UNIVERSITY OF TARTU

FACULTY OF SCIENCE AND TECHNOLOGY

INSTITUTE OF MOLECULAR AND CELL BIOLOGY

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OGG1 inhibition as combination treatment for cancer therapy

Master thesis
Biomedicine
40 ECTS

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TARTU 2016

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Novel anticancer therapies are needed to overcome drug resistance of chemotherapeutic drugs leading to tumor relapse. Combination therapy with target-specific drugs underlies great potential to increase the efficiency of chemotherapy. Increased DNA repair capacity is one of the resistance mechanism of chemotherapy drug cisplatin. Here, inhibitors against DNA glycosylase OGG1 were studied to overcome cisplatin resistance. Combination treatment of OGG1 inhibitor with cisplatin reduced the proliferation of cisplatin resistant bladder cancer cells, whereas sensitive cells did not respond. Increased OGG1 levels in resistant cells and decreased OGG1-GFP mobility upon cisplatin treatment suggests that OGG1 might support cisplatin resistance. Moreover, the activity of OGG1 upon MTH1 inhibition was studied as a side project as both of these enzymes are responsible for 8-oxoG repair, but the findings did not support a role of OGG1 in mediating DNA repair upon MTH1 inhibition. Overall, this study gives insights how OGG1 inhibition could be exploited in anticancer combination therapy.

Keywords: base excision repair, cisplatin, OGG1, MTH1, small-molecule inhibitors

CERCS B200 Cytology, oncology, cancerology

DNA reparatsioonivalgu OGG1 inhibitsioon kui potentsiaalne kasvajate kombineeritud ravi

Marianna Tampere

Tänapäeval on suur vajadus uute vähivastaste ravimite arenduseks, sest kasvajate keemiaravi efektiivsus on tihti ajutine ning aja möödudes areneb ravimresistentsus. Kuna suurenenud DNA reparatsiooni maht on üheks keemiaravim cisplatini resistentsusmehhanismiks, uuriti käesolevas töös kuidas on võimalik DNA glükosülaasi OGG1 inhibitsiooniga cisplatini resistentsust ületada. Töö tulemusena selgus, et rakkude elulemus langes cisplatini ja inhibiitori koostoimel. OGG1 kõrgem ekspressioon cisplatinile resistentsetes rakkudes võrreldes tundlike rakkudega ja OGG1-GFP mobiilsuse langus vastusena cisplatinile viitab võimalusele, et OGG1 osaleb cisplatini resistentsuse tekkel. Kõrvalprojektina uuriti kuidas OGG1 valgu aktiivsus mõjutab rakkude vastust MTH1 inhibiitorile, sest need ensüümid vastutavad 8-oxoG elimineerimise eest rakus. Saadud tulemused vihjavad, et OGG1 ei ole MTH1 inhibiitori poolt indutseeritud DNA kahjustamise reparatsiooniga seotud.

Märksõnad: aluse asendamise reparatsioon, cisplatin, OGG1, MTH1, inhibiitor

CERCS B200 Tsütoloogia, onkoloogia, kantseroloogia

ABBREVIATIONS

8-oxoA – 7,8-dihydro-8-oxoadenine

8-oxoG – 8-oxo-7, 8-dihydro-2’-deoxyguanosine

8-oxo-dGMP – 8-oxo-2'-deoxyguanosine-5'-monophosphate

8-oxo-dGTP – 8-oxo-2'-deoxyguanosine-5'-triphosphate

A – adenine

BER – base excision repair

C – cytosine

CETSA – cellular thermal shift assay

Chr – chromatin fraction

Cl – chlorine

Cyt – cytoplasmic fraction

DMSO – dimethylsulfoxide

DDR – DNA damage response

dsDNA – double stranded DNA

dNTP – deoxyribonucleotide

EMSA – electrophoretic mobility shift assay

ERCC1 – excision repair cross-complementing 1

FapyG – 2, 6-diamino-4-hydroxy-5-formamidopyrimidine

FDA – Food and Drug Adminstration

FRAP – fluorescent recovery after photobleaching

G – guanine

GFP – green fluorescent protein

GSH – glutathione

γH2AX – phosphorylated histone 2AX

H3 – histone H3

IC50 – half-maximum inhibitory concentration

IR – ionizing radiation

$KBrO_3$ – potassium bromate

MEFs – mouse embryonic fibroblasts

MetOH – methanol

Mono-Ub – monoubiquitinated

MTH1 – MutT homolog 1

MUTYH – MutY homolog

NEIL1 – Nei like DNA glycosylase 1

NER – nucleotide excision repair

NH_3 – ammonia

OGG1 – 8-oxoguanine DNA glycosylase

PARP – poly (ADP-ribose) polymerase

Pol β – polymerase β

Pol η – polymerase η

Pt – platinum

RFU – relative fluorescence unit

RIPA – radioimmunoprecipitation assay

RNAi – RNA interference

ROS – reactive oxygen species

siNT – non-targeting siRNA

siOGG1 – OGG1 siRNA

siRNA – small interfering RNA

SMUG1 – single-strand-specific monofunctional uracil DNA glycosylase 1

Sol. Nuc. – soluble nuclear fraction

ssDNA – single stranded DNA

UV – ultraviolet

XP – xeroderma pigmentosum

XPF – xeroderma pigmentosum complementation group F

INTRODUCTION

Acquired resistance for commonly used anticancer chemotherapeutic drugs is a major problem leading to tumor relapse and increased mortality of the patients. Therefore the development of novel therapies to overcome drug resistance is highly needed. Combination therapy with target-specific drugs underlies great potential to avoid the development of acquired resistance and increase efficiency of chemotherapy.

Cancer is a disease of high proliferation capacity as well as increased oxidative stress load. To maintain their survival, cancer cells harness high replicative potential and DNA protective mechanisms. For that reason, the majority of chemotherapeutic agents, including cisplatin, target DNA replication and induce DNA damage to kill rapidly dividing cancer cells. Besides creating DNA crosslinks, cisplatin induces reactive oxygen species (ROS) leading to oxidative nucleobase lesion 8-oxo-7, 8-dihydro-2'-deoxyguanosine (8-oxoG) in both DNA and its precursor pool. 8-oxoG is eliminated by MTH1 protein from the nucleotide pool and repaired by DNA glycosylase OGG1-dependent base excision repair (BER) in DNA. The initial effective response to cisplatin treatment is often temporary and acquired resistance is developed. Increased DNA repair capacity has been described as one of the resistance mechanisms.

The main aim of this study was to investigate OGG1-dependent molecular mechanisms underlying cisplatin resistance. For that, small molecule compounds developed in the host lab targeting OGG1 activity were used. Target engagement properties of OGG1 inhibitors in cells were evaluated by cellular thermal shift assay (CETSA). In order to study how inhibition of DNA repair might overcome cisplatin resistance, cisplatin resistant and sensitive bladder cancer cells were used. OGG1 inhibitors were applied to cells in combination with cisplatin and viability and colony formation assay was used to investigate the combination therapy effect. Moreover, OGG1-mediated cisplatin DNA damage repair was indirectly studied by fluorescent recovery after photobleaching (FRAP).

According to the CETSA results, all of the tested OGG1 inhibitors stabilize the target protein in HL-60 cells, suggesting their binding to OGG1. Based on the previous *in vitro* results, inhibitor 10 (#10) was chosen for further experiments. Combination treatment of inhibitor #10 and cisplatin sensitized cisplatin resistant bladder cancer cells whereas sensitive cells did not responded. Increased OGG1 levels in cisplatin resistant cells compared to sensitive cells suggests that OGG1 might support cisplatin resistance. Decreased mobility of OGG1 labelled with green fluorescent protein (OGG1-GFP) after cisplatin treatment detected by FRAP suggests increased OGG1-GFP binding to cisplatin induced DNA damage sites. These

findings indicate a supportive role of OGG1 activity for cells upon cisplatin treatment and illustrate OGG1 as a promising anticancer target in cisplatin resistant tumors.

Moreover, the host laboratory recently presented MTH1 inhibition as a novel anticancer strategy, by increased incorporation of 8-oxoG into DNA. As OGG1 is responsible for 8-oxoG repair, the activity of OGG1 upon MTH1 inhibition was studied as a side project. Findings from small interfering RNA (siRNA)-based OGG1 depletion showed no sensitization to MTH1 inhibitor TH588. In addition, findings from FRAP and subcellular fractionation did not support a role of OGG1 in mediating DNA repair upon MTH1 inhibition. Overall, this study was a part of a larger project that aims to elucidate how inhibition of OGG1-dependent DNA repair could be exploited as a combination therapy for cancer treatment.

This study was conducted at Helleday Laboratory, Karolinska Institutet - SciLifeLab, Department of Medicinal Biochemistry and Biophysics, Division of Translational Medicine and Chemical Biology.

1.1 DNA damage

Maintenance of cellular homeostasis during cell division requires coordinated control over numerous key processes including DNA replication, repair and transcription. This ensures error-free duplication, faithful segregation and successful transmission of chromosomes to their daughter cells. Naturally, changes at low frequency during those key processes generate genetic variability and drive evolution (Wray, 2007). However, increased rate of genomic changes can accumulate over time and drive tumorigenesis.

Despite that DNA is the carrier of genetic information, its chemical structure is compromised by numerous factors originating from external sources such as ultraviolet (UV) light and anticancer agents or endogenous cellular processes like oxygen respiration which all result in various types of DNA damage (Figure 1a). If left unrepaired, cells activate cell cycle arrest and cell death or establish irreversible mutations, that contributes to tumorigenesis and ageing (Figure 1b) (Hoeijmakers, 2001). To avoid changes in genetic material, cells activate systems - collectively termed the DNA repair pathways – to recognize and repair the lesions in DNA (Figure 1a) (Rouse & Jackson, 2002).

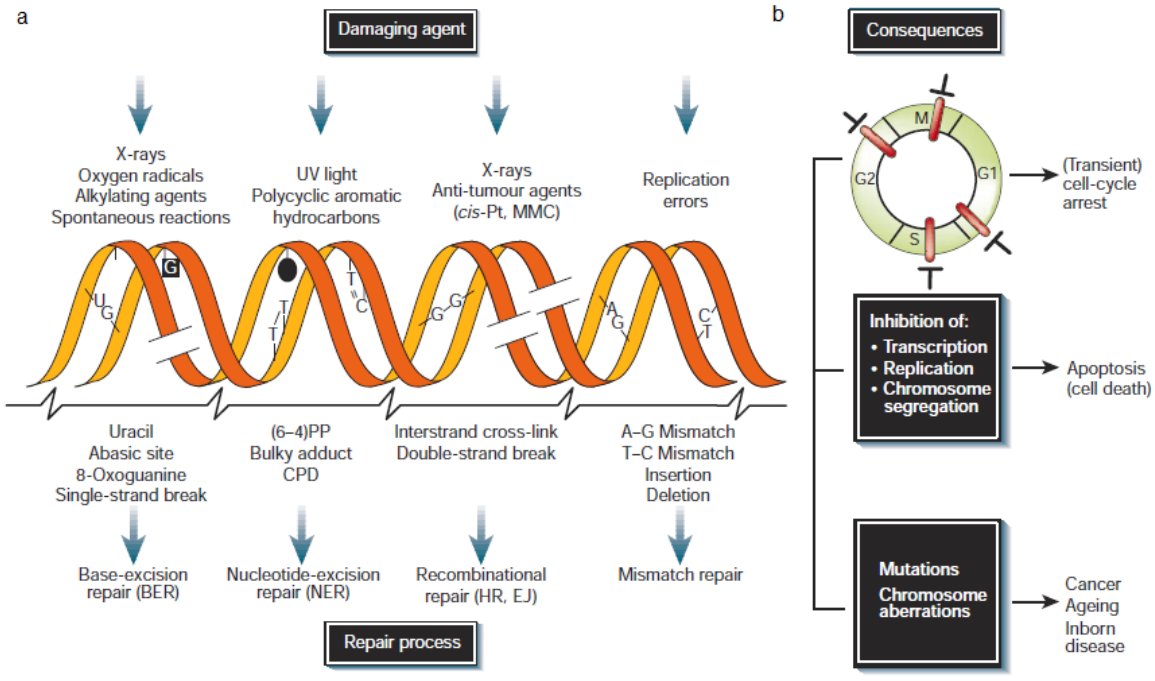

Figure 1. DNA damage types, repair mechanisms and consequences. a) The most common DNA damaging agents (top), induced DNA lesions after their exposure (middle) and repair pathways involved in damage removal (bottom). b) Acute consequences of DNA

damage arisen from blocked cell cycle progression (top) and altered DNA metabolism (middle). Long-term permanent changes followed by unrepaired DNA damage (bottom). (Hoeijmakers, 2001)

1.2 Oxidative DNA damage

Oxidative DNA damage arises from DNA reaction with ROS. ROS can arise as byproducts of normal cellular events such as mitochondrial respiration and lipid peroxidation or from exogenous sources like UV-light or some pharmacological drugs (Bont & van Larebeke, 2004). Therefore cells are continuously exposed to ROS during their life cycle. The most common ROS are superoxide anion ($O_2^{\bullet -}$), hydroxyl radical (HO•) and hydrogen peroxide (H_2O_2), which altogether are shown to generate more than 100 types of oxidative modifications in DNA (Cadet *et al*, 1997).

ROS serve important regulatory function at low concentrations (Hensley *et al*, 2000; Ma, 2010). However, at high levels, ROS pose a challenge to cells due to their high reactivity to proteins, carbohydrates, lipids and nucleic acids, such as DNA and RNA (Berquist & Wilson, 2012). In humans, DNA oxidation damage arises estimably 10'000 times per cell per day (Ames *et al*, 1993; Helbock *et al*, 1998). ROS is believed to be the major driving force of ageing, various diseases and carcinogenesis (Hoeijmakers, 2001; Balaban *et al*, 2005). Oxidized nucleic acids lead to blockage of essential cellular processes such as DNA replication and transcription (Cooke *et al*, 2003). Importantly, cells have evolved antioxidant systems to detoxify ROS and balance the intracellular redox environment. Nevertheless, increased generation of ROS can exceed the antioxidant capacity of the cell, leading to imbalanced redox status and condition termed as oxidative stress (Martindale & Holbrook, 2002).

1.2.1 Oxidation of guanine base

Oxidative stress can pose a constant source of spontaneous damage to DNA as well as to free bases in cytoplasmic and mitochondrial deoxyribonucleotide triphosphate (dNTP) pools. All four bases in DNA are subject to oxidation, but guanine is the most easily oxidized base (Jovanovic & Simic, 1986). Hence, 8-oxoG is the most prevalent type of oxidative damage in DNA (Kovacic & Wakelin, 2001). Many DNA lesions pair with various bases which lead to incorporation of incorrect base during replication, causing a mutation (Robertson *et al*, 2009). The presence of 8-oxoG is mutagenic as it guides the misincorporation of adenine into DNA and therefore causes G to T transversions (Shibutani *et al*, 1991; Michaels & Miller, 1992). Much attention has been dedicated to DNA direct oxidation, however, nucleotide pool is

significantly more susceptible for oxidation compared to double stranded DNA (dsDNA) (Topal & Baker, 1982). A damaged nucleotide pool contributes to the buildup of damaged DNA that leads to spontaneous mutagenesis and cell death (Ichikawa *et al*, 2008; Oka *et al*, 2008). Hence, DNA damage can arise from damaged nucleotides or from direct oxidation of DNA. To combat ROS-induced damage cells have evolved different enzymatic activities and DNA repair pathways.

1.2.1.1 Removal of oxidized guanine base damage

A threat posed by the presence of 8-oxoG in DNA is emphasized by the coordinated removal of the lesion by three enzymes that protect against highly mutagenic properties of 8-oxoG. Human MutT homolog 1 (MTH1) protein is a cytoplasmic and mitochondrial enzyme that sanitizes oxidized dNTP pool by converting 8-oxo-2'-deoxyguanosine-5'-triphosphate (8-oxo-dGTP) into a monophosphate 8-oxo-2'-deoxyguanosine-5'-monophosphate (8-oxo-dGMP), thus avoiding its incorporation into DNA (Figure 2) (Sakumi *et al*, 1993). MTH1 is non-essential to normal cells as they maintain redox homeostasis (Tsuzuki *et al*, 2001). However, MTH1 activity has been associated with efficient cancer cell survival, as the latter suffer from high ROS and oxidative stress, which results in oxidized dNTP pool that requires MTH1 hydrolytic activity (Gad *et al*, 2014). This constitutes MTH1 as a powerful anticancer target. Moreover, the potent and specific MTH1 inhibitor TH588 has been shown to specifically kill cancer cells by incorporation of oxidized nucleotides leading to DNA damage and cell death (Gad *et al*, 2014). In the absence of MTH1 activity 8-oxo-dGTP is being incorporated into DNA during replication (Tsuzuki *et al*, 2001). This creates a substrate to human 8-oxoguanine DNA glycosylase (OGG1). OGG1 is a glycosylase responsible for recognition and removal of 8-oxoG opposite cytosine (C) in DNA (Aburatani *et al*, 1997). The third enzyme that protects cells from the accumulation of 8-oxoG is called MutY homolog (MUTYH). MUTYH specifically recognizes adenine opposite 8-oxodG, when it has been misincorporated into DNA during replication (Shinmura *et al*, 2000). Importantly, OGG1 and MUTYH initiate downstream components of the major DNA repair pathway BER. All three aforementioned enzymes act to avoid mutagenesis by 8-oxoG in either dNTP pool or in DNA.

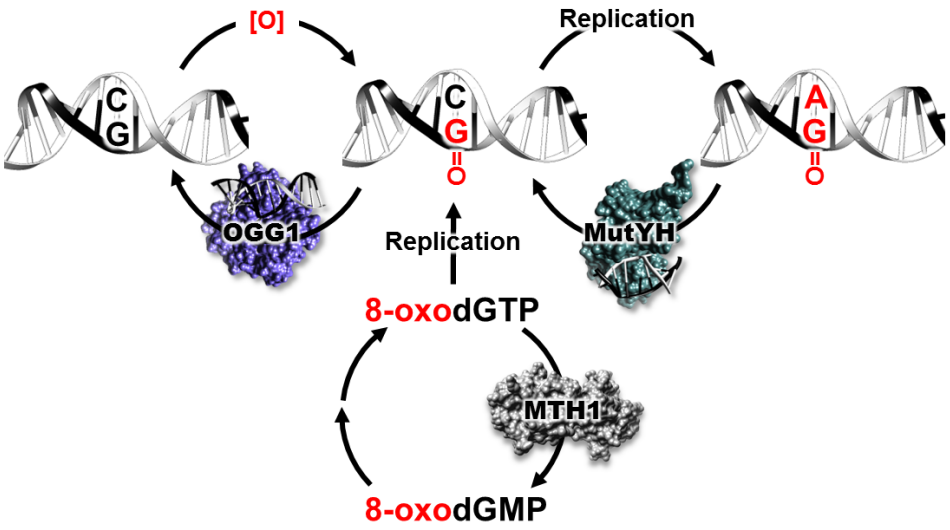

Figure 2. Repair of mutagenic 8-oxoG in DNA and nucleotide pool. MTH1 hydrolyzes 8-oxodGTP from nucleotide pool. OGG1 recognizes and removes 8-oxoG opposite cytosine (C) from DNA while MUTYH guides the removal of adenine (A) opposite 8-oxoG in DNA.

1.2.1.2 Base excision repair guided by OGG1

BER is a major DNA repair pathway responsible for removal of oxidized, deaminated and alkylated DNA lesions that frequently occur in genomic and mitochondrial DNA. High conservation of BER processes between pro- and eukaryotes has led to deep understanding of this pathway. Initial discoveries by Tomas Lindahl revealed fundamental basis of BER (Lindahl, 1974). Several enzymes involved in BER cooperate to recognize, remove and replace damaged nucleotides from DNA. BER comprises of several conserved steps initiated by one of at least 11 DNA glycosylases, depending on the type of lesion (Krokan & Bjoras, 2013). Glycosylases recognize specific lesions and catalyze the cleavage of N-glycosidic bond releasing a free base (Lindahl, 1974). In addition, some glycosylases like OGG1 are bifunctional with additional AP-lyase activity, resulting in cleaved DNA strand (Bjørås *et al*, 1997). Downstream BER enzymes perform further steps to repair the damage such as strand incision, gap filling and ligation (Dianov & Hübscher, 2013).

As already mentioned, OGG1 recognizes and removes the most prevalent oxidative lesion 8-oxoG from DNA. In addition 2, 6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG) and 7, 8-dihydro-8-oxoadenine (8-oxoA) in DNA are also substrates for OGG1 (Jensen *et al*, 2003; Girard *et al*, 1998; Klungland *et al*, 1999).

OGG1 is encoded by *OGG1* gene and expressed in at least 12 different alternatively spliced forms that are located in both nucleus and mitochondria (Kohno *et al*, 1998; Takao *et al*, 1998; Nishioka *et al*, 1999). OGG1 protein expression is considered critical to avoid

potentially miscoding lesions (Sunaga *et al*, 2001). Therefore, its activity has been discussed to be important against carcinogenic processes. In fact, several studies have related defects in OGG1 that affect its repair capacity with increased risk for various types of cancers (Sugimura *et al*, 1999; Audebert *et al*, 2000; Xing *et al*, 2001; Park *et al*, 2001). Repair of mutagenic lesions attribute importance to OGG1 and BER for preventing pathological cellular processes.

1.3 Genomic instability is a hallmark of cancer

Defective DNA repair processes in mammalian cells may lead to accumulation of genomic alterations during the life cycle of cells, collectively named as genomic instability. Genetic alterations include a variety of mutations such as chromosomal rearrangements, point mutations and gain or loss of entire chromosomes. Genomic instability is associated with premature ageing, rare genetic diseases and is a hallmark of most of the cancers (Lengauer *et al*, 1997; Kerzendorfer & O'Driscoll, 2009; Stratton *et al*, 2009; Negrini *et al*, 2010; Vijg & Suh, 2013). In hereditary cancers, mutations in DNA repair genes are believed to cause genomic instability and drive the development of the disease, as described by the mutator hypothesis (Nowell, 1976; Loeb, 1991). For example, hereditary non-polyposis colon cancer is one of the well-documented diseases where the development of the disease is attributed to the mutations in mismatch repair genes (Fishel *et al*, 1993). However, in non-hereditary or sporadic cancers the molecular background of genomic instability is additionally derived from oncogene-induced DNA damage (Gorgoulis *et al*, 2005; Halazonetis *et al*, 2008). This model elucidates the role of activated oncogenes that drive the proliferation of cancer cells. Oncogene activation induces stalling and collapse of replication machinery, which in turn fuels genomic instability (Magdalou *et al*, 2014; Hills & Diffley, 2014). Given that cancer and DNA damage pose an intimate relationship, it opens numerous possibilities for cancer therapy.

1.4 Platinum-based chemotherapy approach in cancer treatment

Cancer is a disease described with uncontrolled cell growth and high proliferation rate (Hanahan & Weinberg, 2000, 2011). Given that one of the major factors to sustain the growth and survival of cancer tissue is limitless replicative potential, chemotherapy targeting DNA replication processes is widely used in cancer treatment (Siddik, 2005). Traditional chemotherapeutic agents are chemical substances with cytotoxic properties that act on rapidly dividing cells. Numerous types of anticancer drugs approved by Food and Drug

Administration (FDA) such as alkylating agents, platinum agents and topoisomerase inhibitors specifically intrude on certain aspects of DNA replication (Chabner & Roberts, 2005).

Platinum-based agents represent a cornerstone of current anticancer treatment. They compose a group of molecules that share a common structural feature of central platinum atom (Sundquist & Lippard, 1990; Ahmad *et al*, 2006). Cisplatin, oxaliplatin and carboplatin are FDA-approved platinum drugs that are used to treat several types of cancers (Kelland & Farrell, 2000). Cisplatin is the first discovered platinum-containing drug that shows clinical potency against wide variety of solid tumors. Since its discovery it has changed the course of treatment of several types of epithelial tumors such as those from testes, ovary and head and neck (Kelland & Farrell, 2000; Galanski, 2006).

1.4.1 Cisplatin mode of action

Cisplatin is a small polar molecule with central platinum (Pt) atom, two chlorine (Cl) atoms and two ammonia groups (NH_3) (Figure 3). It enters the cell by either passive diffusion or active transport (Gale *et al*, 1973; Katano *et al*, 2002; Ishida *et al*, 2002). Passive uptake of cisplatin depends on the concentration of sodium and potassium ions as well as pH inside the cell (Kelland, 2007). Active transport of cisplatin was under major discussion until year 2002 when two research groups elucidated the mechanism by which copper transporter-1 (CTR1) mediates the active intake of cisplatin (Figure 3A) (Katano *et al*, 2002; Ishida *et al*, 2002). Further studies strengthened the idea by showing that *Ctr1*$^{-/-}$ mouse embryonic fibroblasts accumulated only 35% of cisplatin compared to wild-type fibroblasts, leading to increased drug resistance (Holzer *et al*, 2006). After entering the cell, cisplatin becomes intracellularly activated by hydrolysis reaction of one or both of the chlorine residues (Figure 3) and subsequently reacts with nucleophilic centers in biomolecules. Cisplatin has various targets in the cell including DNA, peptides such as glutathione (GSH) and proteins like metallothionein (Lippard, 1983; Knox *et al*, 1986; Ishikawa & Ali-Osman, 1993; Takahara *et al*, 1995). Still, DNA and GSH have been considered the main targets in cisplatin cytotoxicity.

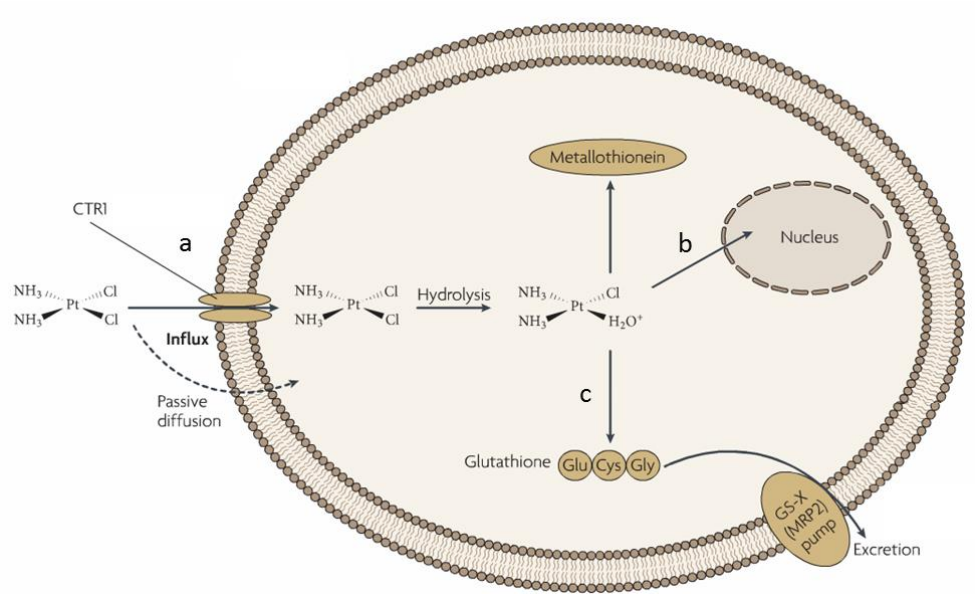

Figure 3. Cellular mechanisms affecting cisplatin accessibility to DNA. a) Cisplatin can enter the cell by passive or active transport where it is activated through hydrolysis. Downregulation of CTR-1 membrane transporter decreases the influx of cisplatin. b) Activated cisplatin is prone to enter the nucleus and form DNA adducts. c) Binding of sulfur-rich molecules like glutathione and metallothionein cause detoxification and excretion of cisplatin. Modified from (Kelland, 2007).

1.4.1.1 Cisplatin-DNA crosslinks

Cisplatin creates various types of covalent bonds with DNA forming DNA adducts that contribute to anticancer effect (Figure 4). *In vitro* studies with salmon sperm DNA introduced to cisplatin showed generation of crosslinks between purine bases in DNA, predominantly between guanines (G) and to lesser extent between adenines (A) (Fichtinger-Schepman *et al*, 1985). Moreover, the majority of cisplatin-induced adducts are being formed within one DNA strand between adjacent nucleobases, so called ApG and GpG intrastrand crosslinks. Other less frequently formed crosslinks are guanine-dependent monoadducts, in which only one of the active groups is bound to guanine base in DNA, and interstrand adducts between guanines in the opposite strands of DNA. These DNA adducts cause distortions and structural alterations in DNA strands attracting recognition proteins of DNA damage response (DDR) (Bellon *et al*, 1991). The final outcome of cisplatin administration is generally p53-mediated apoptotic cell death (Ségal-Bendirdjian *et al*, 1998; Fan *et al*, 1994).

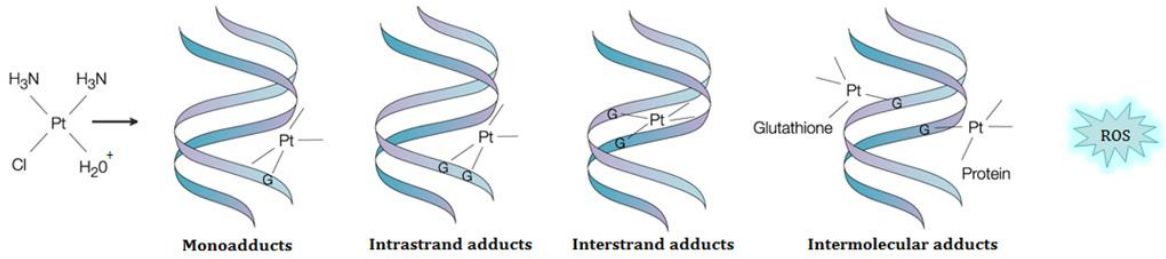

Figure 4. Cisplatin-induced DNA damage types. Reaction with cisplatin induces mono-, intrastrand (~95%), interstrand and intermolecular adducts in DNA. Generation of ROS is accompanied with cisplatin treatment. Modified from (Masters & Köberle, 2003).

1.4.1.2 Cisplatin induced ROS

Apart from generating DNA crosslinks, recent studies have suggested the induction of ROS in cisplatin treated cells. Reports from *in vivo* data suggest that cisplatin-induced ROS mediate severe side effects of this treatment, for example nephrotoxicity, as addition of antioxidants prevented this phenomenon (Satoh *et al*, 2003).

ROS induced by cisplatin is believed to arise at different cellular levels, for example from cytoplasm and cell organelles, predominantly mitochondria and nucleus (Brozovic *et al*, 2010). In a cell free system it has been clearly documented that cisplatin generates superoxide anion through interaction with DNA (Masuda *et al*, 1994). This early indication gave ideas that generated ROS might play a role in cell death induction. Study by Berndtsson and others indicated two dose-dependent mechanisms that both ultimately lead to apoptosis in colon cancer and melanoma cells (Berndtsson *et al*, 2007). Namely, they showed that widely known DDR signaling is activated at lower doses, while higher dose of cisplatin induces superoxide formation that leads to acute apoptosis. Another study gave insight into how ROS is generated through mitochondrial dysfunction that significantly enhances the cytotoxic effects of nuclear DNA damage (Marullo *et al*, 2013). While the importance of cisplatin-induced ROS has been demonstrated in tissue culture conditions, data from primary tumor tissues are lacking. To date, cytotoxic mechanisms of cisplatin-induced crosslinks are well described, yet it is poorly understood to what extent cisplatin-induced ROS contributes to cell death. Activity of BER has been linked to cisplatin-induced DNA repair, as over-expression of OGG1 impairs the cytotoxic effects of platinum drugs, including cisplatin (Preston *et al*, 2009). However, not much is known about detailed molecular mechanisms of how BER mediates the repair of cisplatin induced oxidative damage. More studies are needed to elucidate the role of BER in cisplatin-induced DNA damage repair and its contributions to cell death.

In general, structural and mechanistic insights of cisplatin-induced DNA damage have improved the understanding how cisplatin anticancer potency is created. Also, it offers

valuable insight into novel approaches to avoid cisplatin side-effects without affecting the efficiency.

1.4.2 Mechanisms of acquired cisplatin resistance

As cisplatin can kill cancer cells arising from several tissues, cisplatin treatment is widely used in the clinics for solid tumors. Unfortunately, in many cases the initial response rate to the treatment is not durable and the efficiency of cisplatin treatment is highly limited due to acquired resistance leading to tumor relapse and increased mortality. Cisplatin resistance can be described as acquired upon continuous drug exposure or it can be present as intrinsic set.
As anticancer effects of cisplatin comprise a complex set of intracellular processes, cancer cells can avoid cell death by interfering with any of these steps leading to development of the drug resistance. Cisplatin resistance arises from cellular changes that either alter the intracellular accumulation of the drug, its accessibility to the DNA or subsequent downstream signaling cascades (Siddik, 2003). There are few general processes that determine the sensitivity of the cells to cisplatin treatment (Siddik, 2003). These include the transport of the drug inside and outside of the cell, namely the rate of influx and efflux, the level of thiol-containing species in the cytoplasm, the level of DNA repair capacity, tolerance to cisplatin-induced DNA adducts and the generation of apoptotic signals. Even though many mechanisms of cisplatin resistance have been described in tissue culture studies, it is important to stress the general alignment with clinically relevant investigations (Giaccone, 2000).

1.4.2.1 Resistance mechanisms after DNA-adducts have formed

Once cisplatin-induced DNA damage is formed, cellular survival can be assured by either increased DNA repair capacity or tolerance to form DNA adducts. An example of cisplatin resistance acquired by enhanced nucleotide excision repair (NER) efficiency is seen in ovarian cancer cells (Johnson *et al*, 1994). Since NER is the main pathway repairing cisplatin-induced DNA damage, main research focus has been on the components of this pathway. Excision repair cross-complementing-1 (ERCC1) is an endonuclease involved in NER pathway that forms heterodimer with xeroderma pigmentosum (XP) complementation group F (XPF). This heterodimer is responsible for incising 5' DNA strand from the platinated site facilitating the subsequent repair process. Expression levels of ERCC1 have been used to determine the capacity of NER in cisplatin resistant ovarian cancer cell lines (Ferry *et al*, 2000). This finding is also present in clinical samples, where increased *ERCC1* mRNA levels

correlate with poor outcome of cisplatin treatment (Dabholkar *et al*, 1992). Therefore ERCC1 can be used as a prognostic biomarker to identify patients who would benefit from cisplatin therapy. Indeed, this approach has been used for head and neck cancer patients and is under development for bladder and metastatic colon cancer patients (Sun *et al*, 2012; Choueiri *et al*, 2015; Bauman *et al*, 2013). Alongside with other related discoveries, ERCC1 correlation with treatment outcome has opened a novel concept to develop DNA-repair specific therapies to overcome the issue of acquired cisplatin resistance. Indeed, XPF specific small-molecule inhibitors have been developed that interrupt binding with ERCC1 and synergize with cisplatin treatment (Jordheim *et al*, 2013). Additionally, as BER is the major DNA repair pathway for oxidative damage, it might also have a substantial role in acquired cisplatin resistance, since ROS is generated in cisplatin treated cells (Berndtsson *et al*, 2007). An association study conducted by Peng *et al* connects different mutations in BER genes with earlydeath and hematologic toxicities (Peng *et al*, 2014).

In addition to increased DNA repair capacity, cisplatin resistance can be acquired by enhanced replicative bypass, which is defined as an ability of replication machinery to continue DNA synthesis upon encountering DNA damage. It is important for the cell to proceed with replication process and pass the damage site, since stalled replication forks lead to cell death through various mechanisms. Translesion synthesis by DNA polymerases β (pol β) and η (pol η) have been shown to be responsible for bypass of cisplatin-induced DNA adducts (Bassett *et al*, 2002). Moreover, over-expression of pol η mediates tolerance to cisplatin treatment contributing to cisplatin resistance in ovarian cancer stem cells (Albertella *et al*, 2005; Srivastava *et al*, 2015). These are yet another mechanisms that illustrate the broad DNA specific consequences of cisplatin therapy. Together, tumors are believed to become unresponsive to cisplatin therapy through various alterations that involve several cellular processes which are often combined. New therapies emerge from molecular knowledge about these processes that are being exploited to target bottlenecks of resistant tumors.

1.5 Combination therapy approach

Combination therapy is a medical approach in which more than one therapy is administered to patient with the aim to improve the outcome of treatment. Targeting different cellular components at once has the potential to synergize the treatment efficiency. As outcomes of current first-line therapies are often limited due to various reasons such as intratumor heterogeneity or acquired resistance, application of combinational strategies are often justified and beneficial. Synthetic lethality by definition is a concept in which mutations separately in

two genes have no negative effect on cells, whereas together result in cell death. This concept has provided new approaches for targeted therapy as successfully proven by the concept in DNA repair context with poly (ADP-ribose) polymerase 1 (PARP1) inhibitors in *BRCA1* and *BRCA2* deficient tumors (Farmer *et al*, 2005; Bryant *et al*, 2005). More specifically, PARP1 is involved in DNA repair within BER pathway. *BRCA1* and *BRCA2* are required for DNA double-strand break repair via homologous recombination (Tutt & Ashworth, 2002). However, cancer cells defective in HR fail to repair DSBs arisen from PARP1 inhibition and are therefore selectively killed. In addition to PARP1, DDR pathways comprise of various potential cancer specific targets for combination therapy that can be investigated and exploited for future therapies.

2 EXPERIMENTAL PART

2.1 Aims of the study

This thesis is a part of a project that investigates how targeting DNA damage response can be applied for combinational cancer therapy. As the role of BER and OGG1 in cisplatin-induced DNA damage repair is not established, the main aim of the thesis is to shed light into OGG1-dependent molecular mechanisms underlying acquired cisplatin resistance in bladder cancer cells. Since increased DNA repair capacity is one of cisplatin resistance mechanisms (Kelland, 2007), inhibiting DNA repair by small molecule inhibitors targeting OGG1 activity could sensitize cisplatin resistant cells and therefore be used as combination therapy. Following key questions are addressed in this work:

- Do OGG1 inhibitors show target-binding properties?
- Does OGG1 activity contribute to acquired cisplatin resistance?
- Does OGG1 inhibition overcome cisplatin therapeutic failure?
- Does OGG1 contribute to cellular homeostasis upon MTH1 inhibition?

2.2 Materials and methods

2.2.1 Cell culture and compound treatments

All cell lines used for this study (Table 1) were purchased from American Type Culture Collection and cultivated in suitable medium (all mediums were purchased from Thermo Fischer Scientific) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific) and 10 U/ml penicillin/streptomycin (Thermo Fisher Scientific) using humidified conditions at 37 ^{0}C and 5% of CO_2. All cell lines were tested and proven to be mycoplasma free. Adherent cells were grown on T75 culture flask in 10 ml of culture medium and were passaged when they reached 70-80 % confluency every 2 or 3 days. Passaging was carried out according to manufacturer's protocol by using trypsin-EDTA (Thermo Fisher Scientific). Suspension cells were grown on T75 culture flask in 20 ml of culture medium and maintained by media replacement every 2 or 3 days. Cell concentration was kept between 1 x 10^5 and 1 x 10^6 viable cells/ml. Passage number remained below 10 for the cells used for experiments. TC20 automated cell counter (Bio-Rad), corresponding counting slides and trypan blue was used for cell counting. Cells were seeded into T25 culture flask, 100 mm dish, 96-well plate, 6-well plate or 35 mm dish with glass bottom to perform experiments.

Table 1: Details of cell lines.

Cell line	Origin tissue	Morphology	Culture properties	Culture medium
NTUB1	Urothelial carcinoma, cisplatin susceptible	Epithelial	Adherent	RPMI – 1640
NTUB1P	Urothelial carcinoma, cisplatin resistant	Epithelial	Adherent	RPMI – 1640
U2OS	Bone osteosarcoma	Epithelial	Adherent	McCoy's 5A
HL-60	Promyelocytic leukemia	Myeloblastic	Suspension	RPMI - 1640
HEK293T	Embryonic kidney	Epithelial	Adherent	DMEM high glucose
Mouse embryonic fibroblasts (MEFs)	Embryo	Fibroblast	Adherent	DMEM high glucose

All OGG1 and MTH1 inhibitors were designed and synthesized by my colleagues at Helleday Laboratory. For inhibitor experiments with adherent cells, 10 µM or 25 µM of inhibitor solution was prepared in suitable medium and immediately added to cells. For suspension cells, inhibitor was added to cell suspension with a final concentration of 10 nM, 40 nM, 120 nM, 370 nM, 1.11 µM, 3.33 µM or 10 µM. In all cases, equal amount of dimethylsulfoxide (DMSO) (maximum 0.2% of total volume, VWR Chemicals) was used as a vehicle control. In case of inhibitor and cisplatin (Hospira) combination treatment, inhibitor was added prior to cisplatin for 30 minutes. For cisplatin experiments, various final concentrations between 100 nM and 100 µM were prepared in suitable medium and added to adherent cells. 0.9% saline solution was used as vehicle control for cisplatin. In all experiments, final medium volume between samples was equal.

2.2.2 siRNA transfection

For siRNA transfections, cells were seeded to 30-40% confluency and the day after transfected with 10 nM siRNA using INTERFERin transfection reagent (Polyplus Transfections) according to the manufacturer's instructions for 48 hours. For non-targeting siRNA (siNT) control, All-stars negative control (Qiagen) was used. The following siRNA

sequence was used: OGG1 siRNA (siOGG1) #8: CGGAUCAAGUAUGGACACUGA (Qiagen).

2.2.3 Generation of stable cell lines

To obtain stable over-expression, pLenti PGK Puro vector (Addgene) with GFP-tagged OGG1 insert was used. Constructs were prepared by Torkild Visnes. Stable expression was obtained by lentivirus production and infection. Lentivirus was produced by calcium-phosphate-mediated co-transfection of HEK293T with packaging plasmids and lentiviral construct containing OGG1-GFP overexpression sequence. 16 hours after transfection, medium was replaced with fresh medium. After 24 hours, medium with virus particles was collected, filtered and transduced to OGG1 knockout MEFs or NTUB1/P cells for target cell infection. Infection was done three times with 8-16 hour intervals. Target cells were selected in the presence of 2 µg/ml puromycin (Thermo Fisher Scientific) for three days or until control cells were completely dead. GFP-positive cells were sorted by flow cytometer.

2.2.4 Western blot assay

Cells were grown on 6-well plates, washed once with cold 1x PBS (2.7 mM KCl, 137 mM NaCl, 1.8 mM KH_2PO_4, 10 mM Na_2HPO_4, pH 7.4), lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% NP40, 0.5% Na deoxycholate, 0.1% SDS) supplemented with protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Thermo Fisher Scientific) for 10 minutes and collected into tubes. 4x Laemmli sample buffer (Bio-Rad) with 10% reducing agent β-mercaptoethanol (Sigma) was added and samples were denatured at 95 ^{0}C for 10 minutes. Samples were sonicated using 30 seconds on and 30 seconds off for 10 cycles (Bioruptor Plus, Diagenode). Protein concentration was measured by Pierce BCA protein assay kit (Thermo Scientific) and equal amount of proteins were separated on 4-15% gradient gel (Bio-Rad) using 1xTGS running buffer (25 mM Tris, 192 mM Glycine, 0.1% SDS, pH 8.3, Bio-Rad). Next, proteins were transferred into a nitrocellulose membrane by Trans-Blot Turbo transfer system using 1x Trans-Blot Turbo buffer for 7 minutes (Bio-rad, program 1.3 A and 25 V). This was followed by blocking in 5% milk in TBS-Tween 20 for 1 hour and incubation in primary antibody solution (Table 2) overnight at 4 ^{0}C. Membranes were probed by fluorescently-tagged secondary antibodies (IRDye® 680RD and 800CW, LiCor) and developed with an Odyssey FC machine (LiCOR) with 2 minutes exposure time.

Table 2: Details of antibodies used.

Antibody	Manufacturer	Type	Dilution
Anti-OGG1 (EPR4664(2))	GeneTex	Rabbit monoclonal IgG	1:10'000
Anti-MTH1 (NB100-109)	Novus Biologicals	Rabbit polyclonal	1:1000
Anti-γH2AX (05-636)	Merck Millipore	Mouse monoclonal IgG_1	1:1000
Anti-β-actin (ab6276)	Abcam	Mouse monoclonalIgG_1	1:5000
Anti-γ-tubulin (ab11316)	Abcam	Mouse monoclonal IgG_1	1:10'000
Anti-PARP1 (sc8007)	Santa Cruz Biotechnology	Mouse monoclonal IgG_{2a}	1:1000
Anti-Histone H3 (ab1791)	Abcam	Rabbit polyclonal IgG	1:1000

2.2.5 CETSA

To investigate the target engagement properties of in-house developed inhibitors, general principles from previously published method were applied (Martinez Molina *et al*, 2013).
HL60 suspension cells were seeded into 6-well plate and treated with OGG1 inhibitors. For single-concentration experiment, 10 µM of various OGG1 inhibitors or DMSO control was applied for 2 hours. For dose-response experiment, cells were treated with 10 nM, 40 nM, 120 nM, 370 nM, 1.11 µM, 3.33 µM, 10 µM of inhibitor #10 or DMSO control for 2 hours. Following steps were identical for two experiment setups. Cells were collected by centrifugation (Heraeus Fresco 17 centrifuge, Thermo Scienfitic, also used for following centrifugation steps), washed once with PBS and re-suspended in 50 µl PBS supplemented with protease and phosphatase inhibitors. Samples were placed into PCR tubes and treated with 49.5 ^{0}C for 3 minutes and 20 ^{0}C for 3 minutes, while control sample with no temperature treatment was placed on ice. Cells were lysed by three cycles of freeze-thawing using ethanol and dry ice. Degraded and precipitated proteins were separated from soluble fraction containing proteins of interest by centrifugation at 17'000 g for 15 minutes at 4 ^{0}C. Soluble fractions were stored at -80 ^{0}C until western blot analysis. 14 µl of each sample was loaded on a 4-15% gradient gel (Bio-Rad), transferred into a nitrocellulose membrane and processed for western blot analysis as described above. Images were quantified using Image Studio Lite

(Ver 4.0) software. Relative OGG1 signal was calculated by ratio to γ-tubulin and normalized to DMSO control.

2.2.6 Resazurin viability assay

Cells were seeded into a 96-well plate, 750 cells per well and the following day treated with inhibitor and cisplatin. For combination treatments, 2x concentration of inhibitor or DMSO was added for 15 minutes, followed by 2x concentration of cisplatin, which resulted with desired final concentration of both compounds in 100 µl of medium per well. For siRNA experiments, cells were re-seeded to 96-well plate after 48 hours of siRNA transfection and treated with various concentrations of TH588. Following steps were identical for two experiment setups. After 96 hours, medium was removed and replaced with resazurin (Sigma-Aldrich)-containing medium. Fluorescence intensity was measured at 530/590 (excitation/emission) after 2 hours of incubation with resazurin. Viability was calculated by subtracting average background fluorescence of culture medium from each sample value and represented as percentage of vehicle or siNT control by using Excel software. For statistical analysis, unpaired Student t-test was applied and *p*-value below 0.05 was considered as significant difference.

2.2.7 Colony formation assay

Cells were seeded into 100 mm dish with 500 cells per dish. The day after, 10 µM OGG1 inhibitor and 5 µM or 10 µM of cisplatin or DMSO was added. After 7-10 days medium was removed, plates were fixed and stained with 4% methylene blue in methanol (MetOH) and colonies were counted manually. Survival was calculated as percentage of DMSO or siNT control by using Excel software.

2.2.8 FRAP

Photobleaching was done by using LSM780 confocal laser scanning microscope (Zeiss) with Plan-Apochromat 40x/1.30 Oil DIC M27 objective and heated chamber set to 37 ^{0}C. 100’000 cells were seeded into a 35 mm dish with glass bottom and after 24 hours treated with 30 µM cisplatin for 3 hours or 10 µM TH588 for 18 hours. In order to avoid autofluorescence, CO2-independent medium without phenol red (Invitrogen) supplemented with 10% FBS and P/S was added to cells 2-3 hours prior experiment. The region of interest in the nucleus was selected and photobleached with Argon laser at 100% power for 600 milliseconds. Images were taken at 100 ms time intervals, 20 pre- and 200 postbleach with a frame size of 512x512

pixels and a pixel size of 90 nm. Fluorescence intensities of the bleached region were corrected for background intensity and for whole nuclear loss of fluorescence over the time course and normalized to the mean of the last ten prebleached values. For quantification, intensities of at least five nuclei were averaged and the standard error of the mean calculated and visualized using an Excel software (Microsoft).

2.2.9 Subcellular fractionation

500'000 cells were seeded into a 100 mm dish. Next day, cells were treated with 10 μM TH588 or DMSO and collected after 1 hour, 6 hours and 24 hours. Equal number of cells were centrifuged at 1000 g for 5 minutes, washed once with PBS and gently re-suspended in ice-cold hypotonic buffer A (10 mM HEPES-K^+ pH 7.5, 10 mM NaCl, 0.1 mM EDTA) with freshly supplemented with 1 mM DTT and 1x protease inhibitor (Roche). After 15 minute incubation on ice, 1/10 of volume of 10% NP40 was added to samples for 3 minutes and vortexed vigorously every minute to lyse the cells. Nuclei were collected by centrifugation at 1000 g for 5 minutes at 4 ^{0}C. Supernatant containing cytoplasmic fraction (Cyt) was collected and stored at -80 ^{0}C. Nuclei pellets were washed with buffer A and centrifuged at 1000 g for 5 minutes at 4 ^{0}C. For nuclei lysis, 4 pellet volumes of ice-cold buffer C (20 mM HEPES-K^+ pH7.5, 420 mM NaCl, 0.1 mM EDTA, 20% Glycerol) freshly supplemented with 1 mM DTT and protease inhibitor was added, re-suspended carefully and incubated on ice for 30 minutes, vortexed every 10 minutes. Samples were centrifuged at 17'000 g at 4 ^{0}C for 10 minutes and supernatant containing soluble nuclear fraction (Sol. Nuc) was stored at -80 ^{0}C. Supplemented buffer C was added to pellet containing chromatin fraction (Chr) and sonicated using 45 seconds on and 10 seconds off cycle for 20 cycles. Samples were processed for western blot analysis as described above.

2.3 Results

2.3.1 OGG1 inhibitors have target-binding properties

In order to investigate cellular responses to OGG1 inhibition, small molecule compounds were designed to inhibit OGG1 enzymatic activity. These compounds were developed and synthesized at Helleday laboratory. Numerous in-house OGG1 inhibitors showed inhibition of OGG1 catalytic activity *in vitro* with half-maximum inhibitory concentration (IC50) values of approximately 1 μM (data now shown). After determining IC50 values of newly synthesized compounds *in vitro*, binding properties of 10 inhibitors in cells were validated by CETSA.

Briefly, it is known that inhibitor binding to proteins increases the thermal stability of proteins (Martinez Molina *et al*, 2013). A comparison of soluble protein levels from inhibitor-treated and untreated cells after a temperature treatment is used to unravel target-binding abilities of compounds. This assay does not confirm the inhibition of protein activity, but only provides an indication of the binding properties of the compound into its target protein.

Here, HL-60 cells were treated with 10 different OGG1 inhibitors with a final concentration of 10 µM and vehicle DMSO control (Figure 5a). Importantly, OGG1 and γ-tubulin levels decreased in DMSO control sample upon temperature treatment at 49.5 ^{0}C compared to non-heated sample (Fig. 5a, lanes 1 and 2), confirming a decrease in protein stability upon temperature treatment. All tested inhibitors showed more than two-fold stabilization of OGG1 protein compared to DMSO treatment (Figure 5b), suggesting that the inhibitors are binding to OGG1. In addition, as inhibitors were added to intact cells one can conclude that all inhibitors were able to penetrate through the cell membrane.

Inhibitor number 10 (#10) showed the most promising properties based on an electrophoretic mobility shift assay (EMSA) and OGG1 *in vitro* activity assays (performed by Torkild Visnes, data not shown) and therefore further experiments were done by using the OGG1 inhibitor number 10 (#10). To evaluate a dose response stabilization of OGG1 by inhibitor #10, CETSA was performed upon decreasing inhibitor concentration (Figure 5c). A thermal stabilization of OGG1 was detected at lowest concentration of 1.11 µM, while no stabilization was detected using lower concentrations (Figure 5d). These results indicate that inhibitor #10 binds to OGG1 in a dose-dependent manner and approximately 1 µM of inhibitor stabilizes OGG1 in HL-60 cells. 10 µM treatment with #10 resulted in a 4-fold increase in OGG1 protein levels. In order to determine the maximum level of OGG1 stabilization, higher concentrations of inhibitor #10 will be tested. Taken together, this data confirms binding of all inhibitors to its intended target OGG1 at 10 µM and a dose-dependent engagement of the main inhibitor of interest #10.

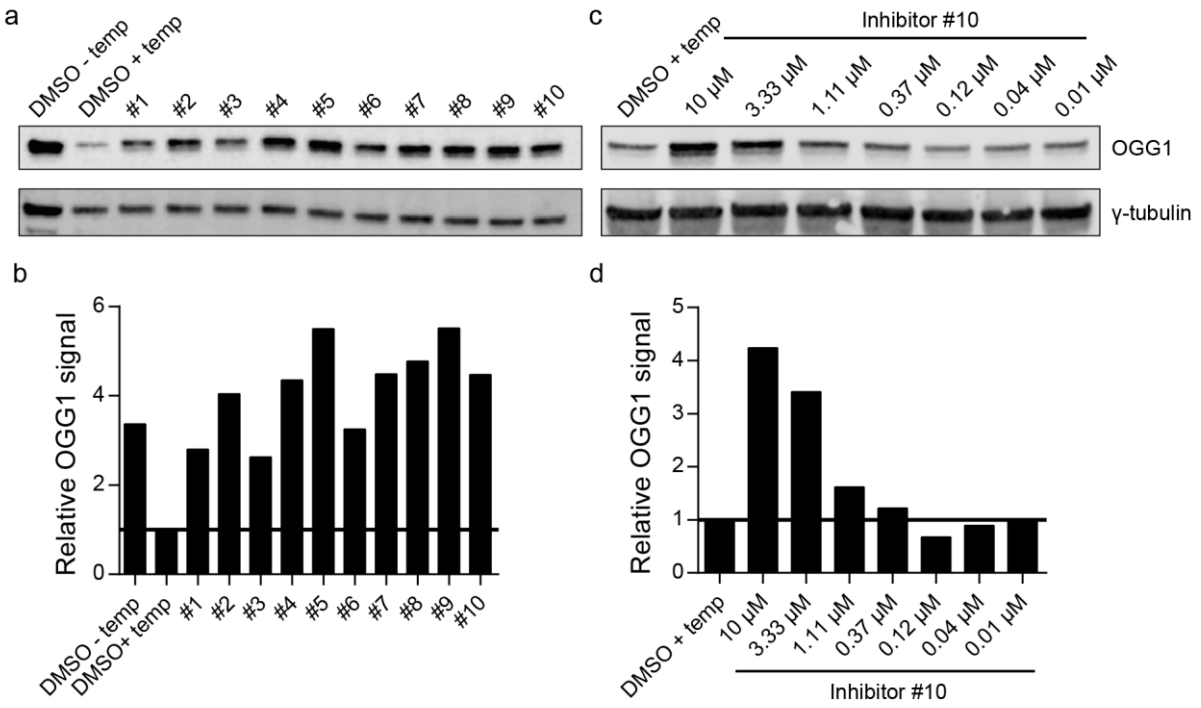

Figure 5. OGG1 inhibitors engage OGG1 in HL-60 cells. a) HL-60 cells were treated with vehicle control DMSO or 10 µM of 10 different OGG1 inhibitors for 2 hours, followed by thermal treatment at 49.5 ^{0}C for 3 minutes. Cells were lysed by freeze-thaw cycles and western blot assay was used to detect OGG1 and γ-tubulin protein levels. **b)** Quantification of OGG1 protein levels in OGG1 inhibitor and DMSO treated cells. Relative OGG1 signal was calculated by a ratio to γ-tubulin protein levels and normalized to DMSO + temp control. **c)** HL-60 cells were treated with vehicle control or indicated concentrations of inhibitor #10 followed by CETSA protocol as described in a. **d)** Quantification of OGG1 protein levels in indicated concentration of inhibitor #10 treated cells calculated as described in b.

2.3.2 OGG1 inhibition increases cisplatin sensitivity in cancer cells

OGG1 together with the downstream BER pathway components recognize and remove oxidized nucleotides from DNA (Krokan *et al*, 2000). As some anticancer therapies, which act through DNA damage induction, suffer from limited therapeutic efficiency (Srinivasan & Gold, 2012), I hypothesized that inhibition of OGG1 catalytic activity could possibly increase the efficacy of oxidant drug therapies. To investigate the effects of OGG1 inhibitor #10 to potentiate clinically relevant drugs, many combination treatments were carried out in various cell lines (data not shown). Resazurin viability assay was used as a read-out to evaluate cytotoxicity of inhibitor #10 and drug combination compared to drug alone. Among other clinically relevant drugs, cisplatin was included in the experiment as prolonged clinical treatment with cisplatin often leads to the development of resistance and therefore limited anticancer efficacy of the treatment (Galluzzi *et al*, 2014). To investigate whether OGG1 inhibition could overcome cisplatin resistance, cisplatin sensitive NTUB1 and resistant

NTUB1/P cell lines were used as a model cell line (Yu *et al*, 1992; Hour *et al*, 2000). Cisplatin resistance of NTUB1/P cells were developed by progressively increased exposure of cisplatin to parental NTUB1 cells in culture (Hour *et al*, 2000), resulting in IC50 values of approximately 100 times higher compared to their parental NTUB1 cells.

To test the cytotoxic effects of OGG1 inhibitors on cisplatin resistant cells, resazurin viability assay and colony formation survival assay were performed in NTUB1/P cells treated with inhibitor #10 and cisplatin (Figure 6). NTUB1/P cells treated with 25 µM of inhibitor #10 alone showed approximately 75% viability compared to vehicle-treated control, whereas 10 µM of inhibitor #10 did not affect cellular viability (Supplementary figure 1a), illustrating a slight cytotoxic effect of inhibitor #10 at higher concentrations. NTUB1/P cells treated with 25 µM cisplatin showed a 20% reduction in cell viability upon 10 µM inhibitor #10 treatment and a 60% reduction upon 25 µM inhibitor #10 treatment (Figure 6a). This potentiating effect was also detectable at 12.5 µM of cisplatin, but not remarkably at higher cisplatin concentrations. Overall, NTUB1/P cells became more sensitive to cisplatin after OGG1 inhibition, suggesting the importance of OGG1 activity to cisplatin resistant cells. Interestingly, OGG1 inhibitor #10 did not additionally decrease the viability of cisplatin sensitive NTUB1 cells in combination with cisplatin (Supplementary figure 2) suggesting that acquired resistance of NTUB1/P cells could be dependent on OGG1 activity.

Importantly, RNA interference (RNAi)-based OGG1 depletion sensitized colon cancer SW480 cells to cisplatin treatment (data not shown), supporting the importance of OGG1 activity in response to cisplatin. To consolidate this in the context of cisplatin resistance, RNAi-based depletion of OGG1 will be done in NTUB1/P and NTUB1 cells.

Next, colony formation assay was performed to study how OGG1 inhibitor #10 in combination with cisplatin affects the proliferation rate and survival of NTUB1/P cells. Figure 6b shows that co-treatment of inhibitor #10 and cisplatin remarkably reduced the survival of NTUB1/P cells. To note, 10 µM inhibitor #10 treatment alone did not compromise NTUB1/P proliferation (Supplementary figure 1b). Cells treated with 10 µM of inhibitor #10 together with 5 µM cisplatin showed approximately half of the proliferative capacity compared to cells treated with cisplatin alone (Figure 6b). Importantly, this effect amplified as the dose of cisplatin increased, supporting the findings from viability assay described in Figure 6a. This reduction in survival demonstrates the ability of OGG1 inhibitor #10 to decrease cisplatin resistance and abrogate proliferation in NTUB1/P cells. Taken together, this data provides insight how inhibition of DNA repair capacity, namely OGG1-dependent DNA repair might stimulate the anticancer effects of cisplatin in resistant cells.

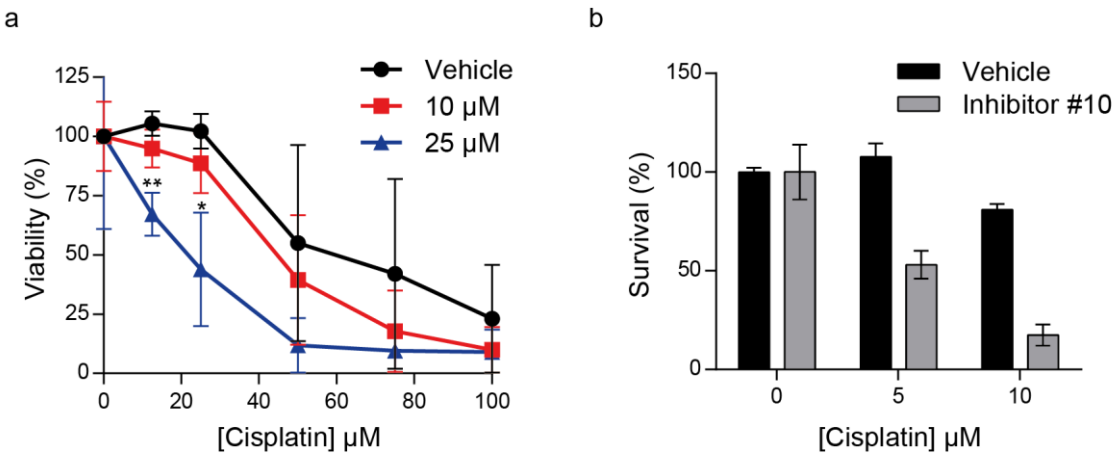

Figure 6. OGG1 inhibitor #10 sensitizes cisplatin resistant NTUB1/P cells. a) Viability of NTUB1/P cells treated with 12.5 µM, 25 µM, 50 µM, 75 µM or 100 µM cisplatin combined with 10 µM or 25 µM inhibitor #10 for 72 hours. Saline and DMSO were used as vehicle controls for cisplatin and inhibitor #10, respectively. Values are presented as mean viability normalized to vehicle control, error bars represent SD from three independent experiments. Asterisks mark a significant difference compared to vehicle control (**P*<0.05, ***P*<0.005, unpaired t-test). **b)** NTUB1/P cells treated with 5 µM and 10 µM of cisplatin together with 10 µM of inhibitor #10 for 10 days. Saline and DMSO were used as vehicle controls for cisplatin and inhibitor #10, respectively. Formed colonies were fixed and stained with 4% methylene blue in MetOH and counted manually. Values are indicated as survival compared to vehicle-treated controls and error bars represent SD from technical replicates from one experiment.

2.3.3 OGG1 is a potential therapeutic target in cisplatin resistant bladder cancer

Results from viability and survival assays illustrated how OGG1 inhibitor #10 potentiated the cytotoxic cisplatin effect in cisplatin resistant cells but not in cisplatin sensitive cells. As increased DNA repair capacity has been described as one of the cisplatin resistance mechanisms (Kelland, 2007), this potentiation upon OGG1 inhibition in resistant cell line was expected. To get insight into OGG1-dependent DNA repair capacity, OGG1 protein levels were detected in cisplatin sensitive NTUB1 and resistant NTUB1/P cell lines (Figure 7). OGG1 protein levels were higher in resistant cells compared to sensitive cells, illustrating OGG1 over-expression in cisplatin resistant NTUB1/P cells. As NTUB1/P cells were developed from NTUB1 cells, increase in OGG1 protein levels seems to correlate with acquired cisplatin resistance (Yu & Wang, 2012). Interestingly when sensitive and resistant cells were treated with IC50 cisplatin doses (0.5 µM for NTUB1 and 50 µM for NTUB1/p cells) for 24 hours, OGG1 protein levels stayed constant in both cell lines, suggesting that short exposure to cisplatin does not induce OGG1 expression.

To compare DDR in sensitive and resistant cells, IC50 doses and constant cisplatin dose of 10 µM were applied on both cell lines for 24 hours. Administration of IC50 doses resulted in

similar DDR induction in both cell lines as seen by increased phosphorylated histone 2AX (γH2AX) levels, while unmodified H2AX levels remained constant (Figure 7). Treatment of two cell lines with 10 µM cisplatin resulted in notably higher DNA damage levels measured by γH2AX levels in NTUB1 cells compared to NTUB1/P cells. This illustrates that NTUB1 cells are more sensitive to cisplatin compared to NTUB1/P cells and indicates more efficient DNA repair capacity of cisplatin resistant cells. In addition to phosphorylation, histone variant 2AX seems to be monoubiquitinated (mono-Ub) after cisplatin treatment in NTUB1 cells indicating DDR activation.

All in all, higher OGG1 levels in cisplatin resistant NTUB1/P cells support OGG1 being a promising therapeutic target to battle against cisplatin resistance. However, more cell lines as well as patient-derived samples need to be tested to draw solid causal conclusions between OGG1 activity and cisplatin resistance.

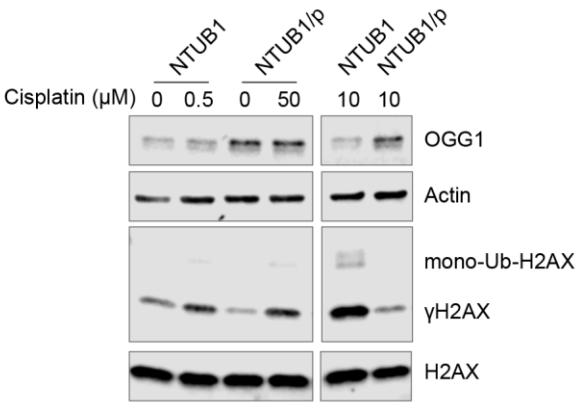

Figure 7. Cisplatin resistant NTUB1/P cells show higher OGG1 protein levels compared to parental cisplatin sensitive NTUB1 cells. NTUB1 and NTUB1/P cells were treated with cisplatin for 24 hours and immunoblotted against OGG1, γH2AX, H2AX and actin proteins. Representative immunoblot of two independent experiments.

2.3.4 Cisplatin treatment reduces OGG1-GFP mobility in the nucleus

As cells that express higher levels of OGG1 tolerate higher cisplatin concentrations, OGG1 activity could be needed to sustain cellular homeostasis upon cisplatin treatment. OGG1 is involved in the repair of oxidative lesions and cisplatin treatment induces oxidative damage, OGG1 might therefore be involved directly in cisplatin-induced DNA repair. One way to indirectly study this is to determine OGG1 protein dynamics in living cells by using fluorescence microscopy. Chromatin-interacting proteins, such as OGG1, are believed to be highly mobile in the cell nucleus until they reach their target sites. It has been shown that OGG1 immobilizes to chromatin fraction upon oxidative DNA damage, suggesting active recruitment of OGG1 into damage sites (Amouroux *et al*, 2010; Campalans *et al*, 2013).

FRAP is an approach to study the mobility of fluorescently labelled proteins in single live cells by using high intensity laser (Houtsmuller & Vermeulen, 2001; van Royen *et al*, 2009). Changes in mobility of a protein of interest can be interpreted as changes in its DNA binding. Briefly, a high intensity laser is used to bleach-pulse fluorescently tagged proteins in pre-determined area in the nucleus. As the majority of fluorescently tagged proteins within the region of interest have irreversibly lost their signal this process is termed as photobleaching. Photobleaching results in loss of fluorescent signal in pre-determined area of the nucleus, which is then recovered over short time due to mobility of the protein from non-photobleached area of the nucleus to the bleached area (Figure 8a). To quantify the mobility of fluorescently tagged protein, images were taken sequentially over period of time before (Figure 8a, prebleach) and after photobleaching (Figure 8a, photobleaching and after recovery of 2 s and 20 s) to capture and measure the fluorescent signal within the nucleus. Decreased mobility upon DNA damage induction indicates a functional role of the protein in the repair of DNA damage.

To study whether OGG1 might be involved in cisplatin-induced DNA damage repair, OGG1-GFP was stably expressed in cisplatin resistant NTUB1/P cells. To investigate whether cisplatin treatment affects the mobility of OGG1-GFP, FRAP analysis was performed after 30 min of 30 µM cisplatin treatment (Figure 8b). Mobility presented as relative fluorescence unit (RFU), illustrates the GFP signal recovery in photobleached region in cell nucleus. The results from the preliminary experiment show a slight mobility shift of OGG1-GFP upon cisplatin treatment compared to saline treatment which was used as a vehicle control (Figure 8b). A 15% decrease in OGG1-GFP mobility was notable between 2.5 and 10 seconds after photobleaching, suggesting that OGG1-GFP have prolonged binding to DNA after cisplatin induced damage.

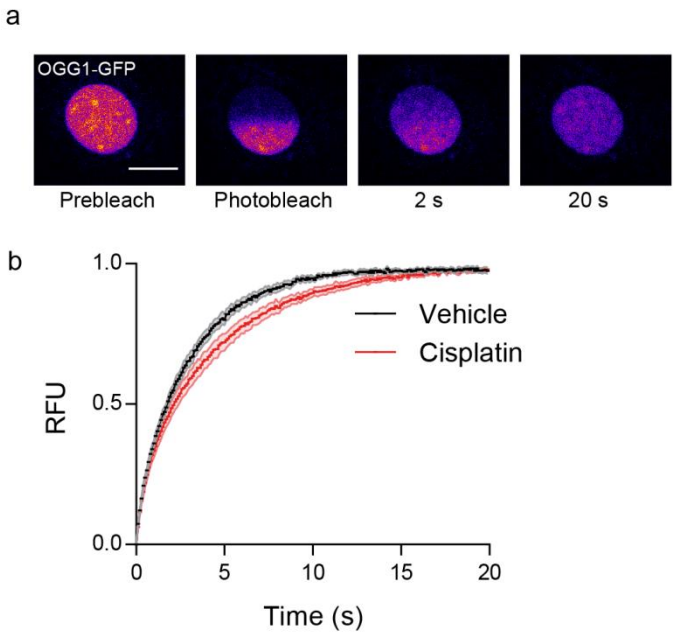

Figure 8. Cisplatin decreases OGG1-GFP mobility in cisplatin resistant cells. a) Representative microscopic images of OGG1-GFP signal in NTUB1/P cell nucleus before, during and after photobleaching. Scale bar size 10 µM. **b)** Mobility described as RFU of OGG1-GFP in NTUB1/P cells treated with vehicle control saline or 30 µM of cisplatin for 30 min. The mobility of OGG1-GFP was detected by photobleaching a pre-determined region of the nucleus and measuring the recovery of fluorescence. 220 images were captured during 22 seconds with 100 millisecond interval per each cell. Error bars represent average SEM of 5 cells from one experiment.

2.3.5 Limited interplay between OGG1 protein and MTH1 inhibition

Applying OGG1 inhibition for anticancer therapy might possess many opportunities for combination therapy with approved chemotherapeutics. Recently, the Helleday Laboratory showed that MTH1 is essential for cancer cell survival while non-essential for normal cells, making it an interesting anticancer target (Gad *et al*, 2014). MTH1 sanitizes oxidized dNTP pool and prevents incorporation of damaged nucleotides, such as 8-oxoG, into DNA. MTH1 inhibitors were developed in the laboratory and they seem to have potential anticancer activities. Since 8-oxoG levels are shown to increase in DNA after MTH1 inhibition (Gad *et al*, 2014), OGG1 activity might be necessary to remove those lesions and potentially underlie resistance mechanisms for novel MTH1-inhibition based therapy. To further investigate potential applications of OGG1 for combination therapy in cancer treatment, interplay between OGG1 and MTH1 was studied.

In order to investigate how essential is OGG1 activity for cancer cells after MTH1 inhibition, recently developed MTH1 inhibitor TH588 was used together with RNAi-based OGG1 depletion. First, the aim was to specifically investigate OGG1-mediated DNA repair which

would be followed by using OGG1 inhibitor. For that reason, siRNA-based depletion was chosen over OGG1 inhibition to obtain specific knockdown. As shown in Figure 9a, OGG1 depletion does not affect the viability of U2OS cells treated with various concentrations between 1 µM and 15 µM of TH588. To investigate whether OGG1 depletion might affect proliferation rather than viability after MTH1 inhibition, colony formation assay was performed in U2OS cells in the presence of TH588. No difference was seen in the survival of OGG1 depleted cells compared to OGG1 proficient cells when treated with 2 µM, 4 µM or 6 µM of TH588 (Figure 9b). OGG1 depletion alone slightly decreased the survival, but not viability rate in U2OS cells (Supplementary figure 3a, b). Importantly, siRNA-based depletion of OGG1 in U2OS cells resulted in substantial decrease of OGG1 protein levels, while γ-tubulin levels remained unaffected (Figure 9c). This data illustrates that U2OS cells acquired no additional cytotoxic effects to TH588 treatment after OGG1 depletion, which could be explained by non-complete depletion of OGG1, as low OGG1 protein levels were detected after siRNA-based OGG1 depletion (Figure 9c).

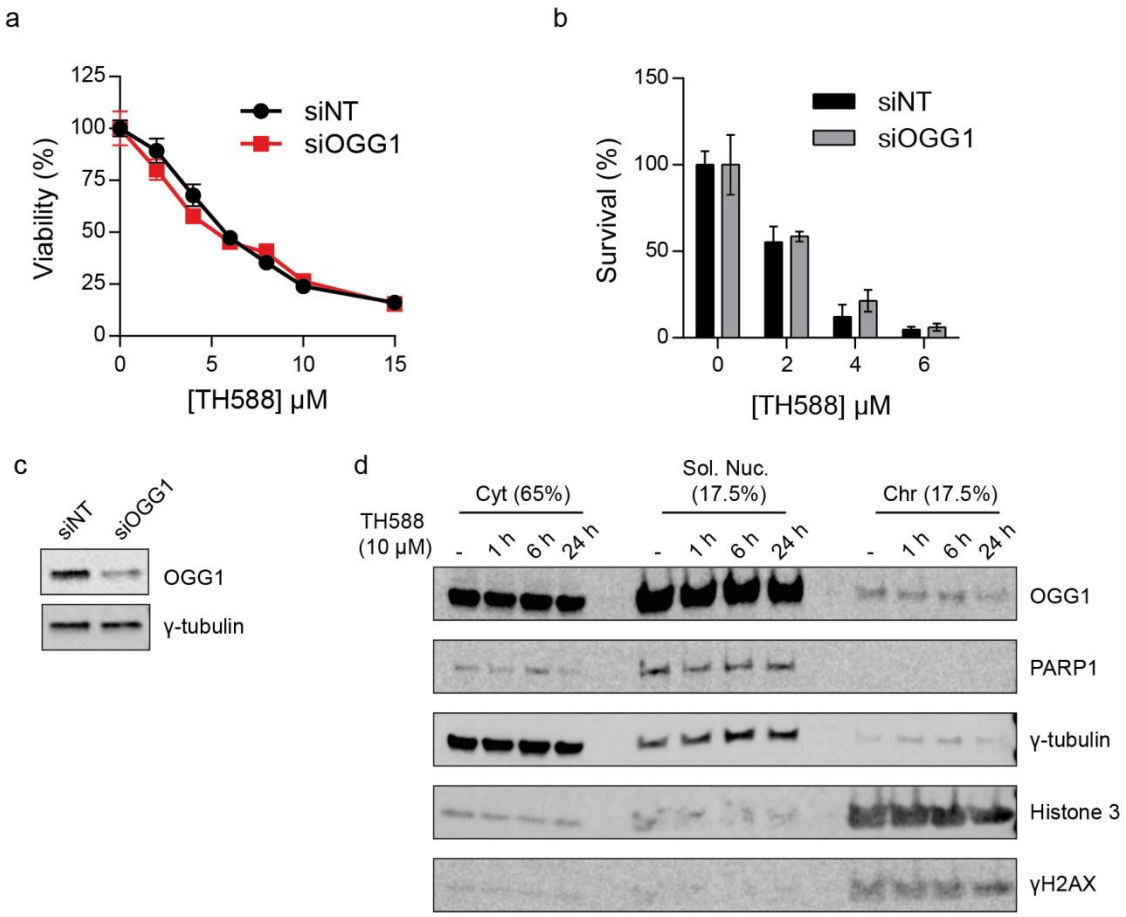

Figure 9. MTH1 inhibitor TH588 cytotoxic effects are not potentiated by OGG1 depletion and TH588 does not affect OGG1 subcellular localization. **a)** Viability assay of U2OS cells transfected with siNT or siOGG1 for 48 hours, re-seeded and treated with 2 µM, 4 µM, 6 µM, 8 µM, 10 µM and 15 µM TH588 for 72 hours. Values are indicated as viability

compared to non-treated controls and error bars represent average SD from technical replicates from one experiment. **b)** Survival assay of U2OS cells transfected with siNT or siOGG1 for 48 hours, re-seeded for colony formation and treated with 2 µM, 4 µM and 6 µM TH588 for 10 days. Values are indicated as survival compared to non-treated controls and error bars represent average SD from technical replicates from one experiment. **c)** After NT and siOGG1 transfection for 48 hours, western blot assay was used to detect OGG1 and γ-tubulin protein levels. **d)**. U2OS cells were treated with vehicle control DMSO or 10 µM of TH588 for 1 hour, 6 hours or 24 hours followed by fractionation protocol and western blot assay to detect indicated proteins.

In order to investigate OGG1 responses to MTH1 inhibition, subcellular fractionation and FRAP was performed (Figure 9d, 10). OGG1 protein levels were detected in different cellular compartments in U2OS cells after 10 µM TH588 treatment for 1, 6 and 24 hours (Figure 9d). Since OGG1 is performing its functions on DNA, the main focus was to look at the localization of OGG1 protein between cytoplasmic (Cyt), soluble nuclear (Sol. Nuc.) and chromatin (Chr) fractions upon MTH1 inhibition. OGG1 protein was detected in all three subcellular fractions: the highest OGG1 level was detected in soluble nuclear (Sol. Nuc.) fraction, whereas chromatin (Chr) fraction exhibited surprisingly low OGG1 levels. Substantial OGG1 levels were also seen in cytoplasmic (Cyt) fraction, which can be explained by nuclear fraction leakage to the cytoplasm fraction. However, no changes in OGG1 localization between any fractions were detected in response to TH588 treatment. This might be due to insufficient TH588 incubation time, as DNA damage marker γH2AX levels showed only marginal increase after 24 hours of TH588 treatment. To confirm the purity of fractions, PARP1 was used as a control for soluble nuclear fraction, γ-tubulin for cytoplasmic fraction and Histone H3 (H3) for chromatin fraction.

In addition, mobility of OGG1-GFP was measured in OGG1 knockout MEFs stably over-expressing human OGG1-GFP by FRAP to investigate whether OGG1-GFP has changed binding to DNA upon TH588 treatment (Figure 10). No change in mobility of OGG1-GFP was detected after 10 µM TH588 treatment for 18 hours, which exhibited similar fluorescence recovery as vehicle treated control suggesting that TH588 does not induce prolonged binding of OGG1-GFP to DNA. Importantly, treatment with 40 mM potassium bromate ($KBrO_3$) for 30 minutes resulted with remarkable decrease in OGG1-GFP mobility, suggesting prolonged binding to $KBrO_3$-induced damage sites in DNA (Figure 10).

All in all, interplay between OGG1 protein and MTH1 inhibition could not be confirmed in U2OS cells as OGG1 depletion did not sensitize U2OS cells and no change in OGG1 localization was seen upon TH588 treatment. In addition, OGG1-GFP binding to DNA in OGG1 knockout MEFs was not prolonged upon TH588 treatment.

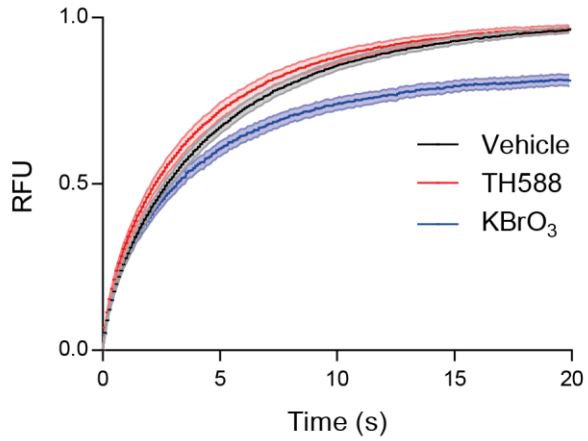

Figure 10. TH588 does not decrease OGG1-GFP mobility in OGG1 knockout MEFs. Mobility described as RFU of OGG1-GFP in MEFs treated with vehicle control DMSO, 10 µM of TH588 for 18 hours or 40 mM $KBrO_3$ for 30 min. The mobility of OGG1-GFP was detected by photobleaching a pre-determined region of the nucleus and measuring the recovery of fluorescence. 220 images were captured during 22 seconds with 100 millisecond interval per each cell. Error bars represent average SEM of 30 cells (vehicle and TH588) from three independent experiments or of 10 cells ($KBrO_3$) from one experiment.

2.4 Discussion

The aim of current study was to investigate whether OGG1 inhibitors designed by medicinal chemists in the Helleday Laboratory could overcome cisplatin resistance in bladder cancer cells by compromising their DNA repair capacity. First, the target binding properties of OGG1 inhibitors were determined in human cells. Then the most potent OGG1 inhibitor was tested in combination with cisplatin in cisplatin sensitive and resistant cell lines and the role of OGG1 in response to cisplatin treatment was studied. Additionally, to get insight how OGG1 responds to cisplatin- and MTH1 inhibitor-induced DNA damage, the effect of MTH1 inhibition on OGG1 protein levels were investigated. Overall, this study was a part of a larger project that aims to elucidate how inhibition of OGG1-dependent DNA repair could be exploited as combination therapy for cancer treatment. The importance of this lies behind the limitations of clinical anticancer treatments, as prolonged cisplatin exposure often leads to acquired resistance.

OGG1 is a major DNA glycosylase in the BER pathway responsible for recognition and removal of 8-oxoG, the most abundant oxidative DNA base damage. The current thesis provides first description about the binding properties of potent OGG1 inhibitors in cells by CETSA. A remarkable stabilization of OGG1 protein by various inhibitors corroborates their binding abilities to its designed target. While these inhibitors are designed and confirmed by *in silico* docking method to bind OGG1 active site (data not shown), the most plausible explanation for CETSA-based OGG1 stabilization by inhibitors is their binding to the active site. This is also supported by inhibition of OGG1 activity *in vitro* by those inhibitors (data not shown). Hence, I presume that the inhibitors decrease OGG1 activity inside cells. This will be confirmed in cell lysates by OGG1-based oligonucleotide cleavage assay. As inhibitor #10 showed remarkable *in vitro* inhibition of OGG1 activity (data not shown), this inhibitor was chosen to be the lead compound for further experiments. To resolve the highly specific interaction between inhibitor and OGG1 active site, collaboration has recently been established to obtain a crystal structure of OGG1 with inhibitor #10. This information would confirm inhibitor binding to OGG1 independently of CETSA assay. However this data do not confirm the selectivity of the inhibitor.

It is well known that DNA glycosylases of the BER machinery have structural similarities and substrate redundancy (Krokan & Bjoras, 2013), explaining why mice with disrupted DNA glycosylases are viable in most of the cases and show only moderate phenotype, including *Ogg1* knock-out mice (Jensen *et al*, 2003; Girard *et al*, 1998; Klungland *et al*, 1999a). OGG1 is the major DNA glycosylase responsible for 8-oxoG repair, yet Nei like DNA glycosylase 1

(NEIL1) is also contributing to 8-oxoG repair to lesser extent (Hegde *et al*, 2008). NEIL1 has a distinct role in repairing 8-oxoG in replicating genome (Hegde *et al*, 2013) and from single stranded DNA (ssDNA) (Dou *et al*, 2003), whereas OGG1 is ubiquitously active throughout cell cycle and only able to repair 8-oxoG from double stranded DNA (Rosenquist *et al*, 1997; Conlon *et al*, 2004). Thus, when developing inhibitors for DNA glycosylase it is important to test their selectivity for other glycosylases. Inhibitor #10 showed high *in vitro* selectivity to OGG1, while low selectivity against various other DNA glycosylases, (tested by Torkild Visnes, data not shown), which excludes those glycosylases as potential off-targets. Importantly, the inhibitor #10 does not inhibit NEIL1 activity *in vitro* (data not shown). However surprisingly, it does inhibit single-strand-specific monofunctional uracil DNA glycosylase 1 (SMUG1) activity with IC50 value approximately 10-fold higher compared to OGG1. This indicates that inhibitor #10 might engage other targets inside cells than OGG1. OGG1 and SMUG1 do not share substrate specificity (Boorstein *et al*, 2001; Wibley *et al*, 2003), so this minor inhibition of SMUG1 could potentially be explained by chemical characteristics of inhibitor #10 interacting with SMUG1 at high concentrations. All in all, it seems that OGG1 inhibitor #10 binds and inactivates OGG1 in cells and does not bind to structurally similar DNA glycosylases.

OGG1 inhibitors developed in our research group are potential clinical drug candidates as well as valuable tools to investigate the role of the BER in various contexts. The activity of BER pathway has been associated with resistance to cisplatin-based chemotherapy in non-small cell lung and ovarian cancer (Wang *et al*, 2009; Zhang *et al*, 2009). Therefore, BER inhibition might potentially reverse the limited outcome of drug-resistant therapies, such as acquired cisplatin resistance. Recently, OGG1 inhibitors were shown to have potent *in vitro* properties (Donley *et al*, 2015), however there are no reports of using OGG1 inhibitors in cells. In this thesis, inhibiting OGG1-specific DNA repair with in-house developed inhibitors overcame cisplatin resistance. My results by combining OGG1 inhibitor #10 with cisplatin treatment demonstrate a significant decrease in cellular viability and survival in cisplatin resistant bladder cancer cells, but not in parental cisplatin sensitive cells, suggesting that cisplatin resistance in these cells might be supported by OGG1-specific DNA repair. These results are in agreement with higher OGG1 expression in cisplatin resistant cells compared to sensitive cells. Based on these results, I propose that OGG1 inhibition upon cisplatin treatment increases 8-oxoG levels in DNA beyond what a replicative cell can handle, leading to cell death. This hypothesis will be further tested by rescue experiments using two strategies: First, to confirm the role of OGG1 in acquired cisplatin resistance of bladder cancer cells, OGG1 will be depleted by RNAi and overexpressed RNAi-resistant OGG1 in

cisplatin resistant cells. Second, by overexpressing OGG1 functional analogue in bacteria Fpg that shares substrate specificity for 8-oxoG but is not inhibited by inhibitor #10 *in vitro* (data not shown). In case these strategies reverse cellular viability and survival in cisplatin resistant NTUB1/P cells treated with inhibitor #10 and cisplatin, increased DNA repair activity can be concluded a major determinant of resistance in the cells.

To explain the effects of OGG1 inhibitor #10 in cisplatin resistant NTUB1/P cells in more detail, DNA damage response was investigated in cisplatin sensitive and resistant cells after cisplatin treatment. As mentioned before, western blot analysis revealed increased OGG1 protein expression in cisplatin resistant cells compared to sensitive cells. This increase is not induced by cisplatin treatment, which indicates stable changes in the cells upon acquired resistance. It has been previously shown that high OGG1 expression promotes esophageal squamous carcinoma cell survival upon cisplatin treatment (Gao *et al*, 2013), which supports the findings here. This supports OGG1 as being a potential biomarker for cisplatin treatment outcome. Cisplatin sensitive NTUB1 cells showed higher DNA damage induction upon cisplatin treatment as seen by increased γH2AX levels compared to cisplatin resistant NTUB1/P cells. This demonstrates higher resistance to cisplatin-induced DNA damage in resistant cells that could be obtained by DNA repair supported by increased OGG1 expression. OGG1 protective role against cisplatin-induced cell death has been reported (Gao *et al*, 2013) and also seen by Torkild Visnes in cells that are not cisplatin resistant (data not shown), which indicates the general protective role of OGG1 in cisplatin-treated cells. More cisplatin resistant and sensitive cell lines as well as patient derived material will be tested to draw conclusions whether OGG1 activity supports cisplatin resistance and shows a general protective role for cisplatin treatment.

On the contrary to my hypothesis of correlating increased OGG1 activity with higher protection against oxidative DNA damaging agents in cancer treatment, it has been previously reported that over-expression of mitochondrial OGG1 leads to increased sensitivity in hepatoma cells upon cisplatin treatment (Zhang *et al*, 2007). This effect was explained by OGG1 over-expression creating an imbalance in the BER pathway that could potentially disturb the repair process. This finding raises questions regarding my observations and investigating mitochondrial and nuclear OGG1 isoform levels and activity would be very interesting. In addition, OGG1 over-expression has also been reported to sensitize cells to ionizing radiation (IR), explained by aberrant base excision repair, where DNA glycosylase activity creates toxic double-strand breaks at clustered DNA lesions (Yang *et al*, 2004). Interestingly, it has been shown *in vitro*, that the lack of other downstream BER proteins is not the reason behind increased cytotoxicity upon IR (Harrison *et al*, 1999). Explanation for

the mechanistic differences upon and cisplatin and IR might be different DNA damage types induced by those treatments, which are therefore differently tolerated by cells. However, it would be interesting to test whether exogenous over-expression of OGG1 in cisplatin resistant cells would lead to increased cell death upon cisplatin treatment. Moreover, repair capacity of downstream BER machinery in cisplatin resistant NTUB1/P cells will be tested, to get insight whether intact BER capacity rather than OGG1 activity is supporting the resistance.

As already noted, there have been various studies investigating OGG1 expression levels and response to cisplatin treatment in cancer cells. Nevertheless, there is no evidence showing that OGG1 is involved in cisplatin-induced DNA damage repair. Indeed, the latter question is not easy to directly address but evaluating protein mobility offers a way to study this indirectly. Here, FRAP method was applied to investigate GFP-tagged OGG1 mobility after cisplatin treatment in living cells. My initial idea to indirectly study cisplatin-induced DNA damage repair by FRAP was supported by previous study, where decreased OGG1-GFP mobility upon treatment with the oxidative agent $KBrO_3$ was detected (Campalans *et al*, 2013). In this study, the presence of cisplatin reduced the fluorescence recovery compared to control cells showing decreased mobility of OGG1 upon cisplatin treatment. This suggests OGG1-GFP increased binding to cisplatin-induced DNA damage sites. In addition, OGG1-GFP mobility will be evaluated in presence of OGG1 inhibitor #10, to investigate the binding properties of OGG1-GFP to DNA upon inhibition. It should be noted that besides interacting with DNA, cisplatin is also known to react with proteins to some extent (Bischin *et al*, 2011), that might affect the mobility of OGG1-GFP in this study. Even though it seems that OGG1-GFP have increased binding to cisplatin-induced DNA damage, unspecific immobilization of OGG1-GFP cannot be ruled out. However, this finding alongside with OGG1 inhibition results reported give strong basis to further investigate the molecular mechanisms of OGG1 in cisplatin-induced DNA damage response in cancers.

OGG1 activity to repair 8-oxoG is tightly coordinated with enzymes such as MTH1 and MUTYH. 8-oxoG in DNA can originate from oxidized nucleotide pool or by direct oxidation of DNA (Nakabeppu, 2014). MTH1, OGG1 and MUTYH minimize the accumulation of 8-oxoG in DNA and prevent mutagenesis induced by 8-oxoG in mammals (Tsuzuki *et al*, 2007). MTH1 inhibitor TH588 has been recently presented as cancer phenotypic lethal, by converting high ROS levels into toxic DNA damage (Gad *et al*, 2014). Here, a side project was designed to study the activity of OGG1 in MTH1-inhibited cells and address the potential limitations of anticancer therapies with MTH1 inhibitors. For that, siRNA depletion of OGG1 was used. OGG1 depletion in U2OS cells did not affect the viability or survival after MTH1 inhibition with TH588. This suggests either that remained low OGG1 levels upon RNAi are

sufficient to repair 8-oxoG from DNA or that another backup activity such as MUTYH is contributing for cancer cell survival upon MTH1 inhibition. CRISPR-based OGG1 and MUTYH knockout cells will be established to further investigate OGG1-dependent responses to MTH1 inhibition. Comet assay will be applied to directly investigate 8-oxoG content in DNA after TH588 in OGG1-depleted cells.

Next, a subcellular fractionation was performed to investigate OGG1 localization upon MTH1 inhibition. It was previously shown that OGG1 is actively recruited to chromatin fraction upon $KBrO_3$ treatment (Amouroux *et al*, 2010). As TH588 induces 8-oxoG levels in DNA in cancer cells (Gad *et al*, 2014), I expected to detect OGG1 accumulation in the chromatin fraction upon TH588 treatment. Interestingly, OGG1 levels remained constant in each fraction after TH588 treatment. Also, most of the OGG1 protein was present in soluble nuclear fraction. The reason behind this might be protocol-specific, as relatively high salt concentrations were used that might dissociate loosely chromatin-bound proteins, such as OGG1. This would explain high OGG1 levels in soluble nuclear fraction, compared to chromatin fraction. Another fractionation protocol with lower salt concentrations will be tested. Also, fractionation findings imply no significant DNA damage induction indicated by γH2AX induction after TH588 treatment. This could also be explained by fractionation protocol, efficient DNA damage repair or too short incubation time of TH588 that was not enough to induce DNA damage. To overcome this issue, immunofluorescence will be applied to visualize OGG1 localization and DNA damage induction upon TH588 treatment after various time points. Chromatin fractionation findings were supported by FRAP, which showed no changes in OGG1-GFP mobility upon TH588 treatment in living cells. The role of OGG1 in mediating DNA repair upon TH588 remains unelucidated and therefore further experiments are needed to directly investigate how OGG1 responds to MTH1 inhibitor treatment and whether it could underlie the resistance mechanism for MTH1 inhibitors. In that case, OGG1 inhibition could be applied as a cancer combination therapy with MTH1 inhibitors. However, my data does not support OGG1 as a potential resistance mechanism for MTH1 inhibitors. To investigate OGG1-dependent mechanisms further, TH588 resistant cell line will be developed.

Taken together, this thesis described first OGG1 inhibitors that sensitized cisplatin resistant bladder cancer cells. Additionally, my results indicate a supportive role of OGG1 activity for cells upon cisplatin treatment. Moreover, these results give preliminary insight that OGG1 might be involved in cisplatin-induced DNA damage repair, which all support OGG1 being as a promising anticancer target. As a side project, OGG1 responses were investigated upon MTH1 inhibition, which provide information about 8-oxoG repair. Future outlook for this

project comprises drug development to improve chemical properties and biologic potency of inhibitor #10, rescue experiments to provide specificity to inhibitor effects. Also, more cell lines and patient derived samples will be tested to validate my findings in a broader scale.

SUMMARY

The aim of this study was to investigate DNA glycosylase OGG1 responses to cisplatin- and MTH1 inhibitor-induced DNA damage. Additionally, to evaluate OGG1 inhibitor target binding properties and their use to overcome cisplatin resistance in bladder cancer cells. This study was a part of a larger project that aims to elucidate how inhibition of OGG1-dependent DNA repair could be exploited as combination therapy for cancer treatment. Acquired resistance of commonly used chemotherapy drugs is a major problem for the patients, as this decreases the anticancer efficiency of the treatment and leads to tumor relapse. New targeted-therapy strategies are needed to battle against drug resistance by combination therapy.

Here, early drug development phase OGG1 inhibitors to overcome cisplatin resistance were presented. Evaluation of cellular target engagement of 10 small-molecule compounds designed to target OGG1 catalytic activity revealed more than 2-fold stabilization by CETSA, which indicates the binding of the inhibitors to OGG1. This data alongside with previously obtained results suggested inhibition of OGG1 catalytic activity. Inhibitor #10 was chosen to be the main inhibitor of interest and dose-response stabilization curve revealed thermal stabilization of OGG1 at 1.11 µM. Next, inhibitor #10 reduced the cellular viability and survival of cisplatin resistant bladder cancer cells upon cisplatin treatment. Cells treated with 25 µM cisplatin showed a 20% reduction in cell viability upon 10 µM inhibitor treatment and a 60% reduction upon 25 µM inhibitor treatment. Overall, resistant cells became more sensitive to cisplatin after OGG1 inhibition, suggesting the importance of OGG1 activity to cisplatin resistant bladder cancer cells. Resistant cells exhibited lower level of DNA damage after 10 µM of cisplatin treatment compared to sensitive cells, illustrating their resistant state. Sensitization to OGG1 inhibitor is supported by increased DNA repair capacity being described as one of the cisplatin resistance mechanisms. Moreover, increased OGG1 levels in cisplatin resistant cells compared to cisplatin sensitive cells were found. Elevated OGG1 levels supports OGG1 being a good therapeutic target to overcome cisplatin resistance. Moreover, cisplatin sensitive cells resulted in notably higher DNA damage levels measured by γH2AX compared to resistant cells. Interestingly, preliminary results by using FRAP suggested prolonged binding of OGG1 to DNA upon cisplatin treatment, which indicates OGG1 involvement in cisplatin-induced DNA damage repair.

In addition, OGG1 activity was studied by siRNA-based depletion upon MTH1 inhibition, a novel anticancer strategy, to underlie potential therapeutic limitations of MTH1 inhibitors. No difference was seen in the survival or viability in OGG1-depleted or proficient cells upon MTH1 inhibitor TH588 treatment. Also, no subcellular localization or prolonged binding to

DNA by FRAP was detected upon TH588 treatment, leaving the role of OGG1 in mediating 8-oxoG repair upon TH588 unelucidated. This data gives novel insights into initial development of OGG1 inhibitors for combination anticancer therapy as well as valuable tools to investigate BER in the future.

DNA reparatsioonivalgu OGG1 inhibitsioon kui potentsiaalne kasvajate kombineeritud ravi

Marianna Tampere

KOKKUVÕTE

Kasvajate tundlikkus keemiaravile on tihti ajutine ning kasvaja areneb ravimresistentseks, millega kaasneb haiguse taasteke ning patsientide kõrgem suremus. Seetõttu on suur vajadus uute vähivastaste ravimeetodite arenduseks, mille abil ravimresistentsust ületada. Kombineerides keemiaravi ning sihtmärk-spetsiifilist lähenemist resistentsust tagavate mehhanismide vastu on potentsiaalselt võimalik ravimresistentsuse vastu võidelda.

Vähkkasvajaid iseloomustab piiramatu jagunemisvõime ja kõrge oksüdatiivse stressi tase. Vähirakkude elulemuse tagamiseks nendes tingimustes on nad omandanud võime replitseerida suures mahus DNA-d ning rakendanud mitmeid täiendavaid DNA reparatsiooni mehhanisme. Seetõttu on kasvajarakkudes olev DNA ja selle replikatsiooni protsess sihtmärgiks mitmetele laialdaselt kasutatavatele keemiaravimitele, nagu näiteks cisplatin, mis eelkõige hävitavad kiiresti paljunevaid kasvajarakke. Cisplatin indutseerib erinevat tüüpi DNA kahjustusi. Lisaks laialdaselt kirjeldatud DNA ristsidemete moodustumisele on näidatud, et cisplatini toimel tekivad rakkudes reaktiivsed hapnikuühendid, mis põhjustavad oksüdatiivseid kahjustusi nii DNA-s kui ka selle monomeerides nukleotiidides. 8-okso-7, 8-dihüdro-2’-deoksüguanosiin (8-oxoG) on kõige sagedasem oksüdatiivne lämmastikaluse kahjustus, mis elimineeritakse nukleotiidide seast MTH1 valgu hüdrolüütilise aktiivsuse toimel. DNA aluse asendamise reparatsiooni signaalirada ning DNA glükosülaas OGG1 eemaldavad 8-oxoG kahjustuse DNA-st ning asendavad selle korrektse lämmastikalusega. Cisplatini raviga kaasneb tihti resistentsus, mille ühe mehhanismina on kirjeldatud suurenenud DNA reparatsiooni mahtu. Käesoleva töö peaeesmärgiks oli uurida kas OGG1 inhibitsiooniga on võimalik cisplatini ravimresistentsust ületada ning kuidas panustab OGG1 valk cisplatini ravimresistentsuse tekkele.

Töö tulemusena selgus, et Helleday teadusgrupis sünteesitud inhibiitorid seonduvad OGG1 valguga rakkudes ning potentsiaalselt inhibeerivad ka OGG1 aktiivsust. Uurimaks inhibiitorite mõju ravimresistentsusele kasutati OGG1 inibiitorit number 10 (#10) ning cisplatinile tundlikke ja resistentseid rakke. Cisplatini ja 25 µM inhibiitori #10 koostoimel langes resistentsete rakkude elulemus kuni 60%. Lisaks sellele langes cisplatini ja 10 µM inhibiitor #10 juuresolekul resistentsete rakkude proliferatsioon kuni 75%. Need tulemused kirjeldavad kuidas OGG1 aktiivsus võib resistentsetele rakkudele vajalik olla, kuna

inhibitsioon muutis resistentsed rakud cisplatinile tundlikumaks. OGG1 kõrgem ekspressioon cisplatinile resistentsetes rakkudes võrreldes tundlike rakkudega viitab võimalusele, et OGG1 osaleb cisplatini resistentsuse tekkel, mis selgitaks OGG1 inhibiitorite efekti rakkude elulemusele ja proliferatsioonile. Uurides 10 µM cisplatini poolt tekitatud DNA kahjustust resistentsetes ja tundlikes rakkudes on näha, et fosforüleeritud histoon 2AX (γH2AX) tase resistentsetes rakkudes on märkimisväärselt madalam. See viitab cisplatini resistentsusele, mis võib olla tagatud suurenenud OGG1 vahendatud DNA reparatsiooniga. FRAP meetodiga elus rakkudes teostatud esialgse katse tulemused viitavad, et GFP-ga märgistatud OGG1 (OGG1-GFP) mobiilsus rakutuumas langeb cisplatini toimel, mis vihjab OGG1 tugevnenud seondumisele DNA-ga. Sellest saab kaudselt järeldada, et OGG1 võib osaleda cisplatini indutseeritud DNA kahjustuste parandamises. Need tulemused toetavad OGG1 valku kui sobivat sihtmärki cisplatini resistentuse kõrvaldamiseks nendes rakkudes.

Kõrvalprojektina uuriti selle töö käigus kuidas OGG1 valgu aktiivsus mõjutab rakkude vastust MTH1 inhibiitorile. Hiljuti kirjeldati Helleday teadusgrupi poolt MTH1 inhibiitori TH588 vähivastane mehhanism, mis toimib 8-oxoG suurenenud inkorporeerumisel DNA-sse ning seeläbi DNA kahjustuse indutseerimisel. Kuna OGG1 elimineerib 8-oxoG mis on DNA-sse inkorporeeritud, uurisin kuidas mõjub TH588-ga töödeldud rakkudele OGG1 siRNA töötlus. OGG1 siRNA transfektsioon ei mõjutanud rakkude elulemust ega proliferatsiooni vastusena TH588 töötlusele, vihjates, et OGG1 ei ole TH588 poolt indutseeritud DNA kahjustamise reparatsiooniga seotud. Teisalt, OGG1 siRNA transfektsiooni järgselt oli OGG1 valk siiski detekteeritav, mistõttu on võimalik, et minimaalne hulk OGG1 valku on piisav, et TH588 poolt tekitatud DNA kahjustus elimineerida ning tagada rakkude eluvõime. Rakkude fraktsioneerimine ja FRAP meetod ei kajasta OGG1 tugevnenud seondumist DNA-le pärast TH588 töötlust, erinevalt positiivse kontrollina kasutatud kaalium bromiidi ($KBrO_3$) korral. Nende tulemuste põhjal jääb OGG1 roll TH588 indutseeritud DNA kahjustuse parandamisel minimaalseks, kuid seda tuleks lisauuringutega kinnitada või ümber lükata.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisors Professor Thomas Helleday, Marjo-Riitta Puumalainen, Torkild Visnes and Viljar Jaks for support, engagement and motivation throughout the research and thesis writing. I would also like to acknowledge all of my colleagues from the Helleday Laboratory, especially OGG1 project and targeted biology team members. Finally, I thank my parents, sister and grandparents for unconditional support and love.

This research was partly funded by Kristjan Jaak scholarship.

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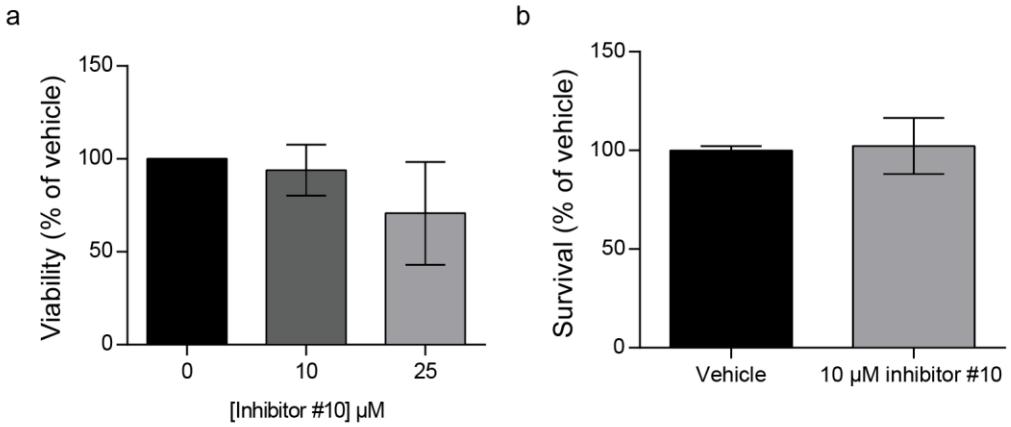
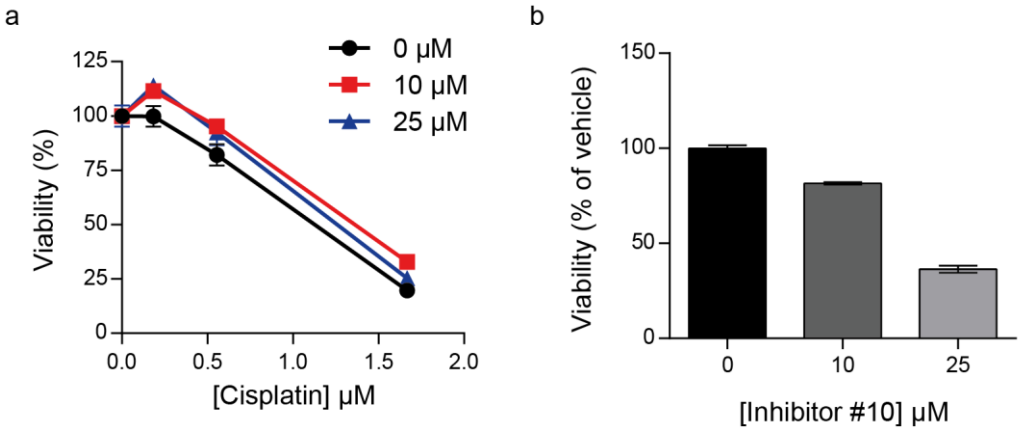
Appendix 1



Supplementary figure 1. Inhibitor #10 shows slight cytotoxic effects at 25 µM, but not at 10 µM in NTUB1/P cells. **a)** Viability of NTUB1/P cells treated with 10 µM or 25 µM inhibitor #10 or DMSO vehicle for 72 hours. Values are presented as mean viability normalized to vehicle control, error bars represent SD from three independent experiments. **b)** NTUB1/P cells treated with 10 µM inhibitor #10 or DMSO for 10 days. Formed colonies were fixed and stained with 4% methylene blue in MetOH and counted manually. Values are indicated as survival compared to vehicle-treated control and error bars represent SD from technical replicates from one experiment.

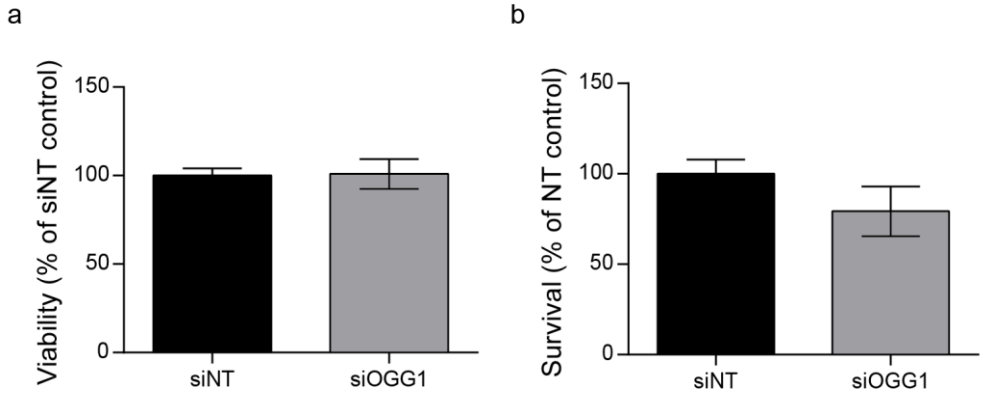
Appendix 2



Supplementary figure 2. Inhibitor #10 does not sensitize parental NTUB1 cells. a) Viability of NTUB1 cells treated with 0.18 µM, 0.55 µM or 1.67 µM cisplatin combined with 10 µM or 25 µM inhibitor #10 for 72 hours. Saline and DMSO were used as vehicle controls for cisplatin and inhibitor #10, respectively. Values are presented as mean viability normalized to vehicle controls, error bars represent SD from technical replicates from one experiment. **b)** Viability of NTUB1 cells treated with 10 µM or 25 µM inhibitor #10 or

DMSO for 72 hours. Values are presented as mean viability normalized to vehicle control, error bars represent SD from technical replicates from one experiment.

Appendix 3



Supplementary figure 3. Slight decrease in survival, but not in viability in OGG1-depleted U2OS cells. a) Viability assay of U2OS cells transfected with siNT or siOGG1 for 48 hour, re-seeded and treated with DMSO for 72 hours. Values are indicated as viability compared to NT control and error bars represent average SD from technical replicates from one experiment. **b)** Survival assay of U2OS cells transfected with siNT or siOGG1 for 48 hours, re-seeded for colony formation and treated with DMSO for 10 days. Values are indicated as survival compared to siNT control and error bars represent average SD from technical replicates from one experiment.

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