 (IV) presents data of CdCl₂ (200µM) from experiments I-III, showing that cadmium at this concentration decreases formazan production to a maximum of ~60% of controls, irrespective of the length of the three exposure periods.

The concentration dependence of MTT reduction seen in exp.9.25 (I-III) are dissimilar to the concentration curve described in Exp.9.2. This is presumably due to the difference in experimental setup, with experiment 9.25 including a washing step, removing most extracellular excess cadmium before MTT addition. In experiment 9.2 cadmium was co-incubated with the MTT reagent.

In these experiments cadmium entry into the cells is fast (under 1 hour), but can only inhibit a part of the reductases responsible for reducing MTT to its formazan. The result shows that inhibition of mitochondrial reductases reducing MTT, are inhibited in less than an hour.

Different reductase systems responsible for reduction of MTT may have different sensitivities towards cadmium. After cadmium is removed from the system, the enzymes responsible for ~60%
of the formazan production, recover their functions fast, while the remaining reductase systems responsible for ~40%, remain inhibited by cadmium.

10.9. Visual Identification of A6 cells:

Experiment 9.28 shows images of the A6 cells, taken at confluent growth with or without the characteristic dome-like formations. A6 cells were grown as per the standard procedure described in the Methods chapter for 1 or 2 weeks before images were taken.

Visual Identification of A6 cells exposed to CCCP and cadmium:

Experiment 9.29. shows several images of A6 cells exposed to cadmium and/or CCCP (a-e).

A6 cells under normal growth conditions have a very distinct morphology as observed in image (a). The images (a) and (b) show there are few visibly discernible changes in A6 cell structure, with incubation in 5mM Glucose-NaCl ringer. Image (b) shows A6 cells at t=0, before CCCP has had a chance to affect them. Image (a) shows the A6 cells in agent free medium after 3 hours. Some cells are less rounded and may have lost volume compared to cells at t=0. There does not appear to be many dead cells, with would be visible as cells with diffuse or disrupted membrane integrity and increased cell volume. The few light cells in image (a) are dead or dying cells detached from the bottom of the wells, these showing as lighter and rounder than attached cells.

Image (d) illustrates the effect of Cd (200µM) on A6 cells after 3 hours. Cadmium exposure of A6 cells leads to a biochemical perturbation with easily discernable and distinct cytopathic effects. Several cells show signs of swelling, when compared to image (a) of the uncontaminated cells. Disruption of the normal membrane structure is prominent, seen as a "fuzzying" of the edges of the cells. These cytopathic effects, are classic hallmarks of cadmium induced toxicity[4;20;62;92;184]. The morphological changes observed resembles necrotic cell-death in the disordered breakdown of cell membranes, more than that which would be expected in an activation of apoptosis [47].

Image (c) shows A6 cells exposed to CCCP (2,5µM) for 3 hours. The cells have decreased in size relative to the controls (a). Image (e) shows the effects of co-incubation of CCCP with CdCl2 (200µM). The number of damaged cells and the severity of damage to the membranes is much smaller than that observed in image (d) for cadmium exposed cells. Instead of the swelling observed with cadmium toxicity, cells decrease in size and become more rounded than cells at the beginning of experiments, seen in image (b). CCCP, an uncoupler of the mitochondrial electron transfer chain abrogates cadmium induced morphological changes.

Experiment 9.16 described the effects of co-incubation of cadmium and CCCP with A6 cells, on the reduction of MTT to its formazan. The experiment showed shat CCCP was very effective in maintaining a high activity of MTT-reducing enzymes, compared to cadmium-only exposed cells. The images (d) and (e) confirm that this is probably partly due to CCCP preventing cadmium mediated cellular dysfunction.

Experiment 9.2 estimated cell-death induced by CdCl2 measured by the vital dye, Trypan blue exclusion assay. The Cadmium mediated changes to plasma-membrane structure observed in image (d) of exp.9.29 indicate a higher degree of cell death, than what was determined with the Trypan Blue Exclusion assay. The two methods have a very important difference in their experimental procedure that could explain part of this observation. The MTT assays used cells at confluent growth, with fully differentiated cells, many in the G0 growth phase. The visual identification series
of experiments used dividing cells, as cells were trypsinated and transferred at a low cell-density, to
the microplate the day before the experiments were executed. Fully differentiated A6 cells can have
a different expression of receptors and channels than cells at confluent growth, resulting in a
somewhat different response to toxicants. The relative "oversensitivity" towards cadmium of the
growing cells in the visual identification series of experiments, could be explained by this.
The visual identification series of experiments were purely qualitative, with regards to estimations
of cell-death, making a direct comparison of cell-death frequencies with the quantitative results
obtained in experiment 9.2 difficult.

Discrepancies between what may observed by the visual identification, vital dye assays and
enzymatic assays, was also noted by Smee et al. They compared the results of using a Neutral Red
vital dye assay, MTT-assay and visual identification and concluded that there is not always a
correlation between these three types of assays. They suggested that despite visual identification of
morphological alterations, sometimes cells would not be stained by vital dyes and showing values
as for untreated cells. Likewise enzymatic based assays such as the MTT-assay might give wrong
estimates of toxicity if the enzymes responsible for conversion of the MTT-reagent were targets of
the drugs tested [153]. These points have all been observed and addressed a number of times in the
literature[15;24;35;67;74;104;153;155;172].

Belyaeva et al. have investigated the effect of cadmium on the viability of Cd\textsuperscript{2+}-exposed hepatocytes,
and when this is modified by agents such as rotenone, antimycin a, oligomycin and CsA. The article
investigating reactive oxygen species produced by mitochondrial respiratory chain after Cd\textsuperscript{2+}-
induced injury of rat ascites heptoma AS-30D cells, a series of experiments use the Trypan Blue
Exclusion assay to investigate changes in viability, expressed as a percentage blue stained cells of
the total number of cells counted[9].

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control (3h)</th>
<th>100µM CdCl\textsubscript{2} (3h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>9±1</td>
<td>27±2*</td>
</tr>
<tr>
<td>CsA (5µM)</td>
<td>8±1</td>
<td>14±4**</td>
</tr>
<tr>
<td>Rotenone (1µM)</td>
<td>28±2</td>
<td></td>
</tr>
<tr>
<td>Antimycin a (1µM)</td>
<td>14±1</td>
<td>17±2**</td>
</tr>
</tbody>
</table>

Table.10.1 (A selection of data from experiments by Belyaeva et al.) The cells were incubated with or without CdCl\textsubscript{2} for 3 hours and the additions specified in the table under treatment. The numbers in the columns express percentage of trypan blu-positive cells. The data are mean values for 4 experiments ±SE. Statistical significance: *P<0,05 with respect to the corresponding control; **p<0,05 with respect to Cd alone[9].

Compared to the Trypan Blue assays in this project, the table shows that the rat hepatocytes are
more sensitive than A6 cells towards CdCl\textsubscript{2}. CsA shows no change compared to the control unless
co-incubated with Cd were it reduces cell-death. Rotenone treatment does change Cd-induced cell
death. Antimycin a may increase cell death compared to cadmium free controls, but with co-
treatment decreases it relative to CdCl\textsubscript{2} alone.

The results of this projects MTT-assays show similar to above that, CsA (5µM) (exp. 9.22) and
antimycin a (2µM) (exp. 9.12) ameliorate the negative effects of CdCl\textsubscript{2}. In the experiments by
Belayeva et al. there was no significant change in treatment with rotenone (1µM) compared to
CdCl\textsubscript{2} alone. The results of this project indicate a small positive effect of co-treatment with
rotenone (exp. 9.14), though not as effective as antimycin.
An important finding by Belyaeva et al. reported in this article was that CCCP reduced the generation of ROS induced by Cd to the levels of controls. CsA and to an even lesser degree antimycin, also decreased ROS-production. Rotenone did not show any effect upon the viability of the rat hepatocytes, as described in the table above. These observations and others were used to conclude that ROS generation was an early event of cadmium toxicity in AS30-D cells, related to cadmium disruption of the normal function of complex III of the ETC. [9].
11. Summary

This project presents a novel way of using the classic MTT viability assay, originally described by Mossman, to estimate changes in mitochondrial function [116]. The project used the A6 cell model, examined and found discrepancies between cell viabilities after a three hour cadmium exposure period, when this was determined by either the Trypan Blue Exclusion assay or the MTT assay. The MTT assay severely underestimated cell viability, possibly due to cadmium acting as an inhibitor of the enzymes responsible for MTT reduction. A series of experiments then used the modified MTT assay, to examine the effects of various mitochondrial inhibitors and compounds affecting calcium signalling, on reduction of the MTT reagent.

The experiments done in this project have not led to a definite conclusion as to the specific coupling points of MTT-reducing enzymes in regards to the electron transport chain constituents. The observations described here lead to some conclusions.

The MTT assay measures the combined output of a number of reductases. The results lead to making a very plausible argument for MTT-reduction in A6 cells being at least partly relegated to the province of the mitochondria. MTT reduction very likely takes place at several locations on the ETC.

MTT reduction very likely takes place downstream of the target for rotenone inhibition (ETC complex I) at one or more points between it and the target site of NaN₃ (complex IV).

The ETC inhibitor antimycin displays an unusual effect on reduction of MTT, compared to the experiments using other ETC inhibitors, in that it increases formation of MTT-formazan. It makes it unlikely that MTT reduction takes place directly at complex III. It is indicative of a coupling point of MTT-reduction with the ETC between complexes I and III.

The ATP-synthase inhibitor oligomycin was very effective in inhibiting MTT reduction, while the uncoupler CCCP increased it three-fold or more. CCCP likely achieves this through uncoupler stimulation of the ETC, while oligomycin has an opposite effect by indirectly blocking all ETC activity.

The results of experiments with ETC inhibitors were complemented by experiments with compounds affecting calcium signalling. They revealed that a decrease in mitochondrial calcium levels decreased reduction, while an increase in calcium increased production of formazan. ETC enzymes are regulated by and very sensitive to changes in mitochondrial calcium. These observations lent further credit to the hypothesis that MTT reduction in A6 cells is associated with mitochondria.

The MTT assay was used to describe cadmium impairment of the mitochondria. It was found that cadmium asserted its toxic effects at more than one site on the ETC and at the mitochondrial ATP-synthase complex.
12. Perspectives

The experiments done here certainly serve to illustrate potential problems and shortcomings of the MTT-assay in determining cell-death and changes in viability, when induced by cytotoxic drugs targeting and affecting activity of mitochondria.

The MTT assay used in this project shows promise as an inexpensive method of determining the status of the mitochondrial electron transport chain. The original assay has seen much debate as the location of MTT reducing enzymes, much less the specific enzymes responsible, has been disputed [19;103]. A number of articles claim reliance on mitochondrial enzymes with nearly as many citing a location distinct from mitochondria to be the site for reduction of MTT. MTT reduction may very well prove to be cell-specific, and even though it sees ubiquitous use as a cell viability assay, there are many aspects of it that should be investigated and described.

While the most common theory holds that MTT-reduction is primarily located in mitochondria, a number of researchers have questioned this view or concluded otherwise[1;13]. It could be interesting to continue the work done here with the modified assay, with fractionation studies using gradient centrifugation. Of particular interest would be performing experiments on free mitochondria, and non-mitochondrial fractions. This would make it possible to compare the effects of ETC inhibitors on the MTT assay, with other classical assays measuring specific ETC activity.

The following condenses the material described in previous chapters into a single figure, giving an overview of the A6 cell. The figure is a generalized scheme of the organelles, ion channels and other items of primary interest to this project, with focus on the toxic mechanism of cadmium. The proposed location for reduction of MTT is shown, namely at the mitochondrial ETC. What should be obvious even from this simplified figure are the complications that can arise as feedback mechanisms and interactions between different cellular components affect the mitochondria. Interpreting the data obtained using the MTT assay, either as a viability assay or especially in an effort to monitor specific ETC activity, is necessarily a very complex affair. Cadmium asserts its toxic effects at many levels not shown in this model. It can however serve as the basis when contemplating further experiments using the MTT assay to study cadmium toxicity in A6 cells. Refer to the preceding chapters for specific details about one of the components or the interaction between elements of the figure.
Fig. 12.1 An overview of the A6 cell focussing on mitochondria, calcium signalling and cadmium activation of the “intrinsic” apoptosis pathway. The figure summarizes material presented in the previous chapters with focus on calcium signalling of the mitochondria. The figure shows the hypothetical location for the reduction of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at the mitochondrial electron transport chain (ETC) and deposition of MTT-formazan crystals at the plasma membrane. The activity of the ETC is inhibited by Rotenone, Antimycin A and Cadmium. Cadmium mediates opening of the SOC’s via CsR signalling the release of calcium from the ER IP3-R. Cadmium enters the cell through SOC’s and the mitochondria through calcium transporters. In the mitochondria calcium inhibits ETC enzymes responsible for reduction of MTT.

Calcium-sensing receptor (CSR); Store operated calcium channels (SOC); Mitochondrial Na+/Ca2+ exchanger (mNCE); Rapid mode calcium channel (RaM); mitochondrial calcium uniporter (mCU); Mitochondrial permeability transition pore (MPT-P); Mitochondrial permeability transition (MPT); Adenine nucleotide translocase (ANT); Voltage-dependent anion channel (VDAC); Cyclophilin-D (Cyp-D); Cytochrome c (Cyt-c); Apoptosis activating factor-1 (Apaf-1); Carbonyl cyanide m-chlorophenylhydrazone (CCCP); Reactive oxygen species (ROS); Cadmium (Cd2+); Calcium (Ca2+); inositol 1,3,4 triphosphate (IP3); Inositoltriphosphate-receptor (IP3-R); Sarco-/Endoplasmic reticulum calcium ATPase (SERCA). The figure is combined from two primary sources, a figure in the master thesis of Søren S. Søgaard[156] and an article by Brookes et al[27].
References


induced acute toxicity in LLC-PK1 cells. *Toxicology, 183*, 211-20.


13. Appendix

13.1. Appendix I

Light Microscopy Imaging and photography.

The Leica light microscope dm_irb was used in the Visual Identification experiments and in establishing cell-count and cell-death in Trypan Blue exclusion assays. The experimental procedures for the two types of experiments is described in the method chapter. This appendix will give an overview of the basic function and settings of the light microscope, including camera settings and IM50 imaging software used in the project.

Images were taken of A6 cells in 1ml x 24 well microtiterplates or 25 ml growth-flasks at 10 or 20 times magnification. The occular increases this magnification an additional x10 for a total magnification of x100 or x200.

![Fig. a schematic of the parts of the Leica dm_irb light microscope, without the camera. The image shows the location of the sample holder (6) and occular (11). The image shows the placement of fine/course focus wheels (6), condenser focus (5), light power adjuster (5), dichroic filter wheel (15), lenses (7) and other adjustable settings.](image)

The sample being examined is placed under the occular in the sample holder. The intensity of the light source is adjusted, the focus ring is reduced to the minimum needed for a complete image to be seen through the occulars. The course and fine image focus wheels (6) are adjusted to focus on the A6 cells.

When the image seen through the occular is sharp the camera is turned on and the imaging software IM50 is started. The image software includes a number of options for archiving and organizing images and viewing existing photographs. The camera is activated by selecting the Acquire option. This will show the image seen through microscopes occulars on-screen. The software and camera includes options for adjusting exposure length, colour scheme, contrast and other parameters. The microscopes fine adjustment wheel (6) is used to focus the on-screen image. The sample is moved with the sample bench adjusters, to select a specific portion of the sample being examined. Images are then taken and saved with the IM50 program. Each of the images in the archive includes an attached record of settings and time when the specific image was taken.
13.2. Appendix II

**Cell counting**

The Trypan Blue exclusion assay, is based on the counting of living and dead cells, the latter visibly dyed stained with the Trypan dye. The counting of cells was done with the aid of a Bürker-Türke counting chamber depicted below.

The Bürker-Türke counting chamber is lightly moistened on each side of the counting area and a coverglass is slid into place. When the coverglass is positioned correctly, refraction patterns known as Newton's Rings will form due to refraction of light between the two pieces of glass. 10-20µl of the sample being measured is placed at each end of the coverglass, with surface tension distributing the liquid evenly across the counting grid and ensuring an equal volume is deposited in each of the counting chambers. This allows for accurate comparison of different samples and most importantly the calculation of cell-densities, as the volume is known for each area of the counting chamber. The counting chambers are examined using the same microscope settings used for visual identification of cell-death described in the **Methods** chapter. The grid-lines seen in the chamber allows one to calculate the exact volume of the sample. This allows for accurate comparison of living and dead cells of different samples.

The image below shows the area of each counting chamber. The number of living and dead A6 cells in each cell of the counting chamber is recorded and the percentage of dead cells is calculated. The cell count is established for each of the squares marked IV below, each of which is divided into 16 lesser squares, marked III. The volume of each square is known, and cell-density can thus be calculated as the number of cells per a known volume, usually transformed into a value of cells/ml.
13.3. Appendix III

Synergy HT Microplate reader.
The Synergy HT micro-plate reader is a multi-purpose spectrofotometer able to measure absorbance Fluorescence and luminescence. The software KC4 is used to programme the reader, record and export the measured data.

KC4 includes a "Wizard" function that gives a simple and intutive way to select and program parameters used by the microplate reader, including wavelength of emission or measurement, interval between measurements, the well(s) being measured. The software allows programming of the Synergy HT's internal sampleholders and injectors to add reagents to the wells without removal of the testplate. Many other parameters, such as incubation temperature can easily be managed using the KC4 software.
The above screenshot shows the window where choices are made for a specific measurement protocol. For the standard MTT assay, one would usually choose: Endpoint measurement of Absorbance values at 595 nm. The plate type and the specific wells being measured can be chosen. Temperature control and optional shaking is usually unnecessary as the platereader is only used for measurement of absorbance values and not used as an incubator. The program includes options for calibrating the equipment for a particular testplate.

The KC4 program includes options for exporting data or performing calculations directly on the data measured. This specific function was not used in this project, but data was stored in the original format and then exported and analyzed using the Microsoft Office program Excell.
The above image shows the output scheme from measuring a typical MTT assay. The value in each cell corresponds to the absorbance values of a single well on the testplate, measured according to the protocol generated with the KC4 wizard. Measurements should include controls, test-reagents, blinds and the solvent.
13.4. Appendix IV

Applied Statistics
The Microsoft software package Excel including its statistical add-ons, was used in this project to
determine means and standard error of the experimental data.

The **Standard Error** of a measurement is the standard deviation in the error from the mean, of the
measuring method. It is the standard deviation of the difference between the measured or estimated
values and the true values. SE is calculated as the Standard Deviation (σ or SD) divided by the
square root of the replicate number (n).

\[ S_E = \frac{\hat{\sigma}}{\sqrt{n}} \]

The **Standard Deviation** is equal to the square root of the variance of the samples and calculated as
follows:

\[ s = \sqrt{\frac{1}{N-1} \sum_{i=1}^{N} (x_i - \bar{x})^2} \]

- s = standard deviation
- N-1 = degrees of freedom
- xi = the value of a given observation from x_i to x_n
- \( \bar{x} \) = the mean of the observations

**Student's t-test**
This is a statistical method to determine if the means of two sample populations are different.
H_a: \( \mu_1 \neq \mu_2 \), H_0: \( \mu_1 = \mu_2 \). The test determines if H_0 can be discarded. The t value used to test the
hypothesis is calculated as follows:

\[ t = \frac{\overline{X}_1 - \overline{X}_2}{s_{\overline{X}_1-\overline{X}_2}} \text{ where } s_{\overline{X}_1-\overline{X}_2} = \sqrt{\frac{(s_1)^2 + (s_2)^2}{n}} \]

- s is the standard deviation or pooled sample standard deviation, and the denominator is the standard
error of the difference in the two means.
13.5. Appendix V

Example of the calculation of MTT reducing activity.

Below is shown an example of how the values were calculated of the percent of activity of MTT reducing enzymes in the various MTT assays. The calculations are derived from Absorbance values measured by the SynergyHT spectrophotometer measured at $\lambda_{595}$. The average of the absorbance values of Blinds is subtracted from all samples. Percent activity is then calculated by dividing the Absorbance of a sample with the activity of the corresponding control sample and multiplying by 100%.

<table>
<thead>
<tr>
<th></th>
<th>Absorbance ($\lambda_{595}$)</th>
<th>absorbance-blind(avg)</th>
<th>%Activity of Controls</th>
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<tr>
<td>Control - a</td>
<td>0.326</td>
<td>0.282</td>
<td>100</td>
</tr>
<tr>
<td>Control - b</td>
<td>0.36</td>
<td>0.316</td>
<td>100</td>
</tr>
<tr>
<td>Control - c</td>
<td>0.3</td>
<td>0.256</td>
<td>100</td>
</tr>
<tr>
<td>Control - d</td>
<td>0.351</td>
<td>0.307</td>
<td>100</td>
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<tr>
<td>CdCl2 (40µM) - a</td>
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</tr>
<tr>
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<td>0.235</td>
<td>0.191</td>
<td>60.44</td>
</tr>
<tr>
<td>CdCl2 (40µM) - c</td>
<td>0.238</td>
<td>0.194</td>
<td>75.78</td>
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<tr>
<td>CdCl2 (40µM) - d</td>
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<td>0.199</td>
<td>64.82</td>
</tr>
<tr>
<td>RuR (25µM) - a</td>
<td>0.247</td>
<td>0.203</td>
<td>71.99</td>
</tr>
<tr>
<td>RuR (25µM) - b</td>
<td>0.245</td>
<td>0.201</td>
<td>63.61</td>
</tr>
<tr>
<td>RuR (25µM) - c</td>
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<td>0.204</td>
<td>79.69</td>
</tr>
<tr>
<td>RuR (25µM) - d</td>
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<td>0.203</td>
<td>66.12</td>
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<tr>
<td>CdCl2+RuR - a</td>
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<td>0.103</td>
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<tr>
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<td>0.136</td>
<td>0.092</td>
<td>29.11</td>
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<tr>
<td>CdCl2+RuR - c</td>
<td>0.138</td>
<td>0.094</td>
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<td>CdCl2+RuR - d</td>
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<td>0.127</td>
<td>41.37</td>
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<td>Blind</td>
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<table>
<thead>
<tr>
<th>Control</th>
<th>CdCl2 (40µM)</th>
<th>RuR (25µM)</th>
<th>CdCl2+RuR</th>
<th>mean</th>
<th>se</th>
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<tr>
<td>100</td>
<td>65.96</td>
<td>71.99</td>
<td>36.52</td>
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<td>64.82</td>
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<td>41.37</td>
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<tr>
<td>0</td>
<td>3.24</td>
<td>3.57</td>
<td>2.53</td>
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</tbody>
</table>
13.6. Appendix VI


Mitochondrial metabolic function evaluated with use of the MTT assay.

Claes W. H. Schütt and Henning F. Bjerregaard, University of Roskilde, Department of Science, Systems and Models, 4000 Roskilde, Denmark. HFB@RUC.DK

Measuring of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT), is a commonly used method in determining cell proliferation and in evaluating toxicity of a wide variety of compounds. Mitochondria have been considered as the possible primary site of MTT reduction generating colored byproduct. Cells must be viable and capable of endocytosis for the cell to reduce MTT, whereas increased cell numbers generate increased byproduct generation, and thus indicate cell proliferation. MTT is converted to its colored formazan byproduct via a group of nonspecific mitochondrial dehydrogenases, including NADH dehydrogenase, malate dehydrogenase, and succinic dehydrogenase. Byproduct formation represents the enzymatic activity of the mitochondria. Alterations in MTT reduction that cannot be explained by changes in viability or proliferation are most likely explained by alterations in the activity of these enzymes. MTT conversion could therefore be a good indicator of mitochondrial enzyme activity as well as viability and proliferation.

The aim of this study is to evaluate the MTT assay for use in short-term (three hours) measurements in order to avoid the MTT formation caused by cell proliferation, and specifically to determine its suitability in investigating mitochondria function. Studies were done on a cell line from the distal part of the kidney (A6 cells), which exhibit characteristics and morphological features of the epithelia of distal tubules in the human kidney. Free cells were incubated in NaCl-ringer solution along with MTT and other reagents for 3 hours. The incubation period was terminated by centrifugation of the cells, removal of the incubation medium and solubilization of the MTT byproducts by addition of isopropanol, before measuring the absorbance with a spectrophotometer at 595 nm. The activity of the control samples was compared to samples of cells incubated with compounds including cadmium chloride to induce cell death, antimycin A and rotenone, two inhibitors of the mitochondria electron transport chain (ETC), oligomycin an ATP-synthase inhibitor, as well as CCCP, an uncoupler of the proton gradient established by the activity of the ETC.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CdCl₂</th>
<th>CCCP</th>
<th>CCCP+CdCl₂</th>
<th>Oligomycin</th>
<th>Rotenone</th>
<th>Rot.+oligom.</th>
<th>antimycin A</th>
<th>anti.A+CdCl₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>100±0</td>
<td>40,7±7,2</td>
<td>300,2±15,6</td>
<td>216,6±11,9</td>
<td>34,1±6,9</td>
<td>73,6±0,5</td>
<td>17,2±3,1</td>
<td>117,5±2,3</td>
<td>44±5,7</td>
<td></td>
</tr>
</tbody>
</table>

CdCl₂(100µM), CCCP(2.5µM), oligomycin(5µg/ml), rotenone(10µM), antimycin(2µM). The measured absorbance values, expressed as % of control, are means ± SE of 3 to 6 experiments.

Incubation with oligomycin and rotenone decreased the amount of MTT-formazan formed and antimycin A gave a slight increase, showing that mitochondrial function may be affected and measured by the assay. The study showed that CCCP increased the activity by at least 200%, and this CCCP-dependent increase was somewhat tolerant of concurrent incubation with CdCl₂. CdCl₂ alone caused a marked decrease in the production of formazan, caused by the destruction of cellular integrity confirmed by visual microscopic inspection. This cellular destruction was seen to be negated by the additional incubation with CCCP. The results showed that MTT reduction could be modulated with agents that are supposed to interact with mitochondrial metabolic function. However, the specific mechanisms for the effects remain to be elucidated by future experiments.

http://www.ssct.net/
13.7. Appendix VII

Visual Identification of A6 Cell Death from exposure to mitochondrial inhibitors.
The following pages present a selection of images of A6 cells using the technique described in the Methods chapter for visual identification of cell death using light microscopy imaging. The results from a similar set of experiments with CCCP and Cadmium exposure were presented in the Results chapter. The Images in this Appendix will not be discussed, but are presented here as an additional set of observations of A6 cells. The images can be compared with the data from the series of MTT-assay experiments using the same mitochondrial inhibitors.

The images were taken of A6 cells that had been harvested from confluent growth. 10µl of the harvested cell suspension (~1*10^6 cells/ml) was transferred to the bottom of the wells on a 24x1 ml plate. 1ml standard growth medium was added to each well and the cells were incubated overnight. At the start of an experiment, the growth medium was removed from the wells and a premixed series of 1 ml of 5mM Glucose-NaCl Ringer samples containing the various inhibitors, was added to numbered wells on the testplate. Images of the A6 cells were taken at the beginning of experiments and after a three hour incubation period. The images were taken using a Leica dmirb light microscope and associated camera and IM50 imaging software.
A6 cells: Control at time = 3 hours

A6 Cells: Cadmium (100µM) at time = 0 hours
A6 Cells: Cadmium (100µM) at time = 3 hours
A6 Cells: Antimycin A (2µM) at time = 0 hours

A6 Cells: Antimycin A (2µM) at time = 3 hours
A6 Cells: Antimycin A (2µM) + Cadmium (100µM) at time = 0 hours

A6 Cells: Antimycin A (2µM) + Cadmium (100µM) at time = 3 hours
A6 Cells: Oligomycin (5µg/ml) at time = 0 hours

A6 Cells: Oligomycin (5µg/ml) at time = 3 hours
A6 Cells: Oligomycin (5µg/ml) + Cadmium (100µM) at time = 0 hours

A6 Cells: Oligomycin (5µg/ml) + Cadmium (100µM) at time = 3 hours
A6 Cells: Thapsigargin (1,6µM) at time = 0 hours

A6 Cells: Thapsigargin (1,6µM) at time = 3 hours
A6 Cells: Thapsigargin (1.6µM) + Cadmium (100µM) at time = 0 hours

A6 Cells: Thapsigargin (1.6µM) + Cadmium (100µM) at time = 3 hours
A6 Cells: Sodium Azide (2.5mM) at time = 0 hours

A6 Cells: Sodium Azide (2.5mM) at time = 3 hours
A6 Cells: Sodium Azide (2.5mM) + Cadmium (100µM) at time = 0 hours

A6 Cells: Sodium Azide (2.5mM) + Cadmium (100µM) at time = 3 hours
A6 Cells: Rotenone (5µM) at time = 0 hours

A6 Cells: Rotenone (5µM) at time = 3 hours
A6 Cells: Rotenone (5µM) + Cadmium (100µM) at time = 0 hours

A6 Cells: Rotenone (5µM) + Cadmium (100µM) at time = 3 hours