UNIVERSITY OF LJUBLJANA
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FUNCTIONAL GENETIC SCREENS TO IDENTIFY AND ELUCIDATE POTENTIAL RESISTANCE MECHANISMS TO MTH1 INHIBITORS

M. Sc. THESIS
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UGOTAVLJANJE MEHANIZMOV ODPORNOSTI PROTI INHIBITORJEM ENCIMA MTH1 S FUNKCIONALNIMI GENETSKIMI PREGLEDI

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AB Reactive oxygen species (ROS) occur in cells as a natural side-effect of metabolism but can cause major damage to DNA and free nucleotide pool. Damaged nucleotides can get incorporated to DNA and cause mutations and double strand breaks, eventually leading to cell death. MutT homologue 1 (MTH1) sanitizes the nucleotide pool and thus prevents DNA damage caused by incorporation of oxidized nucleotides. Since ROS levels in cancer cells are much higher compared to normal cells, MTH1 inhibition would be expected to selectively kill cancer cells. This is the rationale behind development of novel MTH1 inhibitors as a targeted therapeutics. The major challenge of targeted therapy is the occurrence of resistance. The aim of this research was to investigate if cells can get resistant to MTH1 inhibitors and study the mechanism behind it. We performed a functional genetic screen with clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9) to identify possible resistance mechanisms to MTH1 inhibition. The technical performance of the screen was good, however none of 8 top hits validated to be a true hit. We observed emergence of spontaneous background resistance indicating that cells got resistant by other means than loss of certain gene. We show that resistance phenotype is mild but stable and is not explained by MTH1 overexpression, overexpression of drug pumps or lower ROS levels. Understanding mechanisms of resistance to MTH1 inhibition may help in designing new biomarkers for treatment response or combinational treatments for cancer patients.

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AI Reaktivne kisikove zvrsti (ang. *reactive oxygen species*, ROS) se v celicah pojavljajo kot stranski produkt metabolizma. Povzročijo lahko oksidativno škodo v DNA in prostih nukleotidih, ki se nato lahko vgradijo v DNA in vodijo v mutacije in celično smrt. Encim MTH1 preprečuje vgraditev oksidiranih nukleotidov v DNA. Ker imajo rakave celice veliko višji nivo ROS kot normalne lahko inhibicija MTH1 selektivno cilja rakave celice. Inhibicija MTH1tako predstavlja nov pristop tarčnega zdravljenja. Ker pa je ključni problem takega zdravljenja pojav odpornosti je pomembno odkriti mehanizme, ki vodijo v odpornost. Da bi odkrili, ali celice lahko postanejo odporne proti inhibitorjem MTH1 smo izvedli funkcionalni genetski pregled s tehnologijo CRISPR (ang. *clustered regularly interspaced palindromic repeats*)/Cas9 (ang. CRISPR associated protein 9). Tehnična izvedba pregleda je bila ustrezna, vendar nobeden od osmih najboljših zadetkov ni povzročil odpornosti. Opazili pa smo pojav spontane odpornosti v ozadju, kar nakazuje da so celice postale odporne na drugačen način. Opisana rezistenca je blaga vendar stabilna in ni odvisna od prekomernega izražanja MTH1 ali ATP-odvisnih črpalk in razlike v nivoju ROS. Razumevanje mehanizmov odpornosti lahko vodi v razvoj novih označevalcev ali kombiniranega zdravljenja za bolnike z rakom.

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GLOSSARY

2-OH-dATP	2-hydroxydeoxyadenosine 5'- triphosphate
8-oxo-dGTP	8-Oxo-2'-deoxyguanosine-5'-triphosphate
ALK	anaplastic lymphoma kinase
ATM	ataxia telangiectasia mutated
ATP	adenosine triphosphate
BCA	bicinchoninic acid
BCR-ABL	breakpoint cluster region - abelson murine viral oncogene homolog
BCRP	breast cancer resistance protein
BRAF	B- rapidly accelerared fibrosarcoma
BRCA1,2	breast cancer 1,2
BSA	bovine serum albumin
CAR	chimeric antigen receptor
Cas9	CRISPR associated protein 9
CD8+	cluster of differentiation 8 positive
CLASP2	cytoplasmic linker-associated protein 2
CML	chronic myeloid leukemia
CRISPR	clustered regularly interspaced short pallindromic repeats
CRISPRa	CRISPR activation
CRISPRi	CRISPR interference
CTLA4	cytotoxic T-lymphocyte-associated protein 4
CXorf65	Chromosome X open reading frame 65
dCas9	catalitically dead Cas9
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DSB	double strand break
ECM	extra cellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition
Ets	E26 transformation specific
FACS	flow cytometry
FBS	fetal bovine serum
FDA	Food and Drug Administration
GeCKO	genome-scale CRISPR knock-out
gRNA	guide RNA
GTP	guanosine triphosphate
has-mir-21	homo sapiens micro RNA 21
HDR	homology directed repair
HER2	human epidermal growth factor receptor 2
HRP	horseradish peroxidase
HSP90	heat shock protein 90
hTERT	human telomerase reverse transcriptase
IAP	inhibitor of apoptosis protein

KRTAP3-3	keratin associated protein 3-3
MAGeCK	model-based analysis of genome-wide CRISPR/Cas9 knockout
MAPK	mitogen activated protein kinase
MDR1	multi-drug resistant protein 1
MEK	mitogen-activated protein kinase kinase
MET	mesenchymal-epithelial transition
MiR-145	micro RNA 145
MOI	multiplicity of infection
MRP1	Multidrug resistance-associated protein 1
MTH1	MutT homologue 1
NADPH	nicotinamide adenine dinucleotide phosphate
NHEJ	non-homologous end joining
NUDT	nudix type motif-containing
OIS	oncogene induced senescence
P2RX5	purinergic receptor P2X, ligand gated ion channel 5
PAM	protospacer adjacent motif
PARP	poly ADP ribose polymerase
PBS	phosphate buffered solution
PCR	polymerase chain reaction
PD1	programmed cell death 1
PDGFR	platelet-derived growth factor receptor
pDNA	plasmid DNA
PI3KCA	phosphoinositide-3-kinase, catclytic alpha isoform
PITPNA	phosphatidylinositol transfer protein alpha
PPP1CC	protein phosphatase 1 catalytic subunit
PVDF	polyvinyliede fluoride
RAS	rat sarcoma viral oncogene homolog
RIPA	radioimmunoprecipitation assay
RNAi	RNA interference
ROS	reactive oxygen species
shRNA	short hairpin RNA
siRNA	small interfering RNA
SSB	single strand break
STR	small tandem repeats
SV40	simian vacuolating virus 40
TALEN	transcription activator-like effector nuclease
TBHP	tert-butyl hydroperoxide
TIL	tumor inflitrating lymphocytes
UV	ultra violet
VEGF	vascular endothelial growth factor

1 INTRODUCTION

The rapid and fierce advancements medical sciences produced in recent years enabled us to eliminate many infectious diseases and effectively treat others. But the shift of the leading cause of death worldwide from infectious diseases to cardiovascular diseases and cancer shows that there are still diseases we are unable to effectively battle. For example, the number of deaths caused by cancer is expected to rise in the next 20 years, according to the World health organization. However, cancer is not a modern disease contrary to what this may suggest and it has burdened mankind for as long as mankind exists. Its true impact was simply hidden by other diseases that were spreading faster and killing sooner.

With the increased incidence of cancer the pressure to find the cure is also rising. However, the expectations of discovering a single cure for cancer are becoming less and less realistic. The reason for that is that we now know that cancer is not one disease but a set of different diseases with different properties. With the development of new technologies, for example advancements in sequencing, we are now able to understand cellular processes that lead to cancer much better but it is also becoming obvious that the complexity and adaptability of the disease may simply be too big to cure it with a single miracle compound.

One of the major recent breakthroughs in cancer treatment is the use of so-called targeted compounds that are designed to inhibit specific targets crucial for survival of cancer cells. The initial excitement ended quite soon because of emergence of resistance. Because of redundancy of molecular pathways, tumor heterogeneity and the ability of cancer cells to rapidly adapt to inhibition, helped by genomic instability, most of the targeted compounds are not so effective as initially thought. On the other hand, studies of resistance mechanisms uncovered even more information about molecular pathways in cancer cells. This knowledge could lead to discoveries of new synthetic lethal interactions in cancer cells which could offer a new way of targeting cancer cells. In addition to targeted compounds designed to inhibit oncogenic proteins that drive tumorigenesis, other potential targets have been exploited. When comparing the differences between cancer cells and normal cells to search for new targets, one of the most obvious differences is the level of DNA damage and oxidative stress. High levels of ROS in cancer cells lead to DNA damage and damage in free nucleotide pool. However, one of the key enzymes that are involved in sanitization of free nucleotide pool is MutT homologue 1 (MTH1), whose inhibition was described as a novel approach of cancer treatment. As the inhibitors are nearing clinical trials it is of great importance to know if cancer cells can become resistant to MTH1 inhibition and how. These findings could therefore help in finding new targets that can overcome resistance to MTH1 inhibition and enable new effective treatments.

The aim of this work was to identify and elucidate potential resistance mechanisms to MTH1 inhibitors using a novel approach of functional genetic screen. We aimed to perform a genome-wide genetic screen using clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR associated protein 9 (Cas9) technology and validate the top hits. Our hypothesis was that cells can become resistant to MTH1 inhibition and that the resistance is the result of gene inactivation.

2 REVIEW

2.1 BIOLOGY OF CANCER

Even though cancer is often referred to as a single disease, it actually consists of a diverse group of diseases, only sharing a couple of key characteristics. In its essence cancer is a disease of the (epi)-genome, consisting of massive genetic and epigenetic changes (Weinberg, 2013). The start of the malignant process begins with damaged DNA that can be a result of hereditary predisposition or different environmental and lifestyle factors such as tobacco and alcohol use or chronic infections (Jemal et al., 2011).

Because damaged DNA can have such profound consequences, cells have developed mechanisms that can detect and repair it. The source of DNA damage can be endogenous, like replication problems or external, like UV or radiation. These cause DNA lesions such as damaged bases, bulky adducts, mismatched bases, single strand breaks (SSB) and double strand breaks (DSB). The type of lesion determines the repair mechanism that is recruited to the damaged site. There are five different repair pathways: base excision repair which targets damaged bases, nucleotide excision repair which removes larger bulky adducts, mismatch repair targeting mismatched bases, single strand break repair that repairs nicks and double strand break repair that repairs broken double-stranded DNA. Even though each pathway consists of different proteins their general components are the same. The repair starts by sensing the lesion and stabilizing it. Then it signals to recruit other members of repair machinery, which remove the lesion, process the ends, synthesize DNA and ligate the nucleotide backbone. During repair cells arrest in the cell cycle and do not continue until damage has been repaired. However, damage repair mechanisms are not always effective and unrepaired lesions can lead to cell death or cause mutations (Lord and Ashworth, 2012; Weinberg, 2013).

If these mutations occur in oncogenes or tumor suppressor genes, their activity may be altered. Tumor suppressor genes are genes that are often inactivated in cancer and can be involved in restriction of growth, DNA damage repair or other processes. One of the most important tumor suppressor protein is p53, which can activate DNA repair, arrest cell cycle and induce apoptosis (Hanahan and Weinberg, 2000; Weinberg, 2013). Oncogenes, on the other hand, are genes that normally stimulate cell proliferation and are frequently upregulated or activated in cancer. By activating mutations in oncogenes cancer cells can also circumvent apoptosis, which enables them to proliferate infinitely. One of the most important oncoproteins is RAS, a small GTPase often harboring an activation mutation in cancer. This mutation keeps the protein in its active state at all times which results in increased proliferation rates (Weinberg, 2013).

Even though the process of malignant transformation usually begins with a single mutation that can become a driver mutation (Bozic et al., 2010), this is not sufficient for development of cancer. Oncogenic transformation starting with the first driver mutation often triggers a safety response that has to be disabled in order to continue

tumorigenesis. This first safety response is called oncogene induced senescence (OIS) and is characterized by the loss of proliferation capacity, morphological changes and induction of tumor suppressor and DNA damage pathways (Di Micco et al., 2006).

The process of tumorigenesis is therefore a multistep process, involving activation of oncogenes and inactivation of various tumor suppressor mechanisms. The capabilities tumor has to aquire were coined the hallmarks of cancer and summarize the shared characteristics of all cancer cells. The first hallmarks or functional capabilities described included sustaining proliferative signaling, evading growth suppressors, enabling replicative immortality, activating invasion and metastasis and resisting cell death (Hanahan and Weinberg, 2000). Later, another two emerging hallmarks were described, adding deregulation of cellular energetics and avoiding immune destruction to the list. In addition to hallmarks, two enabling characteristics were added that facilitate the acquisition of hallmarks, genome instability and mutation and tumor-promoting

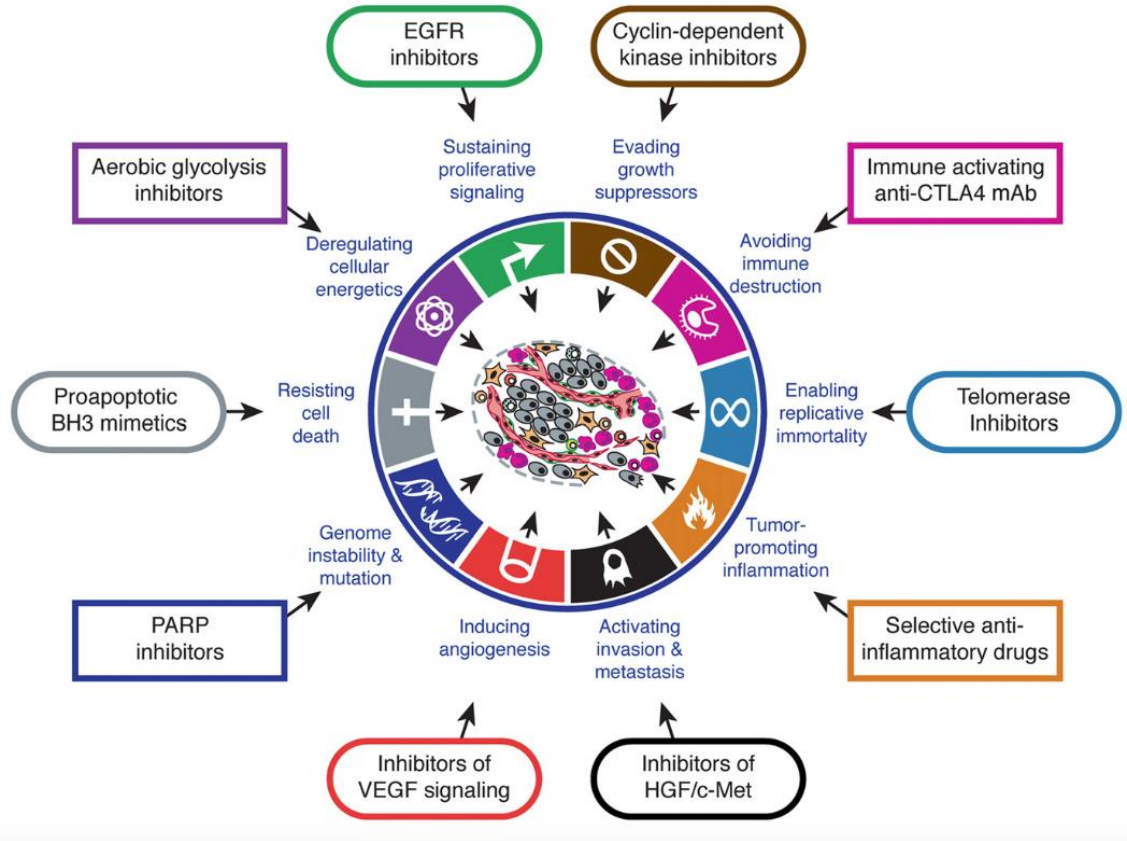


Figure 1: Hallmarks of cancer
All eight hallmarks of cancer and two enabling characteristics are being explored as targets for cancer treatment. Approaches listed here are only representing an example, since a lot of different therapy options are being developet at the moment (Hanahan and Weinberg, 2011: 668)
Slika 1: Lastnosti rakavih celic
Vseh osem lastnosti raka predstavlja osnovo iskanja novih tarč za zdravljenje. Slika prikazuje le primere terapij, ki so trenutno v razvoju. (Hanahan in Weinberg, 2011:668)

inflammation (Hanahan and Weinberg, 2011). Together, these provide a platform for understanding the process of selection and the capabilities that are essential for cancer cells (Figure 1).

The addition of new hallmarks and enabling characteristics is in line with the shift of focus in understanding cancer biology which more and more includes the surroundings of the tumor, the so-called tumor microenvironment. Components of microenvironment, for example immune cells and fibroblasts, secrete various signals that can promote or impair tumor progression (de Visser et al., 2006; DeNardo et al., 2011; Kalluri and Zeisberg, 2006; Qian and Pollard, 2010).

The stepwise progression and acquirement of hallmark capabilities can be divided in two steps: primary tumorigenesis and metastasis. Primary tumorigenesis can occur slowly and last for years before benign primary tumor undergoes an additional changes that transform the cells into invasive cancer cells (Hanahan and Weinberg, 2011). The process of metastasis requires several changes in tumor cells, which have to be able to detach from their surroundings, survive in the circulation and form a micrometastasis at distant organ site (Valastyan and Weinberg, 2011). Epithelial-mesenchymal transition (EMT) was described as a process, which enables tumor cells to begin intravesation to blood vessels and metastasis. Similarly, tumor cells that arrived at the site of metastasis have to undergo mesenchymal-epithelial transition (MET) to form metastasis (Lamouille et al., 2014).

2.2 CANCER THERAPY

Despite the prevalence of cancer, the treatment options are still not as effective as hoped. The most common treatment strategies include surgery, chemotherapy and radiotherapy with immunotherapy and targeted therapy emerging as novel options for particular cancer types (Siegel et al., 2013).

2.2.1 Conventional systemic cancer therapy

While surgery can also be used as a diagnostic or palliative tool, curative surgery consists of excision of tumor and surrounding tissue, for example lymph nodes. It can be really effective for treatment of localized non-hematological and pre-metastatic tumors. It is most commonly used as a treatment of choice for breast, prostate and lung cancers (Siegel et al., 2013). The major drawback of surgery is its ineffectiveness in treating metastatic disease and also the fact that many tumors are inoperable due to various reasons. Incomplete tumor excision due to problems with visualization can also lead to recurrence, so surgery is most commonly used in combination with chemotherapy or radiotherapy.

The effect of both chemotherapy and radiotherapy is induction of DNA damage. Because tumor cells are rapidly dividing and often inactivate checkpoints that would

normally enable cell cycle arrest and DNA damage repair, the damage accumulates until it is no longer compatible with life. But since there are also normal rapidly dividing cells present in the body, these treatments are less specific and they often cause severe side effects and toxicity to normal tissue. Chemotherapy is a common term for cytotoxic drugs interfering with the DNA synthesis, replication and transcription. Chemotherapy is rarely used as the only treatment of choice and is more commonly administered as adjuvant therapy after surgery or radiotherapy to eradicate micrometastases (Chabner and Roberts Jr, 2005; Urruticoechea et al., 2010). Radiotherapy induces DNA damage by ionizing radiation, which causes generation of ROS. These then lead to single or double strand DNA breaks and cell death. Radiotherapy is also mostly used in combination with surgery and/or chemotherapy. While surgery, chemotherapy and radiotherapy alone or in combination can be very effective in certain types of cancers there are still a lot of tumor types that we are unable to cure using only these treatments.

Although specific chemotherapeutics are used to treat specific cancer types and different approaches in radiotherapy are used based on subtype and location of the tumor, these therapies still have a very broad effect. Predictive and prognostic biomarkers have emerged lately to try and predict a response of a patient to these therapies. In addition, new treatments emerged recently with new discoveries about cancer biology.

2.2.2 Immunotherapy

With the realization about how important microenvironment is in progression of cancer, several approaches targeting microenvironment components. Among them immunotherapy holds great promise for, with the goal to shift the balance between pro-tumorigenic and anti-tumorigenic immune cells at the tumor site in favor of the anti-tumorigenic cells. The two main approaches of immunotherapy are blocking the immune checkpoints with monoclonal antibodies thus allowing T-cells to recognize and destroy the tumor (Hodi et al., 2010; Pardoll, 2012) and T-cell based immunotherapy (TIL therapy) (Drakeet al., 2014; Mellman et al., 2011; Nakano et al., 2001). Additionally, combinations between immunotherapy and targeted therapy have been explored recently (Vanneman and Dranoff, 2012).

2.2.3 Targeted therapy

Targeted therapy represents a novel approach to cancer treatment and begun to emerge in the late 1990s. With the advancements in sequencing technology that enabled high throughput generation of data, the view on classification of tumors changed. Even though cancer types are still mainly described based on the organ of origin and histological status, sequencing technology now allows much more important classification based on tumor genotype. When ideas of rationally designed inhibitors that specifically target cancer cell defects were introduced, it seemed that the “magic bullet” to finally win the battle with cancer was discovered. The preliminary results were promising, tumors shrinked, survival rates improved and the scientific community

was busy finding new targets and ways to inhibit them. Unfortunately the success was relatively short-lived. After initial response, the disease most often relapsed and usually killed the patient. Emergence of resistance therefore prevented the success of targeted therapy but provided a new opportunity to study adaptability of cancer cells.

Finding targets for targeted therapy was aided by new technology development and understanding of oncogenic and tumor suppressor pathways (Chabner and Roberts Jr, 2005; Urruticoechea et al., 2010). Most inhibitors designed as targeted therapeutics inhibit kinases that are crucial for driving proliferation pathways (Sawyers, 2004) . Even before the idea of targeting cancer-specific proteins emerged, hormonal therapy gained some attention as a form of targeted therapy. It can be beneficial but is limited to specific hormone dependent cancer types or subtypes (Urruticoechea et al., 2010).

The first successful rationally designed targeted drug was a small molecule imatinib mesylate (Gleevec), designed to inhibit a fusion BCR-ABL kinase in chronic myeloid leukaemia (CML) (Druker et al., 2001). Up to date it is still one of the most successful

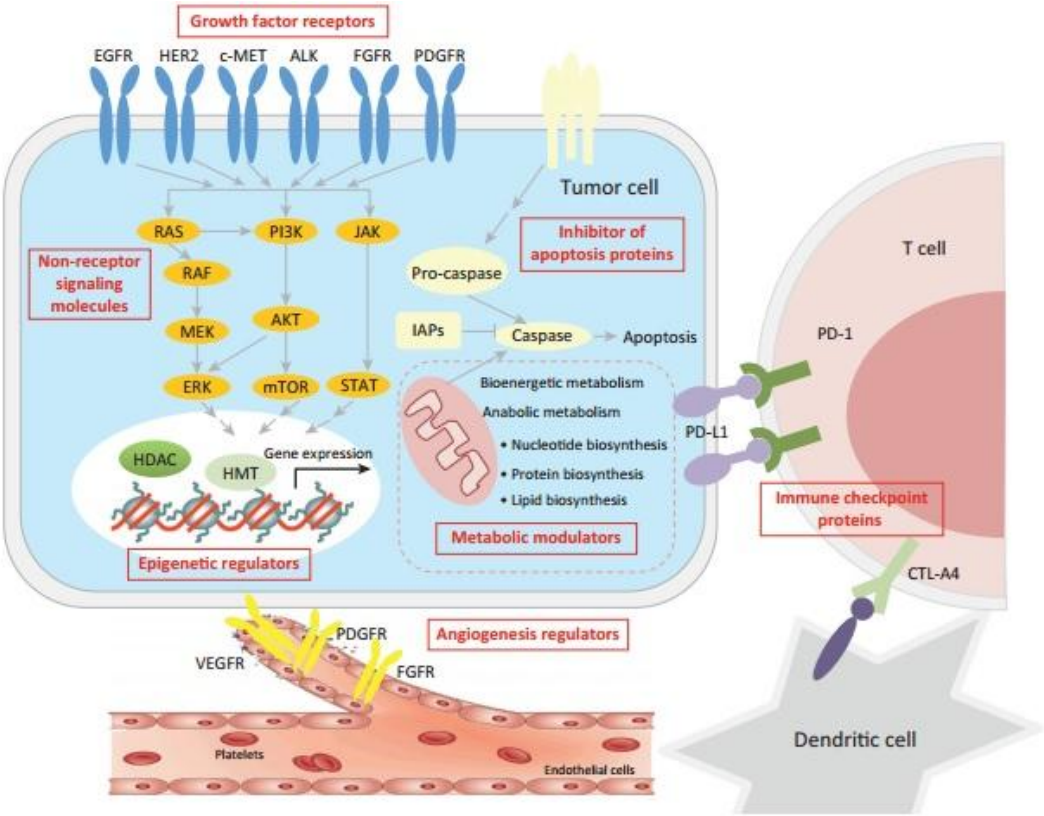


Figure 2: Targets for targeted therapy
Inhibition of growth factor receptors and other signalling molecules has proven very effective. In addition, anti-angiogenic therapy and immunotherapy are another successful treatment options. Targeting epigenetic modulators, inhibitors of apoptosis and metabolic enzymes is emerging as novel therapy opportunity. (Huang et al., 2014:43)

Slika 2: Tarče za tarčno zdravljenje
Inhibicija receptorjev rastnih signalov in drugih signalnih molekul se je izkazala za zelo uspešno. Anti-angiogena terapija in imunoterapija prav tako pridobivata na veljavi. Proteini, ki regulirajo epigenetske spremembe, inhibitorji apoptoze in encimi so prav tako lahko tarče za terapijo (Huang in sod., 2014:43)

targeted therapies available. Driven by the imatinib success, more targeted compounds were developed, inhibiting growth signals, inducing apoptosis and preventing angiogenesis (Figure 2). A monoclonal antibody, trastuzumab, targeting receptor tyrosine kinase HER2 in breast cancer was introduced later and showed promising results in HER2 positive patients. Another story that was successful only in a selected group of patients was inhibition of epidermal growth factor receptor (EGFR) with small molecule inhibitor gefitinib in lung cancer patients, where only around 10% response was observed. It later became clear that responders were patients with activating EGFR mutations (Huang et al., 2014; Lynch et al., 2004). Another drug targeting EGFR, a monoclonal antibody cetuximab, was also approved for treatment of colon cancer (Cunningham et al., 2004). For patients with non-small cell lung cancer without the EGFR mutation, anaplastic lymphoma kinase (ALK) inhibitor crizotinib emerged as a new treatment option (Awad and Shaw, 2014). In malignant melanoma, BRAF inhibition using vemurafenib holds great promise after identification of BRAF V600E as a driver mutation of this cancer type (Chapman et al., 2013).

Other kinases that are being explored as targets for cancer treatments are phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PI3KCA), mitogen-activated protein kinase kinase (MEK) and others. In addition, anti-angiogenic therapy using VEGF and PDGFR as targets is also showing some success. Suppression of inhibitors of apoptosis (IAP) is another treatment option, but had not show remarkable success yet(Fulda and Vucic, 2012; Huang et al., 2014). Recently the focus has also shifted towards epigenetic landscape of cancer and several chromatin modifiers are being researched as potential targets (Figure 2).

Non-oncogenes can also be targets for targeted therapy. For example poly-ADP ribose polymerase (PARP) inhibitors proved to be effective in treatment of breast and ovarian cancers with BRCA1 and BRCA2 deficiency. Due to BRCA gene inactivation these genes rely on a parallel DNA repair pathway and inhibition of this leads to cell death (Davaret al., 2012; Evers et al., 2008).

The biggest challenges of targeted therapy are selectivity and resistance. The problem of selectivity is that some targets are also expressed by normal cells, for example HER2 proved to be important in cardiac function (Force et al., 2007). However, the most severe problem with targeted therapy that prevented its expected success is the emergence of resistance. Because tumors are heterogenous some cells may lack the driver mutation, which is being targeted by the targeted therapy. This innate resistance can therefore lead to low response to the therapy, even though the majority of the tumor is sensitive to the therapy. On the other hand, plasticity of cancer cells can enable them to acquire additional mutations and circumvent the inhibition.

Resistance presents a common problem with no easy solution. One approach of solving it would be with combinational treatment following the idea of synthetic lethality. As demonstrated with the use of PARP inhibitors, synthetic lethality occurs when only a combination of two mutations or inhibitors leads to cell death, while mutation or inhibition of only one is still viable (Kaelin, 2005). By identification of genes that are

synthetically lethal in cancer cells and targeting these it could therefore be possible to selectively target cancer cells.

2.3 RESISTANCE TO TARGETED THERAPY

The response rate of targeted therapy lies low, at around 10 - 20% and it is usually short-lived, followed by disease progression (Huang et al., 2014). The reason for emergence of resistance is complex, but the most important factor seems to be tumor heterogeneity. Tumor heterogeneity refers to existence of multiple sub-populations of cancer cells in one tumor, which differ in their genetic and epigenetic changes. Due to genomic instability that is one of the hallmarks of cancer (Hanahan and Weinberg, 2011, 2000) and different environmental pressures because of changes in tumor microenvironment these different populations can exist at the same time (Fisher et al., 2013). Development of the tumor follows the principles of Darwinian evolution where nature selects for favorable phenotype and changes in a population depend on selection forces in local environment (Gillies et al., 2012). The tumor should therefore be regarded as a heterogeneous population of cells with distinct properties. However, the description of tumors is usually based on their genetic defect, which might not even be present in all cells. The differences in local microenvironment, for example acidosis, hypoxia and reactive oxygen species (ROS) can create independent microenvironmental niches, which favor different sub-clones (Gillies et al., 2012). Even if targeted therapy is effective in killing a bulk of tumor population, but heterogeneity means that there are resistant clones already present in the tumor and change in selection pressure by adding the drug makes them overtake the population (Garraway and Jänne, 2012).

Some examples of the resistance mechanisms observed in response to targeted therapy can be connected to drug transport and metabolism, inactivation of the drug, alteration of the target or compensation of the targeted pathway (Holohan et al., 2013). For example, alterations in form of secondary mutations in the targeted protein have been observed in BCR-ABL (Shah et al., 2002), EGFR (Inukai et al., 2006) and ALK (Katayama et al., 2012). A particularly diverse set of resistance mechanisms have been observed in response to BRAF inhibition (Kemper et al., 2015). They mostly involve re-activation of the pathway by mutations in upstream or downstream proteins (Shi et al., 2014; Trunzer et al., 2013; Villanueva et al., 2013), amplification of the target (Allen et al., 2014; Shi et al., 2014) or switching to CRAF (Villanueva et al., 2010).

Knowledge about these resistance mechanisms provided a better insight in tumor evolution and heterogeneity and can now guide the design of new therapeutic approaches (Garraway and Jänne, 2012). These can be based on combinational therapy or trying to selectively target resistance sub-population and preventing its growth after targeted treatment. In addition, the fact that tumors are more heterogeneous than initially thought has to change the way biomarkers are used to determine treatment.

2.4 MTH1 AS A NOVEL TARGET

2.4.1 Reactive oxygen species in cancer

Reactive oxygen species are a group of highly reactive chemical entities consisting of superoxide, hydrogen peroxide, hydroxyl radical, hydroxyl ion and nitric oxide. Their reactivity can be low (superoxide), medium (hydrogen peroxide) or high (hydroxyl radical). They are produced as a byproduct of mitochondrial oxidation reactions, mostly the tricarboxylic acid cycle and electron transport chain, when free electrons react with oxygen. Most of ROS are either radicals with unpaired electron or highly unstable and can form radicals after decomposition, like hydrogen peroxide and nitric oxide. Free electrons make ROS highly reactive causing oxidation of proteins, lipids and DNA. To prevent oxidative damage from occurring there are several cellular defense systems consisting of enzymes (superoxide dismutase, catalase and glutathione peroxidase) and antioxidants (vitamin C, vitamin E, glutathione) (Sabharwal and Schumacker, 2014).

Deregulation of cellular energetics is one of the emerging hallmarks of cancer (Hanahan and Weinberg, 2011, 2000). Due to rapid divisions and high metabolism rate cancer cells require a lot of ATP for survival. However, with ATP production ROS levels also get higher. ROS can have a dual role in cancer cells, because high levels induce cell death, but presence of ROS increases tumorigenicity and influence activation of several oncogenic transcription factors. For example, cells without superoxide dismutase were shown to have higher degree of DNA double strand breaks, translocations and higher proliferation rate (Samper et al., 2003). Several oncogenes use ROS to mediate their malignant traits. One of those is RAS, which signals through NADPH oxidase, producing ROS that were linked to hyperproliferation, enhanced survival and increased mobility (Ogrunc et al., 2014; Park et al., 2014; Patel et al., 2014). In RAS transformed cells balance of ROS is therefore even more crucial as high levels can lead to cell death, but their presence is essential for malignancy.

In addition to proteins, lipids and DNA, free nucleotides can also be targeted by oxidative damage. The free nucleotide pool consists of DNA precursors 2'-deoxyribonucleotides (dNTPs) and oxidation of nucleotides can be a source of mutagenesis (Kamiya, 2007). Most susceptible to oxidation is guanine, which can cause A:T to C:G transversions in its oxidized form 8-oxo-deoxyguanosinetriphosphate (8-oxo-dGTP) (Hori et al., 2010; Satou et al., 2007). Another frequently oxidized nucleotide is adenosine, which forms 2-hydroxy-2'-deoxyadenosine triphosphate (2-OH-dATP). Incorporation of oxidized nucleotides to DNA during replication can result in mispairing and mutation leading to cell death (Oka et al., 2008). Even though the damage to free nucleotide pool is often overlooked in favor to study DNA damage there are some indications that free nucleotides are more susceptible to oxidative damage than chromatin bound DNA (Mo et al., 1992; Rai, 2010). Although the exact studies are virtually impossible to perform as isolation of nucleotides also causes oxidation it has been shown that free nucleotides are for example 190-13,000 times more susceptible to methylating damage than nucleotides that are built in the DNA already (Topal and Baker, 1982). Whether this is specific to methylating DNA damage or can be also

relevant to oxidative stress still remains unknown. However, cells have to rely on the mechanism to prevent damage to the nucleotide pool, especially when exposed to high ROS levels, like in cancer cells.

2.4.2 MTH1 inhibition as a novel cancer therapy

Damage to the free nucleotide pool is especially prevalent in cancer cells due to increased ROS levels. Since oxidized nucleotides leads to mutations and cell death, these cells have to rely on the mechanism to prevent mutagenic effects of ROS without eliminating them. An enzyme that sanitizes free nucleotide pool and prevents the incorporation of oxidized nucleotides into the DNA is MutT homologue 1 (MTH1).

Because RAS mutated cancers require high ROS levels for their malignant signaling and sustained elevation of proliferation they have to rely on MTH1 to prevent DNA damage. Indeed, elevated levels of MTH1 have been found in these cells, indicating the dual role of ROS (Rai, 2012). There have been multiple consensus sequences for the Ets transcription factor family, which regulate gene expression in response to RAS, found in the promoter of MTH1 gene (Nakabeppu, 2001). This indicates that MTH1 can be upregulated in response to RAS-induced ROS. On the other hand, in RAS transformed cells MTH1 loss induces arrest of proliferation suggesting that ROS produced in these cells massively target free nucleotide pool (Rai et al., 2011). These findings indicate that MTH1 would be an interesting target specifically in cancer cells with RAS mutation.

MTH1 is a nudix hydrolase, encoded by the *NUDT* gene. It selectively binds 8-oxodGTP and 2-OH-dATP and converts them to their monophosphate forms, which are unable to incorporate into the DNA (Figure 3). If overexpressed, it suppresses the mutator phenotype of cancer cells (Russo et al., 2003) and prevents the oncogenic RAS induced DNA damage response and premature senescence (Rai et al., 2011). The importance of MTH1 in cancer cells was demonstrated by depletion of the MTH1 protein by short interfering RNA (siRNA), which resulted in an increase in DNA damage and reduced clonogenic survival and viability of cancer cells (Gad et al., 2014; Huber et al., 2014), but not primary cells (Gad et al., 2014). However, the exact cellular response to MTH1 depletion in cancer cells is still not entirely clear.

Some studies show that MTH1 depletion leads to an increase in double strand break repair and p53 phosphorylation as well as an increase of senescence and apoptosis markers independent of p53 status (Gad et al., 2014). However, others show that p53 status is crucial in determining response to MTH1 loss, at least in RAS mutated background. In p53 competent cells, MTH1 knockdown via short hairpin RNAs (shRNAs) elevated p53 levels and subsequently also p21 expression, which suggests onset of oncogene induced senescence (OIS). On the other hand, in p53 non-functional cells p27 levels were elevated and cells showed reduced but continued proliferation (Patel et al., 2014). Another study found that MTH1 expression is suppressed by MiR-145, causing reduced proliferation but not cell death (Cho et al., 2011). Exact cellular

mechanisms to MTH1 depletion could therefore be cell-type dependent, perhaps because of different ROS levels between cell lines.

By screening compound libraries a small molecule inhibitor TH588 was identified that could selectively and effectively kill cancer cells and was less toxic to primary cells (Gad et al., 2014). In order to improve pharmacological properties a second generation of MTH1 inhibitors was developed, of which TH1579 is a leading compound. Another MTH1 inhibitor was identified quite serendipitously. Crizotinib, a kinase inhibitor already approved for treatment of ALK positive non-small cell lung cancer showed high affinity toward MTH1. Later it was shown that only (S)-enantiomer of crizotinib, but not clinically used (R)- was a strong MTH1 inhibitor (Huber et al., 2014).

Cellular response after MTH1 inhibition with both TH588 and (S)-crizotinib matched the effects observed with MTH1 knockdown, leading to increase in 8-oxodG and DNA damage (Gad et al., 2014; Huber et al., 2014). Furthermore, as xenograft experiments with MTH1 inhibitors showed reduced tumor sizes for various tumor types it seems like targeting MTH1 would be a good approach to target cancer cells (Gad et al., 2014; Huber et al., 2014).

However, for MTH1 to be a good target for targeted therapy its inhibition should not have major impact on normal cells. MTH1-deficient mouse models were established to study the effect of prolonged MTH1 depletion. Even though their survival rates were normal, increased frequency of spontaneous mutations was observed, leading to small but significant difference in incidence of tumors (Tsuzuki et al., 2001). Viability of MTH1 deficient mice confirms the assumption of MTH1 being non-essential in normal cells although the increase in tumor incidence could indicate unfavorable effects of prolonged MTH1 inhibition.

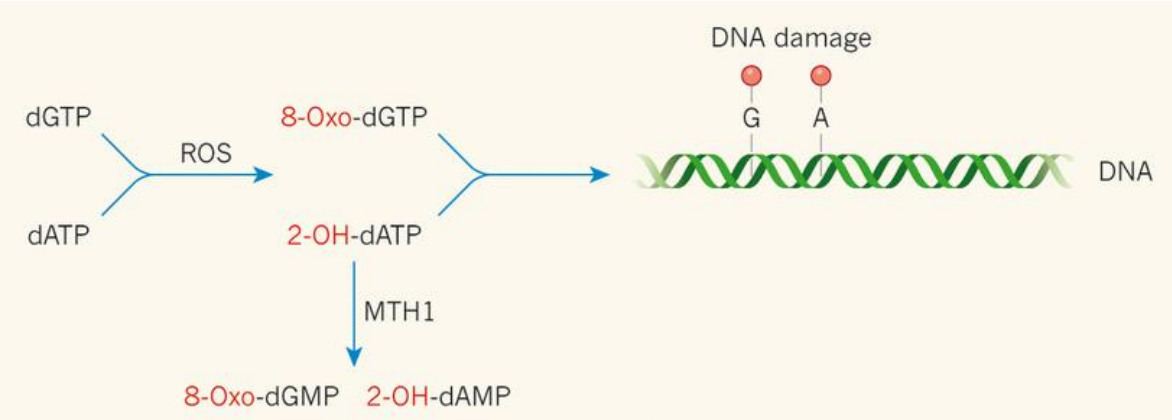


Figure 3: MTH1 prevents DNA damage by sanitizing the free nucleotide pool
Oxidized nucleotides (8-oxo-dGTP and 2-OH-dATP) can cause DNA damage if they are incorporated to DNA. MTH1 recognises them and converts them to monophosphate form. (Dominissini and He, 2014:191)
Slika 3: MTH1 preprečuje poškodbe DNA tako da odstranjuje proste nukleotide
Oksidirani nukleotidi, predvsem 8-oxo-dGTP in 2-OH-dATP lahko ob vgraditvi povzročijo poškodbe DNA. Encim MTH1 jih prepozna in pretvori v monofosfatno obliko (Dominissini in HE, 2014:191).

Taken together, although uncertainties remain about exact consequences of MTH1 inhibition, it may prove an attractive alternative to existing targeted therapies. Since MTH1 inhibitors are now moving into clinical trials and so many other targeted therapeutics lead resistance it is essential to investigate whether there are also mechanisms for cancer cells to become resistant to MTH1 inhibitors.

2.5 CRISPR/CAS9 SCREENING TECHNOLOGY

Clustered regularly interspaced short palindromic repeats (CRISPR) was first discovered as a form of adaptive immune system in bacteria to degrade foreign phage or plasmid DNA. Later, CRISPR associated protein 9 (Cas9) was described as a missing link for CRISPR function. Nowadays this system is used widely to precisely and specifically engineer genomes.

CRISPR/Cas9 system consists of endonuclease Cas9 and guide RNA (gRNA), which targets a specific genomic location. This gives CRISPR/Cas9 a big advantage over other previously used genome-editing tools such as zinc finger nucleases or transcription activator-like effector nucleases (TALENs) as it uses RNA to find homology and not

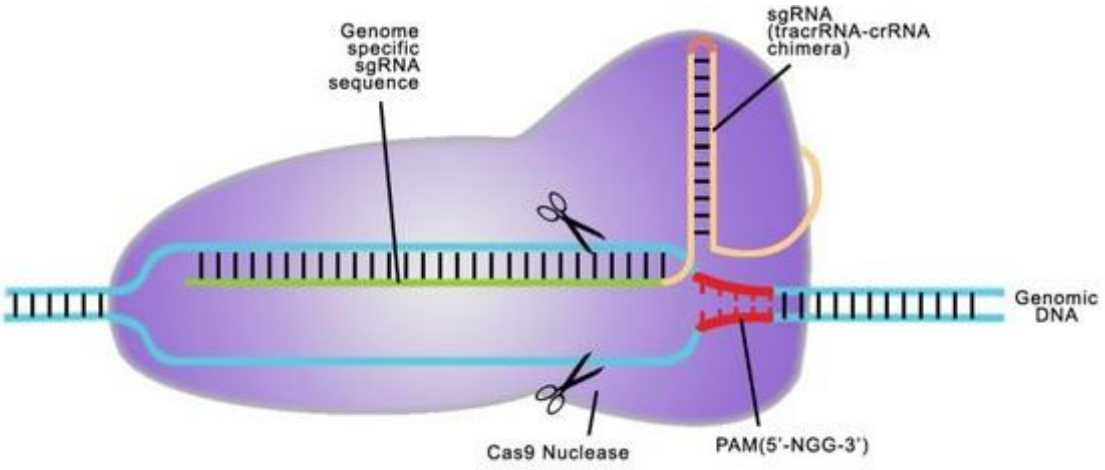


Figure 4: CRISPR/Cas9 system
CRISPR/Cas9 system consists of endonuclease Cas9 and gRNA. When they bind to genomic DNA and find PAM sequence and homology with gRNA Cas9 makes a cut, creating a double strand break in the DNA (Biocompare).
Slika 4: CRISPR/Cas9 sistem
Sistem CRISPR/Cas9 je sestavljen iz endonukleaze Cas9 in vodilne RNA. Ko tvorita kompleks z genomsko DNA in najdeta sekvenco PAM ter homolgno sekvenco z gRNA, Cas9 prereže DNA in tvori dvojni prelom.

proteins. This enables faster, easier and cheaper production of genome editing tools (Hsu et al., 2014; Jinek et al., 2012).

When Cas9 and gRNA are present in a cell they form a complex and start with finding a short protospacer adjacent motif (PAM) in genomic DNA (Figure 4). After finding one, homology between gRNA and genomic DNA is checked and if it is found, Cas9 undergoes a conformational change, which enables it to make a cut through both strands of DNA, creating a double strand break (DSB).

Cells can then repair this break in two ways: either with homology directed repair (HDR) or non-homologous end-joining (NHEJ) (Figure 5). Homology directed repair uses a homologous sequence of sister chromatid to repair the break. Alternatively, the homologous sequence can be provided in form of a short oligonucleotide sequence, enabling introduction of specific mutations to the genome (Hsu et al., 2014). On the other hand, NHEJ is an error-prone mechanism so small deletions and insertions are created around the site of DSB. These can therefore lead to frameshift mutations or premature stop codons and disrupt the gene. This usually result in short, non-functional peptides,creating a stable knockout of desired gene (Hsu et al., 2014; Jinek et al., 2012).

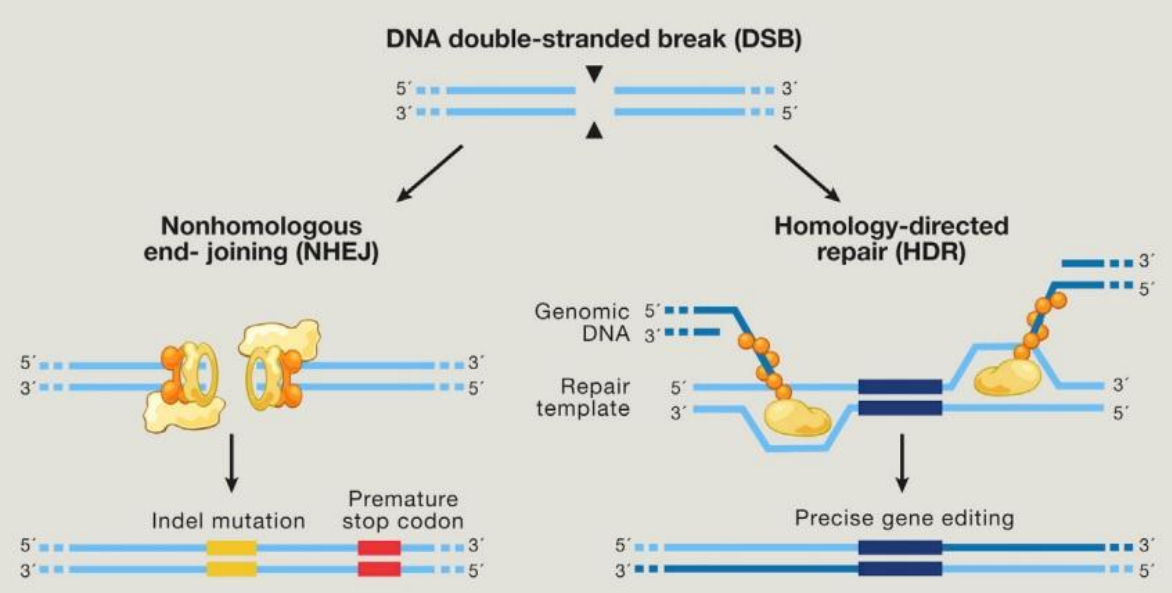


Figure 5: Double strand break repair
After CRISPR/Cas9 mediated double strand break it can be repaired in two ways. Firs approach is non-homologous end-joining which creates mutations and can lead to knockouts. Second repair mechanism is homology directed repair, which recruits a homologous sister chromatid or provided oligonucleotide and can allow precise gene editing. (Hsu et al., 2014: 1264)
Slika 5: Popravljanje dvojnih prelomov
Dvojni prelom, ki nastane po delovanju CRISPR/Cas9 kompleksa, je lahko popravljen z ne-homolognim združevanjem koncev ali homologno rekombinacijo. Prvi način omogoča ustvarjanje izbitih genov, drugi pa natančno urejanje genov (Hsu in sod., 2014:1264)

To create a knockout of a gene with CRISPR/Cas9 system gRNAs are usually designed to target first part of the gene to maximize the probability that frameshift mutation will result in non-functional protein. It is also crucial that target sequence in genomic DNA includes a PAM site in close proximity, to make sure CRISPR system will recognize the correct target.

In addition to creating knockouts by NHEJ, CRISPR can also be designed to alter gene expression. A so called activating CRISPR (CRISPRa) or CRISPR interference (CRISPRi) make use of catalytically dead Cas9 protein (dCas9), which is unable to cut DNA. To activate target gene with CRISPRa, dCas9 is fused to transcription activation domains that recruit the transcription machinery and initiate transcription regardless if gene was previously expressed or not. On the other hand, dCas9 in CRISPRi is fused to a repressor domain and inhibits transcription when recruited to a particular gene (Gilbert et al., 2014).

Functional genome-wide genetic screens are useful tools to identify genes underlying a particular process. Initially loss of function screens were performed using short interfering RNAs (siRNAs) which disrupted translation through RNA interference (RNAi) (Elbashir et al., 2001). Due to only transient knockdown, screening with short hairpin RNAs (shRNAs) was introduced (Berns et al., 2004). Another approach to whole genome screening are insertional mutagenesis screens in haploid cells (Carette et al., 2011) or transposon screens (Holmes, 2003). However, CRISPR/Cas9 technology proved to be an important technology for genome-wide screens because it can be easily engineered to target every gene in a genome in a very efficient way (Shalem et al., 2013; Wang et al., 2014; Y. Zhou et al., 2014).

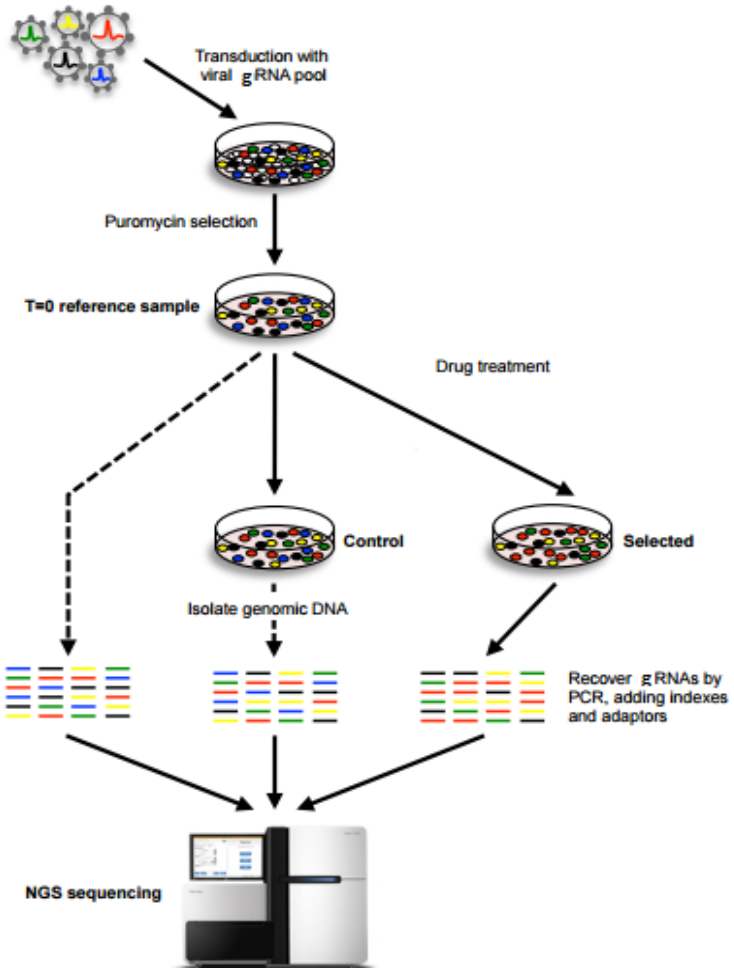


Figure 6: CRISPR/Cas9 genome-wide screen
After infection of the cells with the library and puromycin selection, cells are split into control and test arms. After treatment genomic DNA is isolated, gRNA sequences amplified and sequenced by next generation sequencing. (Adapted from Jastrzebski et al., 2015, submitted).
Slika 6: CRISPR/Cas9 pregled celotnega genoma
Po okužbi celic s knjižnico gRNA in selekciji s puromicinom celice razdelimo na kontrolno in testno populacijo. Po gojenju z zdravilom izoliramo genomsko DNA, pomnožimo sekvence gRNA in sekveniramo (prirejeno po Jastrzebski in sod., 2015, predloženo)

The main idea of CRISPR/Cas9 functional genetic screens is to generate an isogenic population of cells, which only differ in one gene that is knocked out by CRISPR. The gRNAs targeting every gene in a genome (usually multiple gRNAs per gene) are produced and cloned into a vector that can contain also a Cas9 gene (Figure 6). For delivery of this system to cells lentiviral system is commonly used since it ensures stable integration to the genome which later allows identification of gRNA by PCR and subsequent deep sequencing. Furthermore, multiplicity of infection (MOI) can be determined beforehand, ensuring that each cell is only infected by one viral particle (Shalem et al., 2013; Wang et al., 2014).

This system has been used before to identify genes involved in a resistance to targeted therapy for cancer, in that case vemurafenib (Shalem et al., 2013). After infection of the cells with lentivirus containing CRISPR/Cas9 system cells are split to control and treated arm, which is treated with the inhibitor (Figure 6). The majority of treated cells will die, but cells containing knockouts of genes involved in the resistance will be able to grow out and form colonies. Cells of both arms can then be harvested, used for DNA isolation and deep sequenced. To minimize the non-specific effects cells are also harvested right after infection. After bioinformatics analysis of sequences and comparing results of treated and untreated populations we can point out genes that are enriched in a treated population and therefore likely involved in resistance. Due to possible passenger effects top hits need to be individually validated.

The aim of this work was to perform a whole genome CRISPR/Cas9 functional genetic screen to identify possible resistance mechanisms to MTH1 inhibition.

3 MATERIALS AND METHODS

3.1 CELL LINES AND REAGENTS

The following cell lines were cultured in RPMI medium (Gibco) supplemented with 10 % FBS (Thermo Scientific), 1 % penicillin/streptomycin (Gibco) and 1 % L-Gluthamine (Gibco): LOVO, LS174T, 639V, HCT116, RT112 (all ATCC). HEK293T (ATCC) cells were culured in DMEM (Gibco), with the same supplements. NHUC (a kind gift from prof. Margaret Knowles) and NHUC-LT were grown in keratinocytes-SFM (Gibco) supplemented with 0.01 % cholera toxin, 1 % penicillin/streptomycin, 2.5 µg EGF (Gibco) and 25 mg pituitary extract (Gibco). All cell lines were grown at 37 °C at 5 % CO_2. All cell lines were tested for mycoplasma infection and profiled for short tandem repeats (STR) before the beginning of the experiment.

3.2 COLONY FORMATION ASSAY

For the colony formation assay, cells were seeded on 6-well plates at 5000 cells per well in complete medium. After 24h of incubation, medium was removed and cells were treated with compounds: TH588, (S)-crizotinib (Selleck Chemicals), TH1579 (obtained through a collaboration with prof. Thomas Helleday from Karolinska Institute) at different concentrations, or with 0.2 % DMSO. Medium was refreshed every 3-4 days. When the DMSO treated wells were confluent, plates were fixed with methanol and acetic acid (3:1), stained with Coommasie blue (0.1 %) in 10 % acetic acid and 50 % methanol, washed, dried and scanned with the scanner.

3.3 WESTERN BLOT

Resistant cells grown with 0.75 µM TH1579 and parental cells grown with DMSO were washed with PBS, lysed using RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1 % NP-40, 1 % sodium deoxycholate, 0.1 % SDS) with protease inhibitors (Roche) and phosphatase inhibitor cocktails I and III (Sigma). Proteins were extracted by incubating samples on ice for 30 min and debri was removed by pelleting 10 min at 14000 rpm at 4 °C. After quantification of protein concentrations using the bicinchoninic acid (BCA) assay, normalized samples were loaded on 4-12 % BisTris polyacrylamide gels (Life Technologies) and separated by electrophoresis at 180V for 90min. Proteins were then transferred to a polyvinylidene fluoride (PVDF) membrane at 0.3 A for 2 h in transfer buffer (0.01 % SDS, 50 mM Tris, 380 mM glycine, 10 % methanol, 80 % water). After blocking the membranes with 5 % bovine serum albumin (BSA) in PBS with 0.1 % Tween (PBS-T) they were incubated in primary antibody (Table 1) in blocking solution at 4 °C overnight with constant shaking. Membranes were then washed with PBS-T and incubated in HRP conjugated secondary antibody for one hour. The washing with PBS-T was repeated before adding a substrate (Bio-Rad) to the membranes and chemiluminiscence detection on ChemiDoc (Bio-Rad).

Table 1: Antibodies used for Western blot experiments
Tabela 1: Protitelesa uporabljena za prenos po Westernu

Target	Molecular weight (kDa)	Isotype	Dilution
BCRP	72	rat	1:400
MDR1	141	rabbit	1:200
MRP1	171	rat	1:1000
MTH1	18	rabbit	1:500

3.4 GENOME WIDE CRISPR/CAS9 SCREEN

3.4.1 Lentivirus production

To produce lentivirus HEK 293T cells were used. 14 15 cm dishes with 30 million cells were plated out in DMEM (Gibco) without penicillin/streptomycin and transfected after 8 hours of incubation. Transfection was performed in 22.4 ml OPTIMEM (Life technologies) with 896 µL polyethylenimine (PEI). 224 µg 3rd generation packaing mix containing pRSV, pCMV-VSV-G, pMDLglpRRe gag-pol was co-transformed with 224 µg one of two half human GeCKO v2 lentiCRISPR libraries (Addgene) (Shalem et al., 2013). Each half-library conisists of 3gRNAs per gene in the genome, while library A also includes 4 gRNAs per micro RNA. The mixture was incubated for 15 minutes and added to the cells. After 24 hours the medium was refreshed with DMEM complete medium. After additional 24 h of incubation virus was harvested by filtering culture medium through 0.45 µm filters and concentrated using centrifugal filter units (Milipore). Concentrated virus for library A or B was aliquoted and stored at -80 °C.

3.4.2 Determination of viral titer

To determine viral titer, HCT116 cells were infected with serial dilutions of virus containing half library A or B. To each dilution of virus, 50 000 cells in a 6-well plate and polybrene (16 µg/mL) were added. One well was plated without the virus, as the non-infected control. After overnight incubation medium was refreshed and puromycin (2 µg/ml) was added to all except the control well. When the non-infected cells treated with puromycin were dead the titer was determined at 10 µl of concentrated virus as the concentration that allows roughly 25 % of the cells to survive. This approach assures that most of the surviving cells will have only one viral particle integration per cell. The multiplicity of infection (MOI) is in this case 0.3.

3.4.3 Infection, puromycin selection and drug treatment

First the number of cells that needs to be plated for each arm of the screen to maintain the complexity was determined. The complexity to maintain is 100, meaning that 100 cells will have the same gRNA. Since each half-library contains approximately 6 x 10^4 gRNAs and the virus will only allow 25 % cells to survive, the number of cells should be 2.4 x 10^7. To correct for more potent virus and puromycin selection 8 x 10^7 cells were plated out in three independent infections per half-library and once without the libraries.
After the infection, selection for successfully infected cells was performed with puromycin (2 μg/ml). Non-infected cells were taken along as a control for whether the selection procedure is completed. After 48h of puromycin treatment, when the non-infected cells are dead, the cells were harvested, split in treated and untreated arms and time-point zero of the screen was harvested. The untreated arm of the screen was treated with DMSO (0.2 %). Medium with the drugs was refreshed every 3-4 days and non-infected controls were passaged twice a week while maintaining the complexity.

3.4.4 Amplification and identification of gRNA sequences

After a month of treatment with inhibitor, the treated cells were harvested and DNA was isolated in parallel with cells harvested at time-point zero using the DNeasy Blood & Tissue Kit (Qiagen) following manufacturer's instructions. The remainders of the cells were re-plated to same concentration and to 0.75 μM to test their resistance. After DNA isolation the gRNA sequences were amplified using two rounds of PCR to add barcodes and adapters for deep sequencing. After initialization of 2 minutes at 98 °C there are 20 cycles for PCR1 and 15 for PCR2 of denaturation at 98 °C for 30 s, annealing at 60 °C for 30 s and elongation at 72 °C for 30 s. The final elongation is performed at 72 °C for 5 minutes with the hold of 10 °C. The amplification was confirmed on 2 % agarose gel in 0.5x TBE and 0.5 μg/ml EtBr. Purified PCR products were analyzed by deep sequencing facility using one lane of HiSeq 2500 (Illumina).

3.4.5 Statistical analysis

The sequences we obtained were first mapped to the library and the number of gRNAs was counted for every sample. These readcounts were then analyzed by the Model-based Analysis of Genome-wide CRISPR/Cas9 Knockout (MaGeck) algorithm (Li et al., 2014) to normalize readcounts for their relative contribution, check which gRNAs are significantly enriched and produce gene scores from gRNA scores. When making gene scores the program also takes the variation over three replicates in account for calculating p-values. The top hits for validation were chosen as those with a q-value (p-value adjusted for multiple testing) lower than 0.5.

3.4.6 Validation experiments

Oligonucleotides with gRNAs targeting the same gene were pooled together and cloned in a LentiCRISPR v2.1 backbone using NEBuilder (BioLabs) and manufacturer's instructions. Cloning products were inserted into electrocompetent Endura E.coli bacteria (Lucigen) with electroporation (E.coli Pulser – Bio-Rad) at 1.8 kV. Bacteria were plated out (10 %) on LB-carbenicillin plates and grown overnight at 37 °C. The next day, 2 colonies were picked from each plate, grown overnight in 5mL of LB carbenicillin and plasmid DNA was isolated with High pure plasmid isolation kit (Roche) following manufacturer's protocol. Sanger sequencing was then performed using 500 ng DNA, 1 μL 10 μM primer 4 μL BigDye Terminator v3.1 Cycle Sequencing Mix (Life Technologies) in a total volume of 20 μL. After initializing at 96 °C for 5 min, 30 cycles of denaturation at 96 °C for 30 s, annealing at 50 °C for 15 s and elongation at 60 °C for 4 min, were performed. The sequencing facility of the Netherlands Cancer Institute performed the sequencing. Plasmid DNA was also isolated from the liquid overnight culture and used to produce virus. Virus was produced as already described and HCT116 cells were infected with 1 in 3 diluted viral supernatant. After puromycin selection infected cells were plated for colony formation assay with different concentrations of 3 inhibitors.

3.5 INVESTIGATION OF MORPHOLOGY

Resistant clones and parental HCT116 cells were plated at the same time with the TH1579 (0.75 μM) or without and pictures were taken after 4 days of incubation with Axiovert 25 (Zeiss) with Axiocam ICC1 Camera under 20x magnification.

3.6 ROS MEASURMENTS

ROS levels were measured in resistant and parental cells grown on (0.5 μM) or off the TH1579 using the CellROX Green Flow Cytometry Assay Kit (Life Technologies) following manufacturer's protocol. For positive control 400 μM tert-butyl hydroperoxide (TBHP) was used and for negative control 5 mM N-acetylcystine (NAC) and 400 μM TBHP were used, both provided with the kit. Sample used for negative control was first pre-incubated with ROS scavenger NAC, which acts as an antioxidant and protects the cell from generating ROS induced by TBHP. Analysis was performed on BD LSRII Flow cytometer and results were analyzed using FloJo software.

3.7 RNA SEQUENCING

Resistant and parental cells were plated on (0.5 μM) and off TH1579 until sub-confluence. Plates were then washed with PBS, treated with TRIzol (Life technologies) and sequenced by sequencing facility of the Netherlands Cancer Institute.

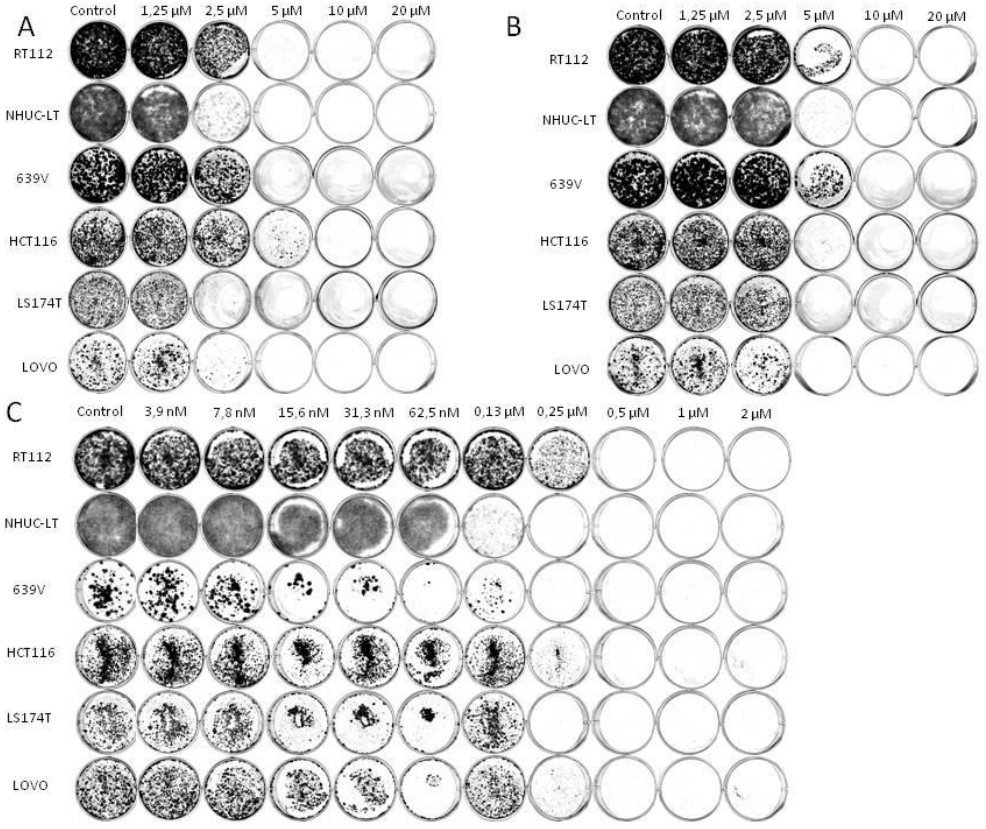
3.8 INVESTIGATION OF POINT MUTATIONS

DNA was isolated from resistant and parental cells using the DNeasy Blood & Tissue Kit (Qiagen) following manufacturer's instructions. Next, PCR was performed with set of primer pairs for each of four exons of MTH1 gene (Supplementary table 1). The reaction started with initialization at 98 °C for 2 min, 35 cycles of denaturation at 98 °C for 30 s, annealing at 58 °C (exon 1), 50 °C (exon 2), 60 °C (exon 3) or 64 °C (exon 4) for 30 s and elongation at 72 °C for 1 min. The final elongation is performed at 72 °C for 5 minutes with the hold of 10 °C. Amplified sequences were confirmed on 1 % agarose gel with 0.5x TBE and 0.5 µg/ml EtBr. Next, samples were purified using PCR purification kit according to manufacturer's instructions and Sanger sequenced as described above. Sequences were analyzed using FinchTV software and BLAT online tool.

4 RESULTS

4.1 RESPONSE OF CANCER CELLS TO MTH1 INHIBITORS

In order to perform a screen to find genes that confer MTH1 inhibitor resistance, we started by determining IC50s of three different MTH1 inhibitors (TH588, (S)-crizotinib and TH1579) in a panel of six cancer cell lines (Figure 7). We chose two bladder cancer cell lines (RT112, 639V), one immortalized urothelial cell line (NHUC-LT) and three colon cancer cell lines (LOVO, LS174T, HCT116). We used a range of concentrations of all three inhibitors, from 1,25 μM to 20 μM for TH588 and (S)-crizotinib and from 3,9 nM to 2 μM for TH1579. All the cell lines tested proved to be sensitive to these compounds, with IC50 for TH588 and (S)-crizotinib between 2.5 μM and 5 μM (Fig. 7A and B) and between 0.13 μM to 0.25 μM for TH1579 as determined from pictures (Figure 7C).



4.2 CRISPR/CAS9 RESISTANCE SCREEN

Figure 7: Cancer cells are sensitive to MTH1 inhibitors
Equal number of cells were plated in colony formation assay. They were treated with MTH1 inhibitors TH588 (A), (S)-Crizotinib (B) and TH1579 (C). Control wells were treated with DMSO.
Slika 7: Rakave celice so občutljive na inhibitorje MTH1
Enako število celic smo gojili s TH588 (A), (S)-crizotinibom (B) in TH1579 (C). Kontrolne celice smo gojili z DMSO.

Based on the colony formation assay with a panel of cell lines we chose the colon cancer cell line HCT116 to perform a CRISPR/Cas9 functional genetic screen to find resistance mechanisms to MTH1 inhibition. This cell line was chosen because it has a mutated RAS background and wild-type p53, which could make it more susceptible to MTH1 inhibition (Patel et al., 2014). Since RAS mutated cells need higher ROS levels to sustain their proliferation they rely more on MTH1 and are therefore more sensitive to its inhibition.

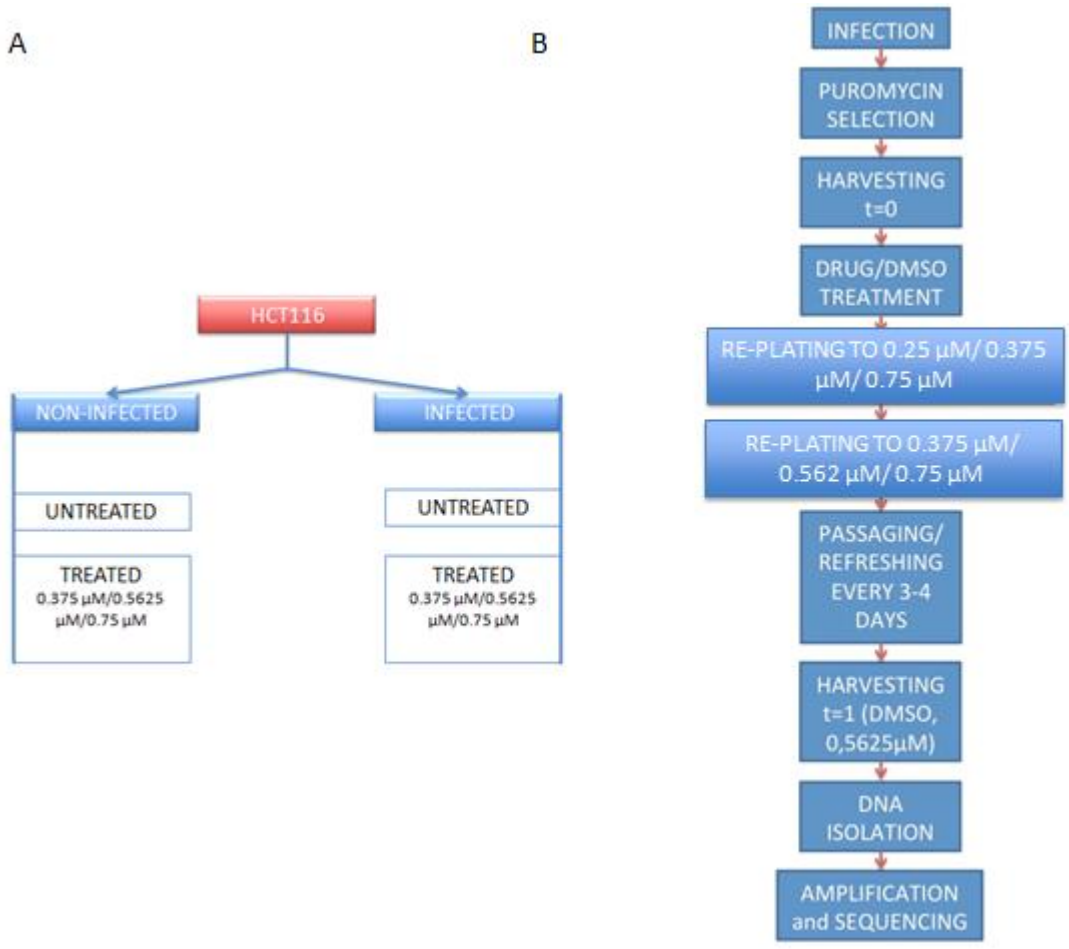


Figure 8: Workflow of CRISPR/Cas9 screen
Layout of the screen is shown in A and timeflow of the CRISPR/Cas9 screen in B.
Slika 8: Potek pregleda z CRISPR/Cas9
Razpored vzorcev (A) in časovni potek pregleda (B) sta prikazana.

We started by infecting HCT116 cells with lentiviral particles containing the genome-scale CRISPR/Cas9 knockout (GeCKO) library and after puromycin selection was completed, we harvested half of the cells for a timepoint 0 and the rest was re-plated for the screen (Figure 8A). The concentration of TH1579 used for the screen was determined by colony formation assay (Figure 7C) to be around 0.25 μM. Since we observed hardly any cell death during first days of the screen, we decided to increase the screening concentration to 0.375 μM and 0.75 μM and after a week 0.5625 μM (Figure 8B). The reason for difference between IC50s between colony formation assay and the screen can perhaps be explained by higher confluence used in the screen, which might play a role in response to the drug.

After a month of drug treatment we observed an occurrence of background resistance in non-infected treated control cells that was virtually as strong as the resistance observed in infected plates (Figure 9A). We harvested cells, isolated DNA and amplified gRNA sequences in two PCRs (PCR1 and PCR2), also adding flanking sequences needed for

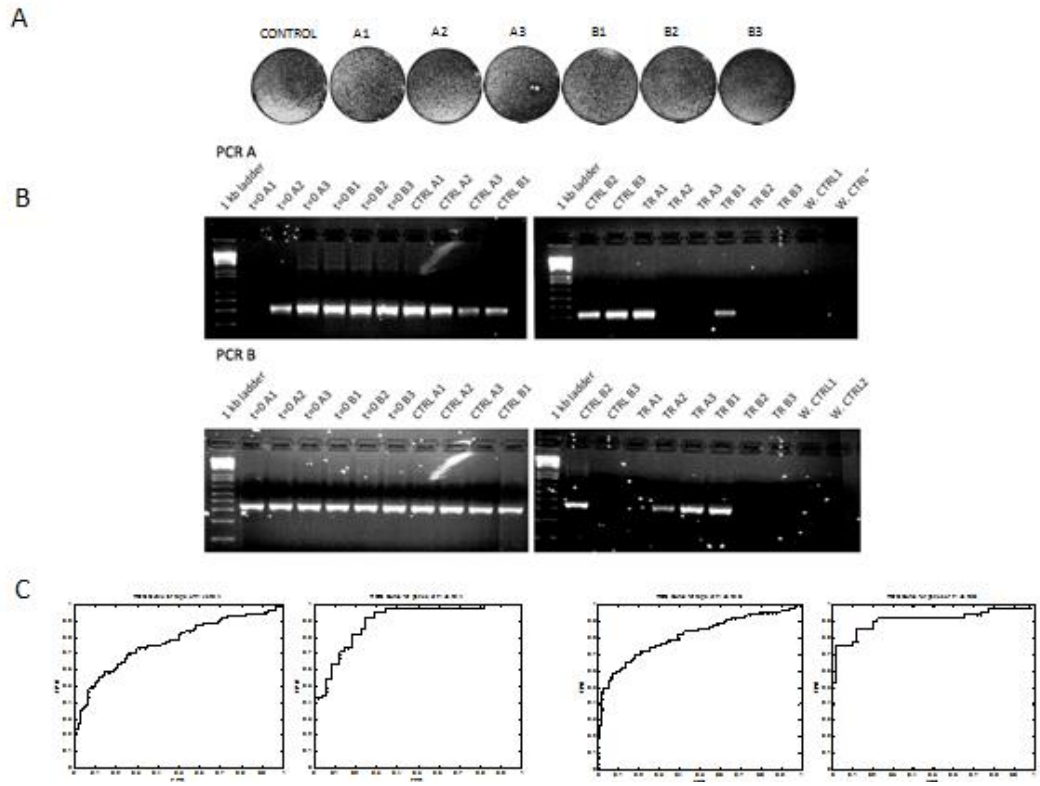


Figure 9: Functional genetic CRISPR/Cas9 screen showed spontaneous background resistance
The occurrence of background resistance in plates treated with 0.56 μM TH1579 for a month (A). Two PCR experiments were performed for gRNA amplification (B) and samples were combined, so that only two samples were missing. Samples labeled with the asterisk (*) were used from PCR A and the rest from PCR B. The ROC curves (C) indicating specificity and sensitivity of screen for half-library A (left) and B (right). First curve uses gRNAS and second genes as hits.

Slika 9: Funkcionalni genetski pregled s CRISPR/Cas9 je pokazal spontano odpornost v ozadju
Pojav odpornosti v ozadju (A) na ploščah z 0.56 μM TH1579 po enem mesecu. Da bi pomnožili sekvence gRNA, smo izvedli dva PCR poskusa (B). Vzorci iz poskusa A označeni z zvezdico so bili uporabljeni za analizo, skupaj z vzorci B. ROC krivulje (C) nakazujejo specifičnost in občutljivost pregleda za polovično knjižnico A na levi in B na desni. Prva krivulja uporablja gRNA kot zadetke, druga pa gene.

deep sequencing. We checked for correct amplification on gel after PCR2 and observed it was not successful for all the samples as 5 samples were missing (Figure 9B). After re-isolation of DNA and repeating both steps of PCR, some of the missing samples were amplified, but not all of them. For clarification, samples from first experiment are marked as PCR A and from the second PCR B. Considering this, we decided to combine samples from both experiments so that only two samples were missing in the end analysis.

With the intention to further evaluate technical performance of the screen, we plotted receiver operating characteristics (ROC) for a list of known essential and non-essential gRNAs and genes of each half-libraries. This analysis determines sensitivity and specificity of screen for essential vs. non-essential gRNAs. Screens with both half-libraries were 100 % specific with no false positives between 40 to 50 % sensitivity (Figure 9C). These results shows that the CRISPR system performed well and the screen was performed long enough to observe depletion of gRNAs targeting essential genes. Even though in the further analysis of the screen we compare treated vs. untreated samples this analysis shows that it is likely that the screen was performed long enough to see enrichment of gRNAs that affect growth of treated samples but not untreated.

To compare different replicates between timepoints and treatments, correlation plots were produced (Figure 10). First, we compared samples that were combined from two amplification experiments to make sure we can use them for subsequent analysis. The correlation plots of replicates from the same PCR round did not differ from the ones made from different rounds with comparable correlation coefficients. Next, we compared all untreated control replicates from the start of the screen (timepoint 0) and the end (timepoint 1, indicating one month). Correlations plots between replicates at timepoint 0 with correlation coefficients around 0.8 were indicating that there were no big differences between replicates at the beginning of the screen for both half-libraries. For timepoint 1, correlation coefficients between replicates are slightly lower but still show that we successfully maintained the complexity. When we compared replicates from timepoint 1 with timepoint 0 we observed lower correlation coefficients which are explained by depletion of essential genes resulting in change in relative representation of some gRNAs. Next, we compared treated with untreated replicates for both half-libraries. We observed better correlations between untreated replicates than treated replicates. The reason for lower correlation can be loss of complexity in treated population at any step of the screen (culturing cells, DNA isolation, PCR). However, it could also indicate that if there is a CRISPR-mediated resistance present, it is not strong and not reproducible between replicates. If CRISPR-mediated resistance would be stronger and reproducible we would expect a cloud of same hits between replicates. Furthermore, the resistance we observed was mild, meaning that it did not occur in every cell. If every cell became resistant due to non-CRISPR mechanism treated samples would correlate better, similar to untreated ones. Since none of these is the case we could speculate that there may be two resistance mechanisms involved, one causing background resistance and other causing CRISPR-mediated resistance. However, mild background resistance alone could also explain our results.

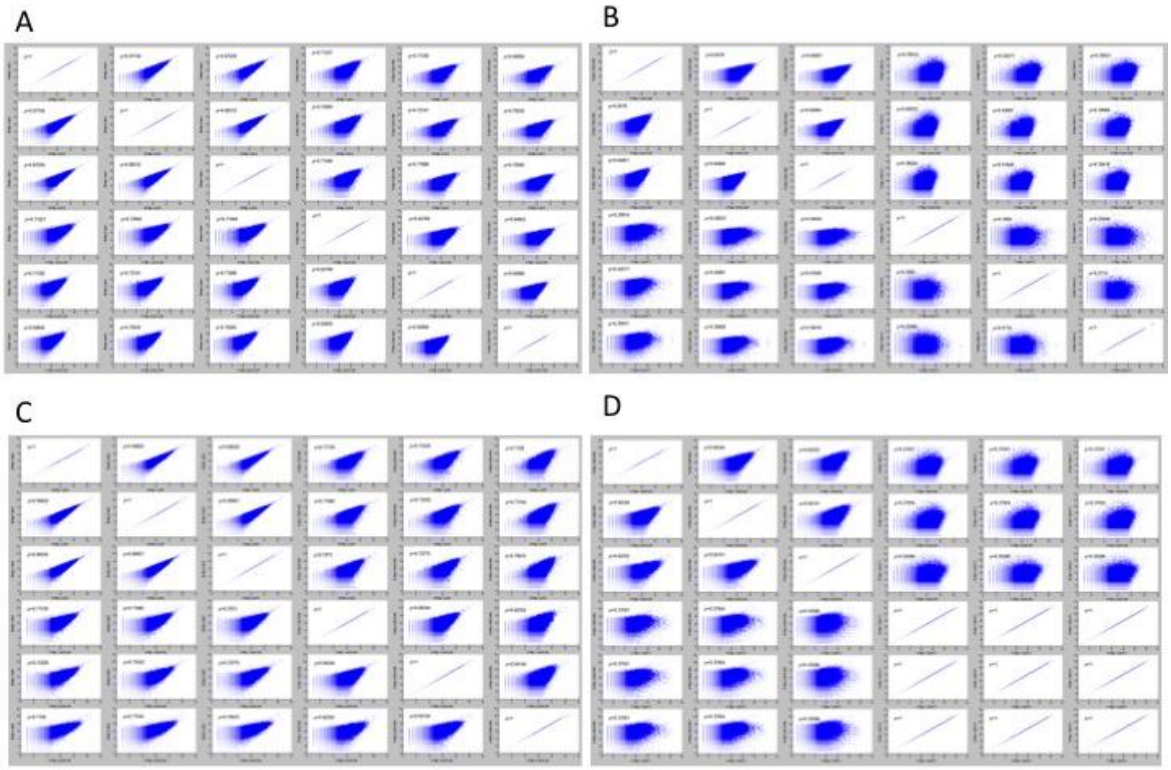


Figure 10: Correlation plots for CRISPR/Cas9 screen
Correlation between untreated samples at timepoint 0 and 1 for half-library A (A) and B (C). Correlation between treated and untreated samples of half-library A (B) and B (D).
Slika 10: Korelacijski graf za CRISPR/Cas9 pregled
Korelacija med kontrolnimi vzorci za začetno in končno točko pregleda za knjižnico A (A) in B (C). Korelacija med testnimi in kontrolnimi vzorci polovične knjižnice A (B) in B (D)

Next, we used MAGeCK (Li et al., 2014) software, to generate gene scores from individual gRNAs. This is a statistical analysis that uses the combined p-value ranks of all gRNAs belonging to the same gene and determines whether these ranks are significantly deviating from what can be expected as a purely random rank distribution. The result is a list of genes with corresponding p-values. We did not observe any statistically strong hits with any of half-libraries (Table 2). However, background resistance could obscure true hits so we chose to proceed anyway.

Table 2: Top ten hits for both of half libraries. Red line indicates validation cutoff.
Tabela 2: Najboljših deset zadetkov vsake knjižnice. Zadetki nad rdečo črto so bili izbrani za validacijo.

A		B	
ID	q-value	ID	q-value
hsa-mir-21	0.044554	JUND	0.044554
PPP1CC	0.15099	P2RX5	0.447195
CXorf65	0.245875	KRTAP3-3	0.447195
CLASP2	0.30396	FOXC1	0.593069
PITPNA	0.30396	SEPHS2	0.593069
GBP3	0.518388	SERPINB6	0.603342
MAPK14	0.518388	CLEC19A	0.603342
APOA1	0.564975	KIR3DL2	0.603342
SETD3	0.867437	SERTM1	0.715122
RHOD	0.881638	TSKU	0.715122

To determine which gRNAs are enriched in treated population we visualized the data with a ratios – M vs. averages – A (MA) plot, where log ratios of treated vs. untreated populations are plotted against average untreated population (Figure 11). There is not a strong cloud of hits visible, which was not expected based on correlation plots and MAGeCK analysis. Even though, we decided to combine the strongest hits from both half-libraries and chose 8 of them with the highest q-values (p-values adjusted for multiple testing) for validation experiments.

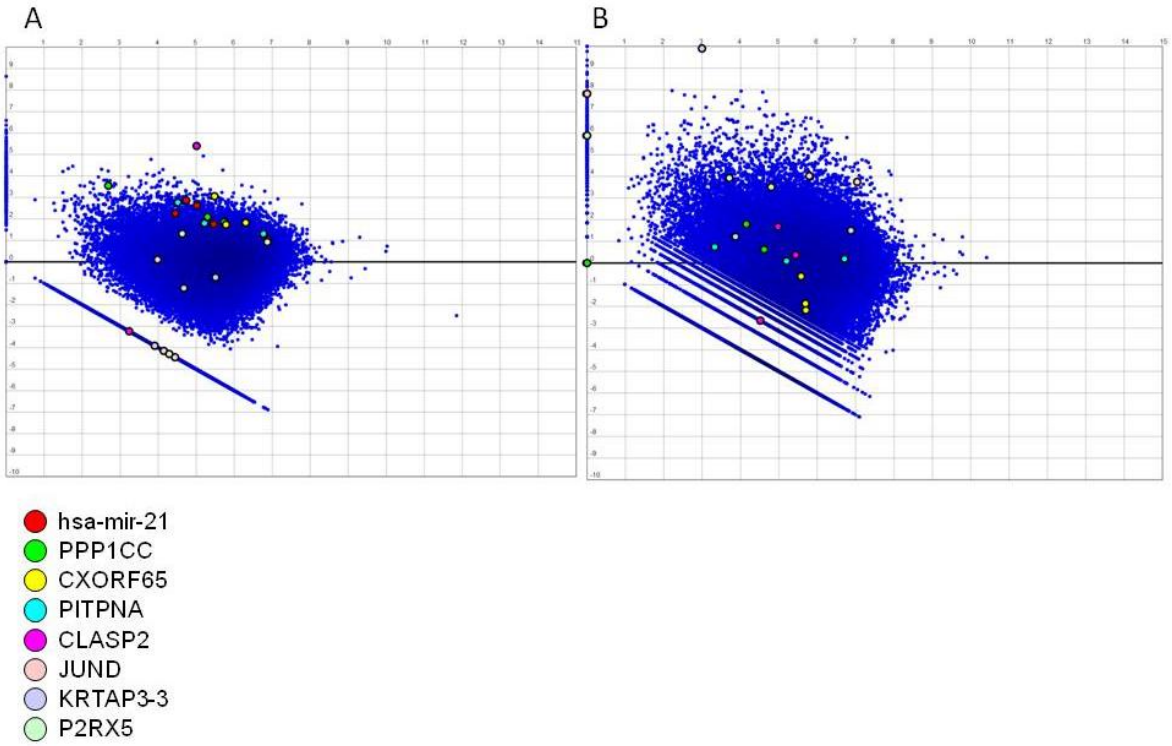


Figure 11: MA plots of eight top hits
Log rations of treated vs. untreated samples for half-library A (A) and B (B) are plotted against average untreated population. Colored dots indicate gRNAs targeting any of the eight hits chosen for validation.
Slika 11: MA graf osmih najboljših zadetkov
Logaritem razmerja med testno in kontrolno populacijo celic je narisan z povprečno vrednostjo kontrolne populacije za polovično knjižnico A (A) in B (B). Barvne pike označujejo gRNA, ki ciljajo osem genov izbranih za validacijo.

For these 7 genes and 1 miRNA we obtained all gRNA sequences from both libraries (6 for each gene and 4 for the miRNA) and cloned them in a pooled approach in lentiCRISPRv2.1 vector. To make sure the cloning product indeed contained gRNAs we picked two colonies for each gene and sequenced them using chain-terminating dideoxy nucleotides (Sanger sequencing). Results (Table 3) indicate that cloning was successful, with only around 19 % of sequences being wrong. Oligonucleotide sequences for each gRNA are shown in Supplementary table 1. Next, we used cloned minipools to produce virus and infected cells to create CRISPR-mediated knockouts for our target genes and test their sensitivity to MTH1 inhibitors. This approach enabled us to distinguish between true hits of the screen and passenger effects from background resistance. We performed colony formation assay with 8 pooled cell lines and parental HCT116 cells using three MTH1 inhibitors (TH588, (S)-crizotinib and TH1579). We used 0.5 μM of TH1579, 5 μM and 10 μM of TH588 and 3 μM and 6 μM of (S)-crizotinib. Control cells treated with DMSO grew out normally and there was also some growth in lower concentrations of TH588 and (S)-crizotinib, but not in any way different from parental cells (Figure 12). However, we observed a mild increase in

growth in cells infected with hsa-mir-21 minipool, treated with 3 μM (S)-Crizotinib and 5 μM TH588, which may be worth exploring further. None of the other constructs used showed any resistance to MTH1 inhibitors.

In addition to the validation, we tried to separate potential CRISPR-mediated resistance from background resistance, so we increased the concentration of Th1579 to 1.5 μM. We hypothesized that CRISPR-mediated resistance is stronger and should therefore be easier to separate at higher concentrations. Unfortunately, we did not observe this. In fact, also control cells became resistant to 1.5 μM after treatment of few weeks. We can therefore conclude that all observed resistance is likely background resistance.

Table 3: Adequacy of cloning products
Tabela 3: Ustreznost rezultatov kloniranja

Sequence number	Gene	gRNA ID	Notes
1	hsa-mir-21	HGLibA_58208	
2	hsa-mir-21	HGLibA_58209	Half sequence missing
3	PPP1CC	HGLibA_38012	
4	PPP1CC	HGLibB_37963	
5	CXorf65	HGLibA_11958	
6	CXorf65	HGLibA_11959	Half sequence missing
7	CLASP2	HGLibB_09786	
8	CLASP2	HGLibB_09786	
9	PITPNA	HGLibB_36693	
10	PITPNA	HGLibB_36693	
11	JUND	HGLibB_23976	
12	JUND	HGLibA_24009	
13	P2RX5	HGLibB_34813	
14	P2RX5	HGLibB_34813	
15	KRTAP 3-3	HGLibB_25710	Point mutation in promoter
16	KRTAP 3-3	HGLibA_25747	

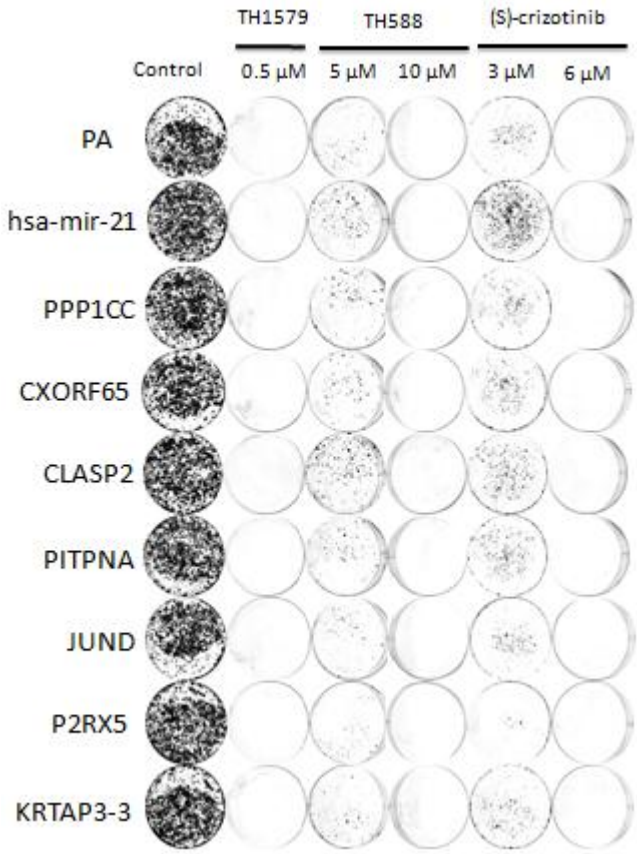


Figure 12: Validation of eight top hits did not show strong resistance
All cells used were HCT116 cell, either parental (PA) or CRISPR-mediated lines. We used all three inhibitors and control cells were treated with DMSO.
Slika 12: Validacija najboljših osem zadetkov ni pokazala močne odpornosti
Vse celice so HCT116, starševske (PA) ali okužene s sistemom CRISPR. Uporabili smo tri inhibitorje in DMSO kot kontrolo.

4.3 INVESTIGATION OF SPONTANEOUS BACKGROUND RESISTANCE

4.3.1 Resistance characteristics

In addition to testing CRISPR-mediated resistance by validation we also decided to investigate the spontaneous resistance. We picked 5 clones from the non-infected control plate of the screen, that was treated with 0.75 μM of TH1579 for a month (RA – RE). We also took along control plate from 0.56 μM screen as RF. First, we tested how resistant the clones we picked are. We performed another colony formation assay using parental HCT116 cells and six resistant cell lines. We used three MTH1 inhibitors, in previously determined concentration ranges. With TH1579 (Figure 13A) we observed small but definite resistance of about 2-fold between parental cells and resistant cells. All the resistant cells also seemed slightly more resistant to TH588 (Figure 13B). Two of the resistant lines – RA and RB were also more resistant to inhibition by (S)-crizotinib (Figure 13C), while other lines were even more sensitive than the wild type cells. This may indicate different resistance mechanisms between the resistant cell lines.

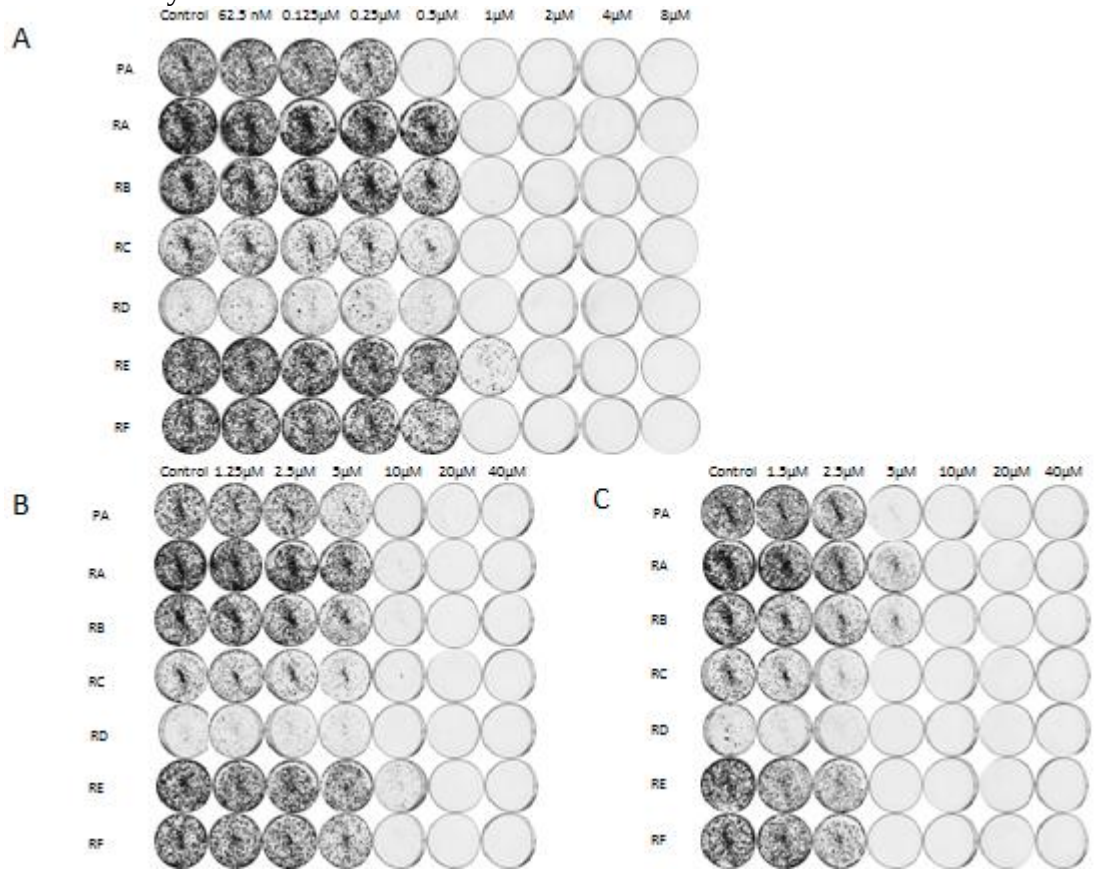


Figure 13: Spontaneous resistant cells show mild resistance phenotype
Resistant cells (RA-RF) and parentals (PA) were treated with TH1579 (A), TH588 (B) and (S)-crizotinib (C).
Slika 13: Spontana odpornost je mila
Odporne celice (RA-RF) in starševske celice (PA) smo gojili s TH1579 (A), TH588 (B) in (S)-crizotinibom (C).

Since we observed that confluence might play a role in cell survival upon treatment with MTH1 inhibitors we decided to test parental and resistant cells using an IncuCyte experiment for tracking cell growth. Identical amounts of cells were plated in DMSO, 0.5 µM TH1579 and 5 µM TH1579. After a week 0.5 µM and 5 µM of TH1579 were added to cells previously grown on DMSO which were then around 50 % confluent. Pictures were taken every 4 hours and confluence was analyzed by the IncuCyte software (Figure 14A).

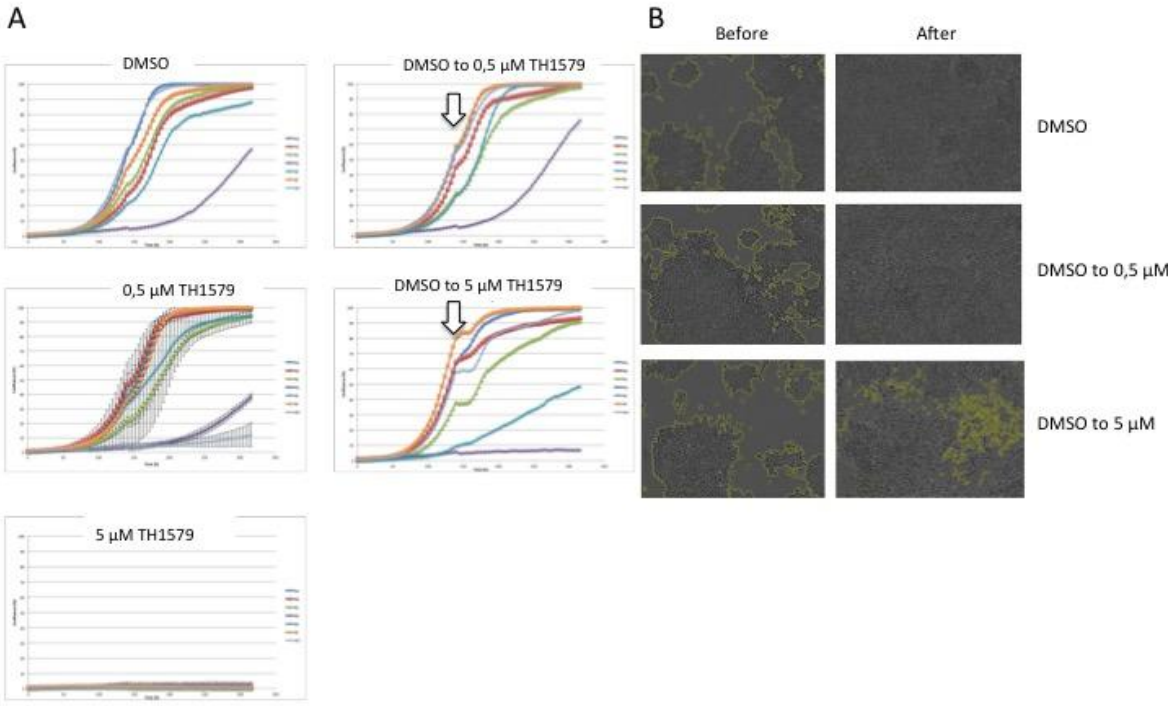


Figure 14: Confluence plays a role in sensitivity to MTH1 inhibitors
Growth curves of resistant cell lines and parental cells (A). Cells were treated with DMSO (top left), 0.5 µM (middle left) or 5 µM of TH1579 (bottom left). Cells in right panels were first treated with DMSO, and later 0.5 µM (top right) or 5 µM (bottom right) of TH1579 was added as indicated with arrows. Average of three replicates was plotted and error bars represent standard deviation. Pictures (B) represent parental HCT116 cells before adding the inhibitor or DMSO and at the end of the experiment. Yellow lines indicate confluence mask used by software to determine confluence.
Slika 14: Konfluntnost igra pomembno vlogo pri občutljivosti celic na inhibitorje MTH1
Rastne krivulje odpornih celic in starševskih celic (A). Celice smo gojili z DMSO (zgoraj levo), 0.5 µM (sredina levo) ali 5 µM of TH1579 (spodaj levo). Celice na desnih grafih smo najprej gojili z DMSO, nato pa dodali inhibitor, kot nakazujejo puščice. Grafi kažejo povprečje treh vzorcev z standardnim odklonom. Slike (B) kažejo starševske celice pred dodatkom inhibitorja ali DMSO in na koncu poskusa. Rumene črte kažejo masko prekrivnosti.

Since resistant line D (RD) grew slower, which was also apparent from colony formation assay so we excluded it from subsequent experiments. When TH1579 was added at the beginning of the experiment the growth of parental, but not resistant cells was impaired. On the contrary, when TH1579 was added to half confluent cells we

surprisingly observed a small stagnation and then growth of the parental cells. When 5 µM of TH1579 was added this effect was explained by change of morphology and cell debris, which mimicked the growth and changing confluence mask (Figure 15B). However, that was not the case with 0,5 µM, indicating that confluence plays a role in response to MTH1 inhibitors.

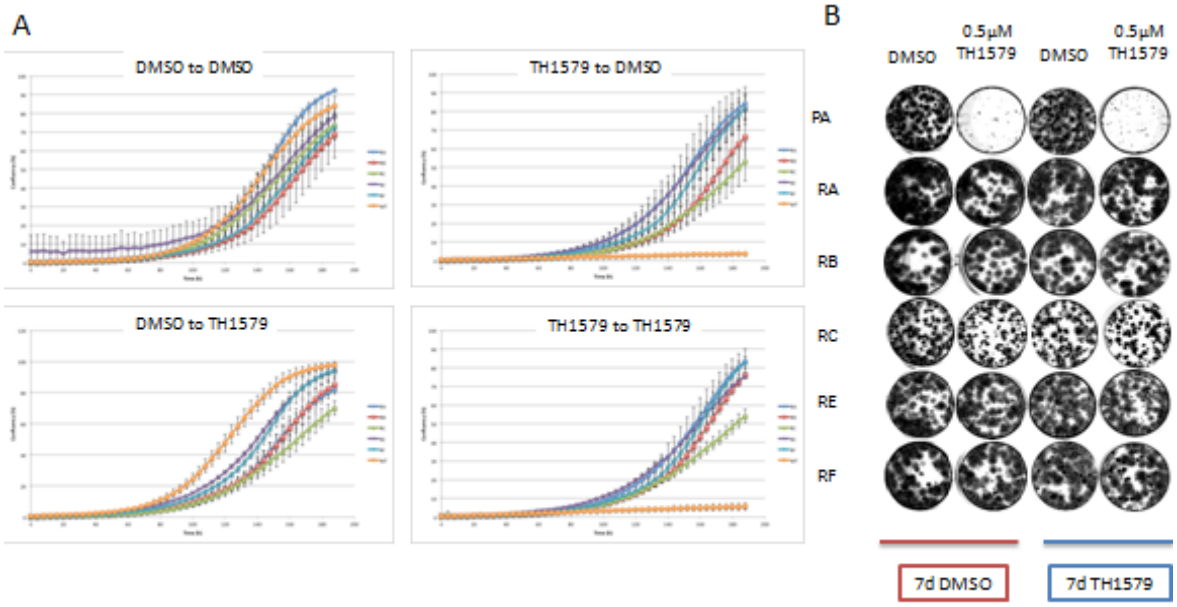


Figure 15: Resistant phenotype is stable and does not convert to sensitive after "drug vacation"
Growth curves of resistant lines and parental cells (A) that were cultured without the inhibitor for a week (top) or with the inhibitor (bottom). Cells were treated with DMSO (left) or 0.5 µM TH1579 (right). Average of three replicate was plotted and error bars represent standard deviation. Plate was stained at the end of experiment (B) and one out of three replicates is shown.
Slika 15: Fenotip odpornih celic je stabilen in se ne spremeni po gojenju brez inhibitorja
Rastne krivulje odpornih linij in starševskih celic (A), gojenih teden dni z (spodaj) ali brez inhibitorja (zgoraj). Celice gojene z DMSO (levo ali inhibitorjem (desno). Graf predstavlja povprečje treh vzorcev in standardni odklon. Ploščo smo na koncu obarvali (B), na sliki je en izmed treh vzorcev.

In order to test how stable the resistance phenotype is, we cultured cells with or without 0.75 µM TH1579 for a week (resistant cells) or 0.5 µM for a day (parental cells). (Figure 15). Then we started an IncuCyte and colony formation experiment either on or off 0.5 µM (Figure 15). Firstly, we can observe that all cell lines used grow comparably without TH1579. On 0.5 µM of the inhibitor parental cells did not grow out, regardless of their pre-incubation with the inhibitor. Resistant cells grew uninhibited whether or not they had been on a "drug-holiday". We therefore conclude that the resistance phenotype is stable.

We observed that the morphology of resistant cells differed a lot from parental cells and also between the resistant lines (Figure 16). To eliminate the possibility of cross-contamination with another cell line during screening, we had them tested for STR profile. STR profiling is a way of cell-line identification that makes use of variable

repeats of a few nucleotides to unambiguously identify a cell line. In total 15 markers were tested for each of the clonal resistant populations (RA, RB, RC, RE) and parental cells (Table 4). Even though we observed some differences between resistant cells and wild type cells they were mostly due to low quality (marked in red or orange) of the analysis. Considering this it seems to be highly unlikely that these cell lines are not in fact derived from the parental HCT116.

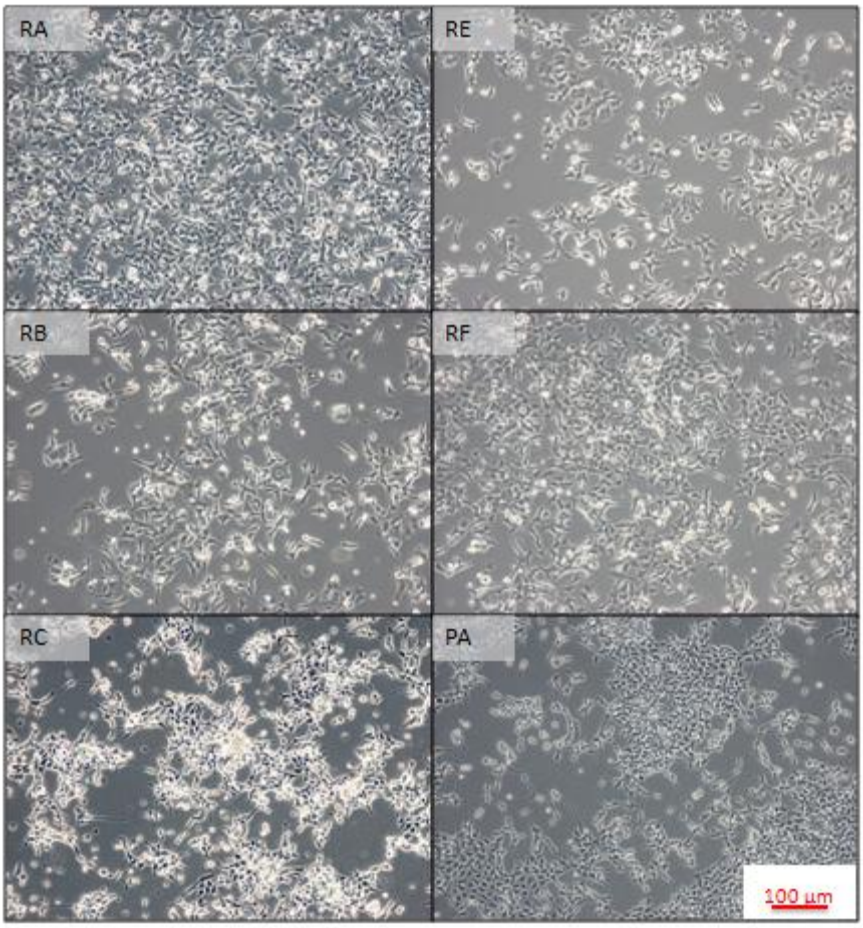


Figure 16: Difference in morphology between resistant cell lines
Different resistant clones (RA-RF) and parental cells (PA) treated with 0.75 µM of TH1579. Pictures were taken under 20x magnification.
Slika 16: Različna morfologija odpornih in starševskih celic
Različne odporne linije (RA-RF) in starševske celice gojene z 0.75 µM TH1579. Fotografije so posnete pod 20x povečavo.

Table 4: STR profiling of resistant and parental cell lines
Tabela 4: STR profil odpornih in starševskih celic

	PA			RA			RB			RC			RD			RE			
Marker/Allele	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	Matching
D8S1179	10	14	15	10	14	15	10	14	15	10	14	15	10	14	15	10	14	15	100%
D21S11	29	30		29	30		29	30		29	30		29	30		29	30		100%
D7S820	11	12	13	12	13		11	13		11	13		12	13		12	13		
CSF1PO	7	10		7	11		7	11		7	10		7	11		7	11		
D3S1358	12	18		12	17		12	17		12	18		12	18		12	17		
TH01	8	9		8	9		8	9		8	9		8	9		8	9		100%
D13S317	10	12		11	12		10	12		10	11		11	12		11	12		
D16S539	11	13	14	10	13	14	10	13		11	13	14	10	13		10	13	14	
D2S1338	15	16		15	16		15	16		15	16		15	16		15	16		100%
D19S433	11	12		12			12			11	12		12			12			
vWA	17	24	25	17			17	23		18	24		17	24		17			
TPOX	8	9		6	8	9	8	9		8	9		8	9		6	8	9	
D18S51	16	17		16	17		16	17		15	18		16	17		16			
D5S818	10	11		10	11		10	13		10	11		10	11		10	11		
FGA	18	23		18	23		18	23		18	23		18	23		18	23		100%

4.3.2 Elucidation of resistance mechanism

With the intention to uncover the mechanism of resistance we decided to test several hypotheses that could explain the occurrence of spontaneous resistance. Firstly, resistant cells could overexpress MTH1 protein and therefore become less sensitive to the inhibition. Secondly overexpression of certain ATP driven efflux pumps could enable cells to get rid of the inhibitor and therefore confer resistance. Thirdly, overall lowering of ROS levels by upregulation of antioxidant mechanisms or lowered ROS production could enable cells to be less dependent on MTH1 and thus resistant to its inhibition. Even though HCT116 have a RAS mutation which makes them dependant on high ROS levels we decided to check if the cells could potentially lower ROS levels and maintain high proliferation. Lastly, point mutations in active site of MTH1 proteins could prevent the binding of inhibitor and make cells resistant.

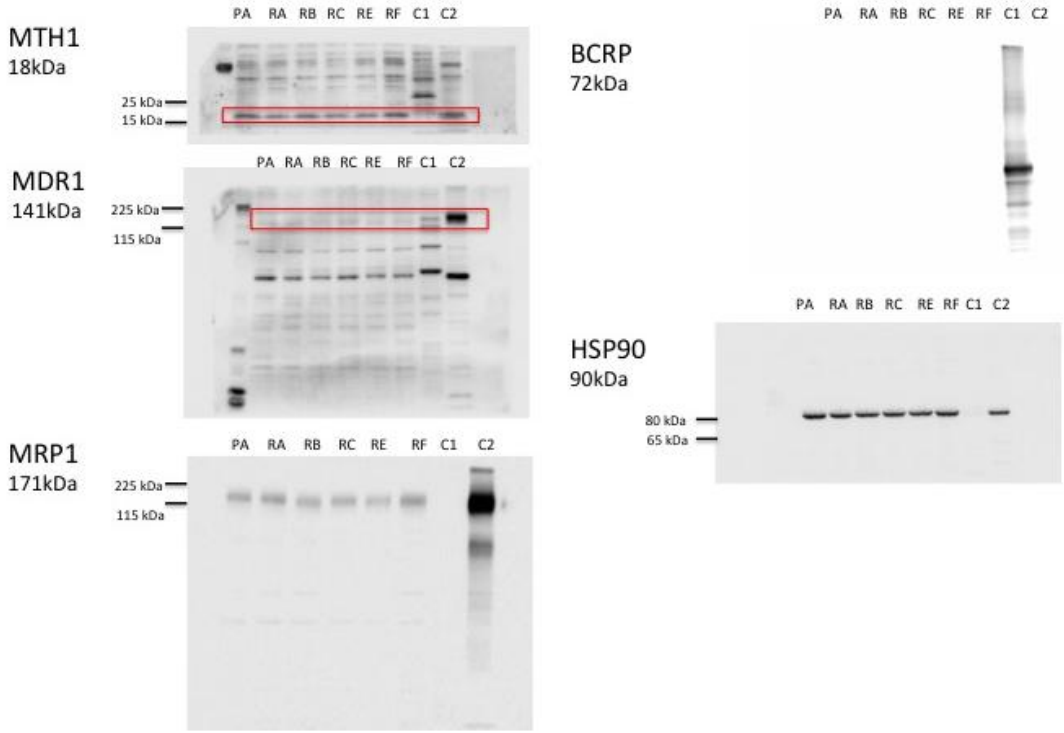


Figure 17: Resistant cells do not show difference in MTH1 or ATP-pumps for exporting drugs expression
Western blot of MTH1, MDR1, MRP1 and BCRP proteins. Red brackets indicate target protein. Controls used were vesicles containing BCRP (C1) and HEK293T cells overexpressing MRP1 (C2). HSP90 was used as loading control.

Slika 17: Odporne celice ne kažejo razlike v izražanju MTH1 ali ATP-črpalk
Prenos po Westernu za protein MTH1, MDR1, MRP1 in BCRP. Rdeč kvadrat označuje tarčni protein. Kot pozitivno kontrolo smo uporabili vezikle z BCRP (C1) in celice HEK293T, ki prekomerno izražajo MRP1 (C2). Kot kontrolo nalaganja smo uporabili HSP90.

To check for upregulation of either MTH1 or drug pumps we performed a Western blot (Figure 17). The 3 most common drug pumps were chosen for the analysis and we controlled for loading with housekeeping gene HSP90. In the blot for MTH1 we observed some background staining which makes it harder to assume which band is the target protein due to the lack of knockout controls. However, based on the size of the protein location of the band we can assume which bands are background bands. We did not observe any differences in MTH1 levels between parental cells and resistant cells. On the MDR1 blot we observed a band at correct weight for both 293T and all the HCT116 lines tested. Levels did not change between resistant and parental HCT116 cells, but were a lot lower than 293T suggesting that this protein is not or lowly expressed in HCT116 cells. While the protein runs at the correct weight, also here we did not have proper negative controls to identify the correct band unequivocally. There were no bands in the BCRP blot except for the positive control, so this protein is probably not expressed in HCT116 cells. This results show that upregulation of either MTH1 or drug pumps does not seem like a likely explanation of resistance.

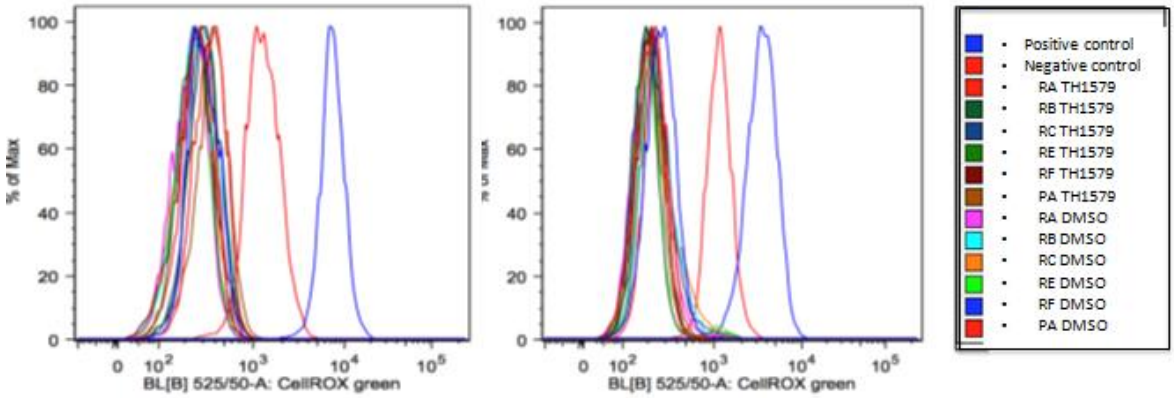


Figure 18: There is no difference in ROS levels between resistant parental cells
Levels of ROS were measured using specific dye and measuring its fluorescence with flow cytometry. TBHP was used as positive control and pre-incubation with NAC and TBHP was used as a negative control.
Slika 18: Med nivojem ROS v odpornih in starševskih celicah ni razlike
Nivoje ROS smo izmerili s specifičnim barvilom in izmerili fluorscenco s pretočno citometrijo. Kot pozitivno kontrolo smo uporabili TBHP in predhodno inkubacijo z NAC in nato TBHP kot negativno

To test if deregulation of ROS levels is a resistance mechanism, we measured ROS with the kit using ROS sensitive dye, detectable with flow cytometry (FACS). We cultured resistant cells with and without the inhibitor for a week exactly the same as for the second IncuCyte experiment. Data of two separate experiments is shown in Figure 18. We did not observe any major difference in ROS levels between resistant cells and parental cells or between cells cultured with or without the inhibitor. Changes in ROS levels are therefore unlikely to be a resistance mechanism.

To check if there was a point mutation present in MTH1 gene we designed primers for each of four exons, amplified the sequences and Sanger sequenced them. Analysis of

the exon 1, 3 and 4 sequences did not show any differences between resistant and parental cell lines. Unfortunately, exon 2 showed unspecific amplification so analysis was not possible. We therefore cannot exclude mutation in exon 2 to be a reason for resistance.

In addition to testing these 4 hypotheses, we also performed a preliminary RNA sequencing experiment, where we compared expression profiles of resistant and parental cells cultured with or without TH1579. We were unable to include complete analysis, but only preliminary results. Figure 18 shows hierarchical clustering of samples based on correlation, meaning that samples with similar expression profiles cluster together. Firstly, we can observe that resistant cell lines cluster together, regardless of being cultured with or without TH1579. However, there is a big difference

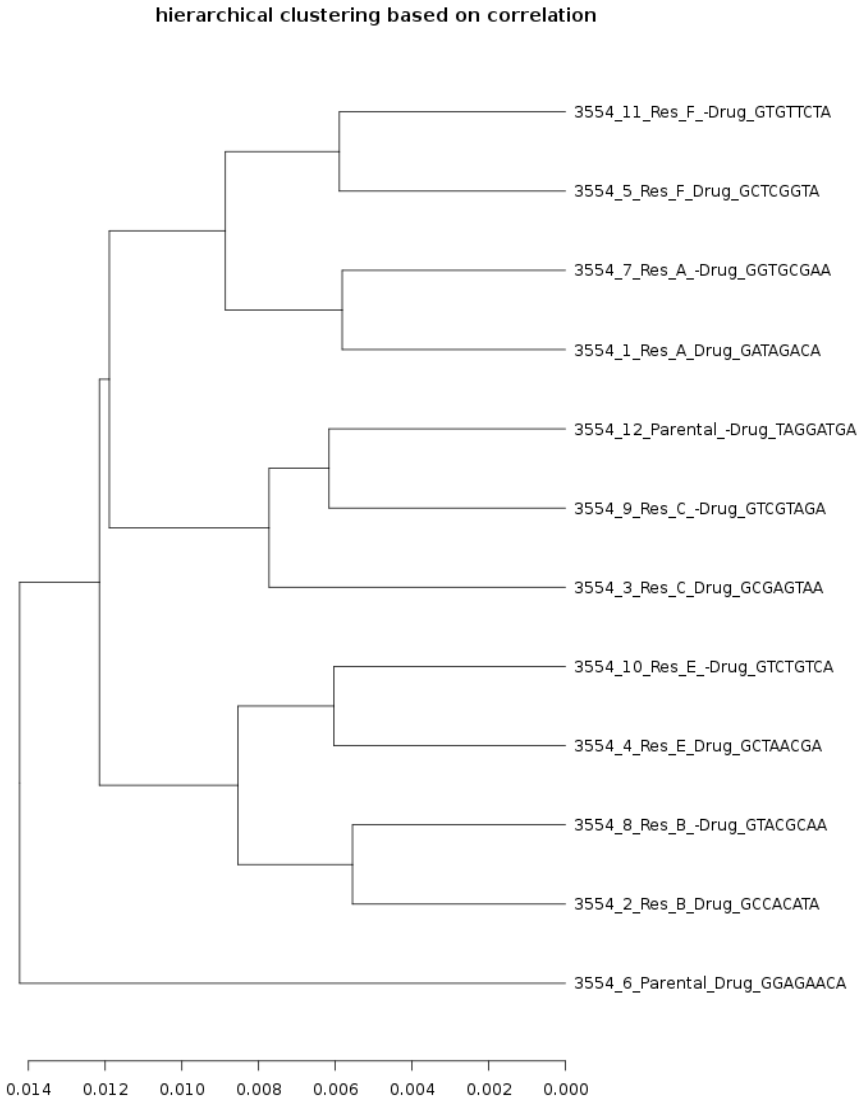


Figure 19: Hierarchical clustering based on correlation
Based on correlation, samples are clustered together and shown as dendrogram.
Slika 19: Hierarhična struktura glede na korelacijo
Glede na podobnost se vzorci združujejo in so prikazani kot dendrogram.

between parental cells cultured with or without TH1579. These results could indicate that the resistance lies upstream in the pathway, making difference in transcription profiles between resistant cells with and without after MTH1 inhibitor minor.

Secondly, all resistant lines are more similar to parental cells without the drug. These observations could indicate that resistant cells do not react to the drug at all. However, more detailed analysis is needed before making conclusions.

4.4 UNEXPECTED SENSITIVITY OF PRIMARY CELLS TO MTH1 INHIBITION

The idea of using MTH1 inhibitors is that cancer cells produce higher ROS levels than normal cells. To investigate the difference in sensitivity to MTH1 inhibitors between primary and cancer cells Gad et al. performed an experiment with primary cells that were transformed in a stepwise fashion by subsequently infecting with hTERT, SV40 large T (LT) antigen and RAS. They noticed that while cells infected with hTERT were

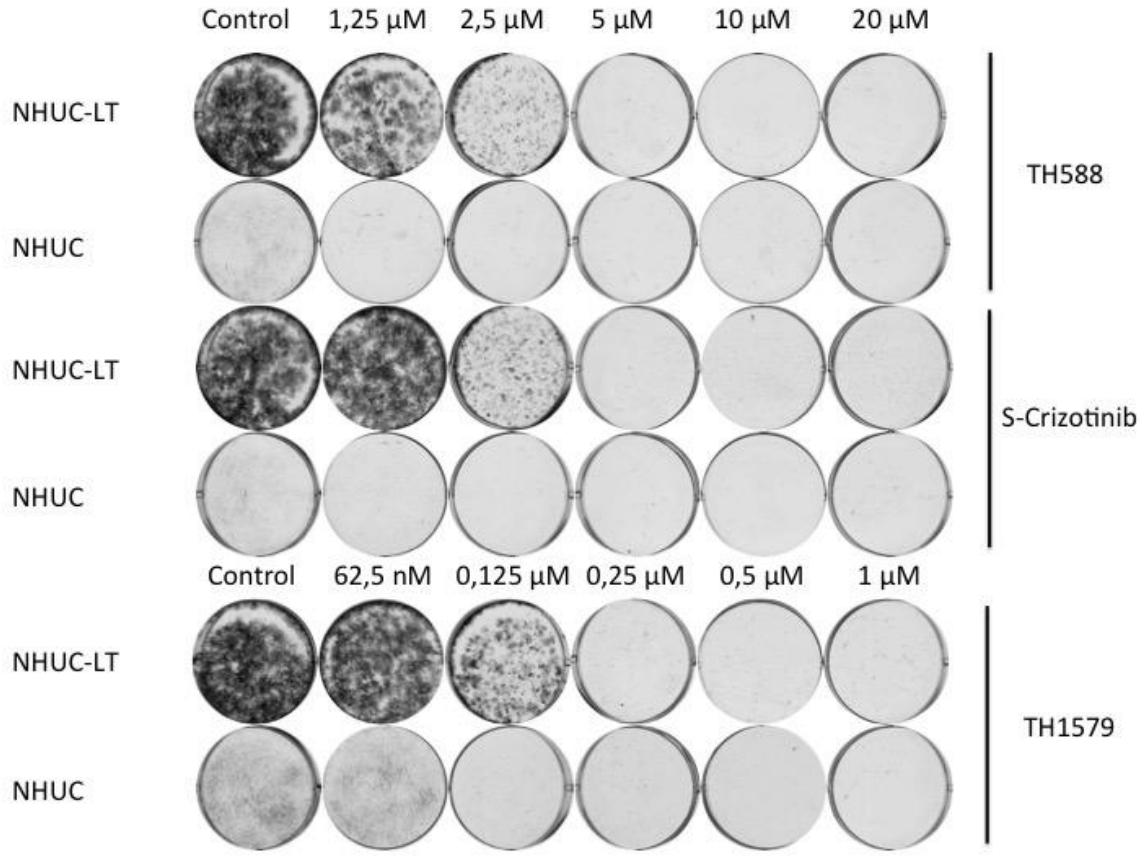


Figure 20: Non-cancer cells are not less sensitive to MTH1 inhibitors
Colony formation assay of NHUC and NHUC-LT cells with three inhibitors. Control wells were treated with DMSO
Slika 20: Normalne celice niso manj občutljive na inhibicijo MTH1 v primerjavi s transformiranimi
Celice NHUC in NHUC-LT smo gojili s tremi inhibitorji. Kotrolne celice pa z DMSO.

resistant to MTH1 inhibition, they become sensitive once infected with LT. However, this and other similar experiments were always done on fibroblasts. In order to test whether we would observe a similar induction of sensitivity when repeating this in epithelial cells, which are much more relevant model for most cancer types, we made use of normal human urothelial cells (NHUCs), which were hTERT immortalized and compared them to the same cells infected with LT (NHUC-LT). Both cell lines were tested in colony formation assay with the three inhibitors, with the concentration range from 1.25 μM to 20 μM for TH588 and (S)-crizotinib and from 62.5 nM to 1 μM for TH1579 (Figure 19). We observed a big difference in growth rate between NHUCs and NHUC-LTs with NHUCs growing much slower. However, we did not observe increased sensitivity to MTH1 inhibition in LT transformed cells, contrary to what we expected. Non-LT transformed cells seemed to be even more sensitive than LT-transformed ones even though the difference in growth rate makes is harder to compare them.

5 DISCUSSION

5.1 GROWTH AND VIABILITY OF CANCER CELLS UPON MTH1 INHIBITION

The rationale behind the development of MTH1 inhibitors was that differences in oxidative stress between cancer cells and normal cells would present a difference in cell's reliance to MTH1 thus making cancer cells, but not normal cells, sensitive to its inhibition (Gad et al., 2014; Huber et al., 2014). Although the exact consequences following MTH1 inhibition are not entirely clear yet, it is known that it induces double strand breaks and DNA damage response in cancer cells, followed by lower proliferation rates and/or cell death (Gad et al., 2014; Huber et al., 2014; Patel et al., 2014).

Indeed, when three MTH1 inhibitors (TH588, (S)-crizotinib and TH1579) were tested on a panel of cancer cell lines they all proved to be sensitive (Figure 7). IC50s of TH588 and (S)-crizotinib were comparable to those observed in other studies (Gad et al., 2014; Huber et al., 2014). The newest version of TH inhibitors, TH1579, was effective in around 10 times lower concentrations as TH588 and (S)-crizotinib, probably due to improved effectiveness or improved permeability to the cells.

5.2 IDENTIFICATION OF RESISTANCE GENES BY CRISPR/CAS9 RESISTANCE SCREEN

To investigate potential resistance mechanisms to MTH1 inhibition we performed the genetic resistance screen. The interpretation of the results from the screen was challenging due to spontaneous occurrence of resistance. The observed resistance was mild, which could mean that it occurred due to underlying genetic variability and heterogeneity of the cell population. Another clue that inherent resistant cells may be heterogeneously present comes from the different morphology of resistant clones that we observed (Figure 15). In this case some cells in the population are more prone to become resistant and grow out or there are already resistant clones present in the population. In any case this could explain the loss of complexity we observed in treated population (Figure 10). If the reason for resistance comes from some other genetic background that is present in some cells but not others, these cells would overgrow the population, leading to loss of complexity.

Although the validation of our top eight hits did not show a clear resistance phenotype we did observe some minor resistance to TH588 and (S)-crizotinib by cells containing gRNAs against micro RNA 21 (hsa-mir-21). However, since colony formation assay takes less time than screen and observed resistance only emerged after a month of treatment prolonged culturing of these cells could potentially show greater difference in resistance. Mir-21 was otherwise one of the first micro RNAs discovered and described as an oncomir. It targets a lot of tumor suppressor genes including PTEN, BCL2 and

PDCD4 (Asangani et al., 2008). The latter was shown to be downregulated in cancers as it inhibits tumor invasion and progression. Furthermore, mir21 was shown to be associated with colon cancer, where it suppresses expression of PDCD4 (Asangani et al., 2008). This finding was also confirmed in HCT116 cells, where high mir21 levels and low PDCD4 levels were observed. If this miRNA proves to be a true hit it would indicate that its loss would induce a tumor-suppressor response. Although this seems counter-intuitive, some tumor suppressors can lower the ROS levels(Vurusaner et al., 2012). This response could therefore make cells less dependent on MTH1 and thus resistant. More experiments are needed to confirm or disprove has-mir-21 as a hit and to explain potential mechanism behind it.

One limitation of CRISPR resistance screens is that they can only detect resistance if the mechanism is loss of a certain gene. Another approach to identify resistance mechanisms would therefore be to perform a CRISPRa whole genome screen, to find if activation of any gene could make the cells resistant. We could also try to identify synthetic lethal interactions with MTH1 inhibition which could disable resistance pathway and therefore circumvent the occurrence of resistance. One approach would be to perform either shRNA or CRISPR dropout screen to find genes that are synthetically lethal with MTH1.

5.3 INVESTIGATION OF BACKGROUND RESISTANCE PROPERTIES AND MECHANISMS

Resistance of picked colonies turned out to be subtle, around 2-fold. Surprisingly, some clones were resistant to all three inhibitors, while others were not. Even though all three inhibitors work by binding to active center of MTH1(Gad et al., 2014; Huber et al., 2014) this could mean different resistance mechanisms. One of the possible explanations would be if only (S)-crizotinib would be a substrate for some drug pump, but not other inhibitors. There were some indications that (R)-crizotinib is a substrate (Tang et al., 2014; Katayama et al., 2012; Zhou et al., 2012), but our Western blot data makes it unlikely that this is the explanation.

When investigating growth rates of resistant and wild type cells we observed that confluence plays a role in sensitivity of wild-type cells to MTH1 inhibition. When 0,5 μM TH1579 was added to 50% confluent cells their growth was not impaired, even though they did not grow out when initially plated in same concentration. This may indicate some kind of cell-cell signaling involved in MTH1 sensitivity, where cells would excrete signals leading to reduced sensitivity to MTH1. However, since the growth curve of these cells is exponential it could also mean that after MTh1 inhibition cells are able to divide once more before the damage accumulates and they stop, which would already lead to confluent dish. IncuCyte experiments also allow us to distinguish between cell death or growth arrest. Dead cells start get degraded or loose adhesion and start to float therefore disappearing from focus layer of the picture. On the other hand, cells in growth arrest stay adherent and just do not proliferate. Our results are therefore more in line with findings of Patel et al. (2014) which state that MTH1 loss leads to

senescence or lower proliferation. On the other hand Gad et al. (2014) observed apoptosis after MTH1 inhibition. A possible explanation for this discrepancy is that MTH1 inhibition induces different responses in different cell types. More experiments are needed to finally confirm exact mechanism of cellular response to MTH1 inhibition.

Stability of resistant phenotype was investigated in second IncuCyte experiment. The fact that we did not observed a conversion to sensitive phenotype after a week of culturing them without the inhibitor could indicate that changes are most likely on a genomic or epigenomic level. Epigenetic modifications could be studied by chromatin immunoprecipitation (ChIP) or investigation of methylation patterns. To detect changes on genomic level, we could perform whole genome sequencing.

Although the western blot experiments were not completely unambiguous, all proteins ran at the correct weights so it seems unlikely that overexpression of either MTH1 or any of the drug pumps tested here would explain resistance. On the other hand, there are several other drug pumps whose overexpression could potentially confer resistance so future experiments should also include those.

The next possible resistance mechanism we tested was lowered ROS levels and therefore less reliance on MTH1. Our results did not show any difference in ROS levels, but it is important to note that sensitivity and specificity of the dye used was not explicitly tested. Furthermore, perhaps fluctuations in ROS can be really fast and therefore non-detectable with this experiment. We could also speculate that very small changes in ROS levels can already be meaningful in terms of response, but are under the detection level of this kit. To verify if this is the case other ROS-detecting methods can be used, such as measurement of carbonylated proteins (Dalle-Donne et al., 2003). Another approach to further investigate this would be to measure the levels of proteins that are known for their antioxidant function like superoxide dismutase.

Although we did not identify mutations in exons 1, 3 or 4 of the MTH1 gene, point mutations in exon 2 could still explain the resistance. In addition, mutations in regulation regions, such as promoters or enhancers or introns could lead to changes in MTH1 expression. However, if that was the case we would be able to detect it with western blot experiment.

To compare expression profiles of resistant cell lines and parental cells with and without TH1579, RNA sequencing was performed. To identify these differences we could compare differences in genes expressed in resistant cells with TH1579 and resistant cells without TH1579. Secondly, comparison of parental cells with and without the drug could point out which genes are involved in response to MTH1 inhibition. Furthermore, we could find a common gene expression signature that may be shared between resistant cells. This analysis could therefore provide more candidate genes, possibly involved in resistance.

We were not able to identify mechanism behind observed resistance. However, another approach worth trying would be to measure DNA damage response to check if

upregulation of repair pathways could confer resistance. For example, base excision repair is known to repair DNA after 8-oxodGTP incorporation (Krokan et al., 2000) and double strand break repair was also shown to be upregulated after MTH1 inhibition (Gad et al., 2014). Furthermore, there are two more nucleotide sanitation enzymes known: MTH2 and NUDT5 (Hori et al., 2010), whose upregulation could also explain the resistance. It was previously shown that MTH2 and NUDT5 prevent mutagenesis caused by 8-oxo-dGTP (Hori et al., 2010), even though most recent data suggests that MTH1 is more efficient in 8-oxo-GTPase activity than the other two (Carter et al., 2015).

Development of MTH1 inhibitors continues. Recently pharmacokinetic studies in mice showed favorable results (Saleh at al., 2015) and overexpression of MTH1 in breast tumors was validated indicating its importance in cancer cells (Coskun et al., 2015). This is why the importance of understanding the problem of resistance and its mechanisms might be crucial for further development. Occurrence of resistance should also be tested *in vivo* as understanding the mechanisms may lead to improvement of treatment.

The resistance we observed was mild and would not necessarily present a problem in clinical setting. However, the fact that cancer cells have a mechanism to modulate sensitivity to MTH1 inhibition can be worrying.Also, upon prolonged culturing of resistant cells in even higher concentrations of up to 1.5 μM TH1579, we observed even stronger levels of resistance coming up.

When we compared the sensitivity to MTH1 inhibition between cancer cells and normal cells the biggest challenge was slow growth of non-LT transformed NHUC cells. Stepwise transformation of BJ cells with hTERT, LT and Ras showed that hTERT transformation does not impair cell survival, but LT and Ras transformation decrease clonogenic survival (Gad et al., 2014). However, we were not able to reproduce these findings in NHUC model as both LT transformed and non-transformed cells were sensitive to MTH1 inhibition. These results may indicate that effect is cell-type specific and therefore not detectable in our model. Another explanation could be that NHUC cells acquired additional mutations during culturing that made them more "cancer-like" and thus sensitive to MTH1 inhibition. Furthermore, BJs are fibroblasts while NHUCs are epithelial cells so it is challenging to compare these two systems. Epithelial cells are more suitable for this kind of studies, as they more accurately represent stepwise transformation to cancer cells. Fibroblasts can possibly be exposed to environment stress that can also lead to different ROS levels. All together our findings seem to indicate that some normal cells may be sensitive to MTH1 inhibition in the same concentration range as cancer cells, which can have a big impact when designing therapy. More primary cell lines should be tested for sensitivity to rule out cell-type specificity and toxicity to normal tissue in mice models should be closely monitored.

6 CONCLUSIONS

In this work we investigated whether cancer cells can get resistant to MTH1 inhibition and if so, figure out the mechanism behind it. The hypothesis was that resistance is conferred by gene loss and therefore detectable with a CRISPR/Cas9 functional genetic screen. Our conclusions are as follows:

1. We observed that a panel of colon and bladder cancer cell lines as well as LT transformed NHUC cells were sensitive to MTH1 inhibitiors TH588, (S)-crizotinib and TH1579.

2. Spontaneous resistance to MTH1 inhibitors occured.

3. Although the CRISPR/Cas9 resistance screen indicated good performance, no validatable hits were found.

4. Further investigation of the background resistance revealed that the resistant phenotype was mild but stable. Cross-resistance of some but not all resistant lines to (S)-crizotinib could indicate different resistance mechanisms present in these cells.

5. Sensitivity of cancer cells to MTH1 inhibition seemed to be connected with confluency, as semi-confluent cells were able to grow with MTH1 inhibitors present.

6. Our results also indicated that MTH1 inhibition did not lead to cell death but only growth arrest.

7. Overexpression of MTH1 was unlikely to be the resistance mechanism.

8. Overexpression of any of drug pumps MRP1 and BCRP was not, and of MDR1 unlikely the explanation of the resistance.

9. Changes in ROS levels did not seem to explain the resistance phenotype.

10. Point mutations in MTH1 were unlikely to be the resistance mechanism.

11. We also showed that NHUC cells with hTERT and LT were not less sensitive to MTH1 inhibition, which could be a cell type specific event or could mean that MTH1 inhibition did not leave normal cells entirely unharmed.

The importance of this study is that we showed that cancer cells can get resistant to MTH1 inhibition. Uncovering the resistance mechanism may therefore lead to discovery of a biomarker that would predict which patients would benefit from therapy or a synthetic lethal interaction which could help in combinational treatment.

7 SUMMARIES

7.1 SUMMARY

Cancer is a common name for a group of distinct and diverse genetic diseases. Even though they are different they share a few common characteristics, the hallmarks of cancer. They include sustained proliferative signaling, evading growth suppressors, enabling replicative immortality, activating invasion and metastasis, resisting cell death, deregulation of cellular energetics and avoiding immune destructions. Another two important characteristics that enable development of cancer are genome instability and tumor-promoting inflammation.

Conventional cancer treatment includes surgery, chemotherapy and irradiation and while being highly effective in certain cancer types it is frequently highly toxic or leads to emergence of resistance. New approaches to cancer therapy are targeting the immune system or specific cancer-related proteins. Immunotherapy focuses on stimulation of the immune system to recognize and attack tumor cells, while targeted therapy aims to block cellular pathways that are essential for proliferation and survival of cancer cells. The major problem associated with targeted therapy is emergence of resistance.

Because of tumor heterogeneity and genomic instability of cancer cells they are able to rapidly adapt to changes in environment therefore causing resistance even if therapy is otherwise effective. Mechanisms of resistance that were uncovered until now are mostly upregulation of targeted protein, point mutations in targeted protein or compensation of inhibition by the same or a parallel pathway.

In the search for new therapeutic targets, MTH1 emerged as a target due to its common overexpression in cancer and it being more essential for cancer cells. Due to high metabolism rates of cancer cells they produce a lot of ROS, which are highly reactive and can cause damage to lipids, proteins and DNA. Until recently, free nucleotide pool was not being described as a target for ROS due to the focus being on DNA damage. However, oxidative damage in the free nucleotide pool can cause mutations, double strand breaks and cell death. MTH1 is a free nucleotide sanitizing enzyme which recognizes oxidized guanine and adenine and prevents them from getting incorporated to newly synthesized DNA, thereby preventing cell death especially in cells with high ROS levels. Additionally, some cancer cells actually require ROS for their survival and tumorigenesis so they are especially in need of a mechanism to prevent nucleotide pool damage while still maintaining high ROS levels. Normal cells should not depend heavily to MTH1 and therefore should not be sensitive to MTH1 inhibition.

Inhibition of MTH1 was indeed shown to be specific for cancer cells and inhibitors that bind MTH1 with high specificity were developed. However, as with all targeted therapy occurrence of resistance could present a major setback to this new therapy when moving into the clinic. Therefore there is a need to identify possible mechanisms of resistance to MTH1 inhibitors.

We used the recently developed CRISPR/Cas9 technology to perform a functional genetic screen by generating a population of cells each harboring a knockout of one gene. Treating this population with MTH1 inhibitors allowed us to investigate resistance mechanisms as knockouts of certain genes could confer resistance to cells.

Firstly, we tested a panel of cancer cell lines in a colony formation assay with three MTH1 inhibitors, finding that they were all sensitive to MTH1 inhibition. To investigate the difference in sensitivity between normal and cancer cells we used NHUC cells transformed with hTERT which were also transformed with LT or not. We found that non-LT transformed NHUCs were not less sensitive to MTH1 inhibition, but the results were not really clear due to differences in growth speed.

Interpretation of screen results proved to be challenging due to spontaneous background resistance. Even though technical performance of the screen was good, validation experiments failed to show any resistance. This indicated that resistance mechanism was not connected to loss of the top-scoring genes of the CRISPR screen.

We continued the study by investigating background resistance. We observed that only 2 out of five resistant lines were resistant to all three MTH1 inhibitors used, while others were sensitive to (S)-crizotinib. These observations could therefore indicate different resistance mechanisms behind these lines. Additionally, we observed that confluence plays a role in sensitivity of cells to MTH1 inhibition as more confluent cells were able to proliferate when more sparsely seeded cells did not grow. With the intent to identify resistance mechanism we tested if resistant cells overexpressed MTH1 or any of three common drug pumps, which turned out to not be the case. We also did not observe any difference in ROS levels between resistant and wild type cells. In addition, point mutations in MTH1 gene seemed highly unlikely to be a resistance mechanism.

Further experiments could include testing if overexpression of MTH2 or NUDT5 could explain resistance, repeating ROS measurements using more specific method and checking for upregulation of antioxidant enzymes such as superoxide dismutase. Another explanation of resistance could be upregulation of DNA repair pathways.

The importance of understanding resistance mechanisms is growing as MTH1 inhibitors are nearing clinical trials. Knowledge about resistance could enable identification of novel predictive biomarkers or emergence of new synthetic lethal interactions used for combinational treatment.

7.2 POVZETEK

Nenaden in hiter razvoj molekularne medicine v zadnjih desetletjih je omogočil popolno izkoreninjenje nekaterih nalezljivih bolezni ter učinkovito zdravljenje kroničnih bolezni. Še vedno pa smo precej nemočni glede zdravljenja raka, čeprav se število bolnikov z rakom povečuje in napovedi kažejo da se bo v prihodnjih desetletjih še povečalo. Težnja po razvoju učinkovitih zdravil je zato vedno večja, vendar pa z vedno globljim razumevanjem mehanizmov poteka bolezni kaže, da eno samo magično zdravilo za raka nikoli ne bo obstajalo. Raziskave zadnjega desetletja, ki temeljijo predvsem na novih tehnologijah sekvenciranja, so namreč odkrile, da rak ni ena sama bolezen, temveč več sto različnih bolezni, ki imajo le nekaj skupnih značilnosti. Rak je pravzaprav bolezen (epi)-genoma z velikimi genetskimi in epigenetskimi spremembami.

Proces razvoja rakavega obolenja se začne s poškodbo DNA, ki je lahko posledica dednih pomanjkljivosti ali vplivov okolja in načina življenja, kot so kajenje, alkohol in virusne okužbe. Kljub številnim mehanizmom, ki uspešno zaznavajo in popravljajo poškodbe v DNA, pa se nekatere lahko izmaknejo in povzročijo nastanek raka.

Če se poškodbe DNA pojavijo v tumor-supresorskem genu ali onkogenu, lahko vodijo do spremembe njegove aktivnosti. Tumor-supresorski geni so geni, ki v normalnih razmerah zavirajo celično rast in je njihova aktivnost pri raku pogosto zmanjšana. Po drugi strani so onkogeni v normalnih razmerah zadolženi za stimulacijo proliferacije in so pri raku lahko čezmerno aktivni. Mutacija v onkogenu je tako lahko prvi korak k razvoj raka, vendar sama po sebi še ni dovolj za maligno transformacijo. Celice imajo namreč varovalni mehanizem, ki povzroči zmanjšanje sposobnosti proliferacije, imenovano z onkogeni povzročena senescenca (ang. oncogene induced senescence OIS). Šele ko celice pridobijo nove mutacije, ki jim omogočajo izognitev senescenci, lahko postanejo maligne. Razvoj raka je torej večstopenjski proces, ki sledi načelom evolucije. Celice postopno pridobijo vse znake raka, ki vključujejo zvišanje signalov proliferacije, izogibanje supresorjem rasti, aktivacijo invazivnosti in zasevanja, omogočeno neskončno podvojevanje, indukcijo angiogeneze, upiranje celični smrti, spremenjen metabolizem in izmikanje imunskemu sistemu.

Raziskovalci biologije raka so se dolgo osredotočali samo na tumorske celice in so se šele v zadnjem desetletju začeli zanimati za tako imenovano mikrookolje tumorja. Signali, ki prihajajo iz neposredne okolice lahko namreč vplivajo na rast in invazivnost tumorskih celic. Najpomembnejši del mikrookolja so imunske celice, katerih vloga je bila dolgo časa dvoumna. Danes vemo, da ima imunski sistem dvojno vlogo, saj T-celice lahko prepoznajo in napadejo tumor in tako služijo kot obramba, druge imunske celice pa lahko spodbujajo stalno vnetje okoli tumorja in izločajo signale, ki spodbujajo rast in preživetje tumorskih celic. Poleg imunskega sistema so v mikro okolju pomembni še izvencelični matriks in fibroblasti, ki prav tako izločajo rastne faktorje in s tem spodbujajo rast tumorskih celic.

Osnovo zdravljenja raka danes predstavljajo kirurška odstranitev tumorja, kemoterapija in radioterapija. Kirurška odstranitev tumorja je v nekaterih primerih zelo učinkovita, vendar je omejena na primarni tumor in neuspešna, ko pride do zasevanja. Večina kemoterapevtikov in radioterapija delujeta tako, da povzročita poškodbe DNA v celicah. Ker se rakave celice delijo izjemno hitro, ne ustavijo celičnega cikla za popravilo poškodb DNA kot zdrave celice, kar vodi v celično smrt. Glavni problem teh terapij je relativno nizka stopnja učinkovitosti kemoterapije in neučinkovitost radioterapije ter problem toksičnosti za druga tkiva, ki vsebujejo hitro deleče se celice.

V zadnjih letih se je pomembno razvila tudi imunoterapija, katere cilj je vplivati na imunski odziv telesa proti rakavim celicam. Eden od mehanizmov delovanja imunoterapije je blokada interakcij receptor-ligand, ki onemogočajo T-celicam, da bi prepoznale in napadle tumor. Tako lahko T-celice tumor spet prepoznajo in ga odstranijo.

Z razvojem tehnologij sekvenciranja je prišlo do odkritja genov, ki so pogosto spremenjeni pri raku. To je omogočilo nov pogled na klasifikacijo tipov raka, ki je doslej upoštevala le organ izvora in ne molekularnih značilnosti tumorja. Odkrivanje ključnih molekulskih poti, ki so spremenjene v rakavih celicah in možnost vplivanja nanje, pa je vodilo v razvoj tarčnega zdravljenja. Prve raziskave so se izkazale za izjemno uspešne, tumorji so se zmanjšali, stopnje preživetja zvišale in znanstveniki so dejavno iskali nove tarče in inhibitorje. Na žalost se je izkazalo, da je bil uspeh kratek, saj se je po začetnem odzivu v večini primerov bolezen vrnila.

Tarče za tarčno zdravljenje so pogosto kinaze, ki sodelujejo v metabolnih poteh za vzpodbujanje proliferacije in preživetje rakavih celic. Do sedaj najbolj uspešen inhibitor, ki so ga razvili je imatinib, za zdravljenje kronične mieloične levkemije in cilja specifičen fuzijski protein BCR-ABL, prisoten pri tem tipu raka. Zelo uspešno monoklonsko protitelo trastuzumab pa se uporablja za zdravljenje raka na dojkah, katerega celice izražajo receptor HER2. Danes se raziskave usmerjajo na veliko število drugih onkoproteinov ter drugih tarč za tarčno zdravljenje, vendar za enkrat še ni prišlo do pomembnega preboja.

Ključni problem tarčnega zdravljenja je selektivnost, saj je večina tarč izraženih tudi v normalnih celicah, zato lahko inhibicija v nekaterih primerih vodi v neželene stranske učinke. Drug, večji problem pa je zmožnost tarčnih celic, da postanejo odporne na tarčno zdravljenje. Prvi razlog za tako imenovano prirojeno odpornost je heterogenost tumorja. Zaradi specifičnih pogojev v mikrookolju tumorja namreč pride do razvoja različnih sub-populacij tumorskih celic, ki imajo lahko različno genetsko ozadje. Tarčna terapija je tako lahko učinkovita pri večini celic, vendar so lahko zaradi raznolikosti populacije odporne celice prisotne že pred zdravljenjem in pod selekcijskim pritiskom prevzamejo večji del populacije. Po drugi strani pa imajo rakave celice izredno nestabilne genome, kar vodi v zelo visok nivo mutacij. Ta plastičnost jim tako omogoča hitro pridobivanje novih mutacij, ki lahko vodijo v razvoj odpornosti. Večina mehanizmov odpornosti, ki so bili opisani do sedaj, temelji na točkastih mutacijah v genu tarčnega proteina, tako da proteinski produkt ne more več vezati inhibitorja,

čezmernem izražanju tarčnega proteina ali proteinov iste ali vzporedne metabolne poti. Druge možnosti pridobljene odpornosti so še spremembe v metabolizmu in transportu zdravila ter njegovi inaktivaciji.

Kljub relativnemu neuspehu tarčnega zdravljenja pa raziskovalci še vedno iščejo nove možnosti tarč za zdravljenje. Ključen pogoj pri iskanju novih tarč je v razlikah med rakavo in normalno celico. Ena od pomembnih razlik je na primer oksidacijski stres. Spremenjen metabolizem v rakavih celicah vodi do nastanka reaktivnih kisikovih zvrsti (ROS). Gre za zelo reaktivno skupino radikalov in molekul, ki imajo prost elektron ali pa tvorijo radikal s prostim elektronom po razpadu. Prost elektron vodi do izjemne reaktivnosti teh spojin, ki lahko reagirajo s proteini, lipidi in DNA in povzročijo oksidativno škodo. Veliko študij je že povezalo visoke nivoje ROS z rakavimi celicami, saj se nekateri onkogeni proteini zanašajo na mutagene posledice ROS in tako ti sodelujejo v celični signalizaciji. Eden takih je mutiran RAS, ki spodbuja proliferacijo in invazivnost celic in za svoje delovanje potrebuje visok nivo ROS. Prav zato pa te celice toliko bolj potrebujejo mehanizem, ki jih ščiti pred negativnimi posledicami ROS, brez zmanjšanja njihovega dejanskega nivoja. Poleg DNA pa lahko reagirajo tudi s prostimi nukleotidi, ki nato tvorijo oksidirane nukleotide. Če se ti nato vgradijo v DNA lahko pride do pojava mutacij, dvojnih prelomov DNA in celične smrti. Celice se zato zanašajo na encim MTH1, ki prepozna oksidirane nukleotide in jih pretvarja v njihovo mono-fosfatno verzijo in s tem preprečuje vgraditev v DNA. Izguba ali inhibicija tega proteina tako v rakavih celicah z visokim nivojem ROS vodi v vgraditev oksidiranih nukleotidov v DNA in posledične poškodbe, v normalnih celicah pa ne predstavlja težave, saj je nivo ROS veliko nižji.

Ta dognanja so vodila v razvoj inhibitorjev MTH1, ki bi učinkovito in selektivno ciljali rakave celice. Inhibitor z majhno molekulsko maso TH588 se je izkazal za učinkovitega, njegove lastnosti pa so še izboljšane pri naslednji generaciji inhibitorjev MTH1, ki jo predstavlja TH1579. Precej po naključju je prišlo do odkritja še enega inhibitorja MTH1, ko so ugotovili da stereoizomer že uveljavljenega zdravila crizotiniba, (S)-crizotinib selektivno deluje na MTH1. Poskusi s temi inhibitorji so pokazali, da se nivo oksidiranih nukleotidov in s tem poškodb DNA v tumorskih celicah poveča ter vodi v celično smrt ali senescenco. Natančni mehanizmi na molekularnem nivoju še niso povsem razjasnjeni, vendar so bili tudi poskusi na mišjih modelih uspešni. Inhibitorji MTH1 tako predstavljajo nov pristop k tarčnemu zdravljenju raka, ko tarča ni onkoprotein, ampak protein, ki je bolj pomemben za rakave celice kot za normalne celice.

Odkritje sistema CRISPR/Cas9 pri bakterijah je omogočilo novo orodje za natančno in uspešno preurejanje genomov. Sistem je sestavljen iz endonukleaze Cas9 in vodilne RNA (ang. *guide RNA*, gRNA), ki tvorita kompleks in iščeta ustrezno zaporedje v DNA. Ko sekvenca gRNA ustreza genomski DNA pride do konformacijske spremembe v proteinu Cas9 tako, da ta prereže obe verigi DNA. Na ta način lahko s spreminjanjem zaporedja nukleotidov v gRNA ciljamo točno določena zaporedja v genomu. Pri popravljanju dvojnega preloma z ne-homolognim združevanjem koncev pride do nekaj nukleotidov velikih insercij ali delecij, ki povzročijo spremembo v bralnem okvirju in s

tem nastanek nefunkcionalnega proteina. Preko sistema CRISPR/Cas9 lahko torej ustvarimo celice z izbitimi geni. Če na ta način ustvarimo populacijo celic, ki imajo vsaka izbit en gen celotnega genoma, ki je kodirajoč lahko izvedemo tako imenovani funkcionalni genomski pregled, ki nam omogoča identifikacijo genov, ki so udeleženi pri nekem celičnem procesu. Ko tako populacijo celic na primer zdravimo z inhibitorjem, bo večina celic umrla, tiste, ki preživijo, pa bodo preživele zaradi izbitega gena. Analiza z visoko zmogljivim sekvenatorjem nove generacije nam omogoča identifikacijo tega gena ter sklepanje o mehanizmu odpornosti.

Namen tega dela je bil preučiti, ali rakave celice lahko postanejo odporne proti inhibitorjem encima MTH1 in odkriti kako. Cilj naloge je bil izvedba funkcionalnega genetskega pregleda s sistemom CRISPR/Cas9, identifikacija genov, ki so kandidati za odpornost ter validacija le-teh s tremi inhibitorji MTH1. Delovna hipoteza magistrskega dela je bila, da celice lahko postanejo odporne preko izbitja določenega gena ter da lahko te gene odkrijemo z visokozmogljivim genetskim pregledom.

Da bi izbrali najbolj primerno celično linijo, smo izbrali skupino šestih celičnih linij: dve liniji celic raka mehurja (RT112, 639V), eno epitelijsko celično linijo spremenjeno s hTERT in SV40LT (NHUC-LT) ter tri linije celic raka debelega črevesa (LOVO, LST147, HCT116). Enako število celic smo tretirali z različnimi koncentracijami vsakega od treh inhibitorjev (TH588, TH1579 in (S)-crizotinib). Gojišče smo zamenjali vsake 3 – 4 dni, poskus pa končali ko so bile kontrolne celice, tretirane z DMSO, konfluentne. Vse testirane celične linije so bile občutljive na vsakega od treh inhibitorjev, TH1579 pa je učinkoval pri dosti nižjih koncentracijah kot ostala dva inhibitorja, verjetno zaradi izboljšanih lastnosti. Za nadaljevanje poskusov smo izbrali celično linijo raka debelega črevesa HCT116, ki nimajo mutiranega p53, imajo pa mutacijo v genu RAS, kar izboljša njihovo občutljivost na inhibicijo MTH1.

Za dostavo plazmidov, ki vsebujejo knjižnico molekul gRNA in protein Cas9 v celice smo uporabili lentivirusni sistem. Prednost tega sistema je, da nam omogoča natančen nadzor nad številom virusnih delcev in s tem števila gRNA v eni celici. Tako se izognemo morebitnim napakam zaradi ciljanja več genov hkrati. Lentivirus smo pridobili s pomočjo celic HEK293T, transfeciranih z mešanico plazmidov za ovojne proteine. Kasneje smo določili virusni titer in okužili ustrezno število HCT116 celic. Po selekciji s puromicinom smo začeli genetski pregled, tako da smo populacijo celic razdelili na kontrolno in testno in ju tretirali z DMSO ali TH1579. Gojišče smo zamenjali vsake 3 do 4 dni in tiste vzorce, ki so bili konfluentni presadili, hkrati pa vzdrževali kompleksnost, tako da je bilo prisotnih vsaj 100 celic z isto gRNA. Po mesecu genetskega pregledovanja smo opazili pojav spontane odpornosti kontrolnih celic, ki niso bile okužene z virusom in niso vsebovale sistema CRISPR/Cas9. To kaže na to, da imajo celice nek drug mehanizem odpornosti, neodvisen od izbitja genov preko delovanja CRISPR/Cas9. Hkrati pojav spontane odpornosti močno oteži analizo rezultatov, saj je brez nadaljnih poskusov nemogoče razlikovati prave zadetke od lažno pozitivnih, tako imenovanega efekta potnikov (ang. *passenger effect*). Po izolaciji DNA iz celic z začetka in konca pregleda smo sekvence za gRNA pomnožili z dvostopenjskim PCR tako, da smo dodali adapterje potrebne za sekvenciranje in

sekvencirali z visoko zmogljivim sekvenatorjem. Sledila je bioinformacijska analiza, kjer smo sekvence mapirali glede na knjižnico genov ter izvedli statistično analizo, ki je upoštevala tri ponovitve vzorcev. Končni podatki tako vsebujejo seznam gRNA in število le-teh prisotnih v vsakem vzorcu. Glede na statistično najbolj pomembne gene, katerih gRNA so bile največkrat prisotne, smo izbrali osem genov za validacijo. Validacijski poskus je vseboval kloniranje sekvenc za gRNA v vektor in ustvarjanje celic z izbitimi geni. Te smo nato testirali s tremi inhibitorji, da bi potrdili ali so res odporne ali ne.

Interpretacija rezultatov pregleda se je izkazala za težavno predvsem zaradi spontane odpornosti. Tehnična izvedba pregleda je bila dobra, kar so potrdili visoki koeficienti korelacije med tremi ponovitvami. Kljub temu pa nismo opazili zadetkov, ki bi bili statistično zelo pomembni in bi torej povzročili odpornost. Prav tako eksperimenti za validacijo niso pokazali močne odpornosti, kljub temu da smo opazili rahlo odpornost pri celicah, ki smo jim preko sistema CRISPR/Cas9 izbili gen za microRNA 21. Ta je že bila opisana kot onkomir, saj zmanjša izražanje številnim tumor supresorskim genom. Zato bi lahko nadaljevali poskuse, ki bi pokazali ali izguba tega gena res vodi k odpornosti ter pojasnitvi mehanizma. Validacijski poskus bi bilo potrebno izvajati dlje časa, saj se je odpornost pri pregledu pojavila šele po enem mesecu.

Poleg raziskovanja odpornosti preko sistema CRISPR/Cas9 pa smo se odločili raziskati tudi spontano odpornost v ozadju, ki smo jo opazili pri pregledu. Zato smo izbrali nekaj kolonij, ki so se pojavile pri kontrolnih celicah tretiranih s TH1579. Za začetek smo preverili obseg odpornosti, tako da smo odporne celice testirali z vsakim od treh inhibitorjev. Izkazalo se je, da so vse odporne celice odporne na TH1579 in TH588, vendar je bila odpornost šibka, saj so rasle le pri enkrat višji koncentraciji kot starševske celice. Zanimivo je tudi, da sta bili le dve od odpornih celičnih linij odporni na (S)-crizotinib, kar lahko kaže na različne mehanizme odpornosti.

Ker smo med različnimi poskusi opazili, da se celice odzivajo na inhibitorje različno, glede na stopnjo konfluence, smo izvedli poskus z "IncuCyte". Gre za kamero znotraj inkubatorja, ki preko fotografij celic določa stopnjo konfluence. Odporne celice in starševske celice smo v treh ponovitvah tretirali z DMSO in dvema koncentracijama TH1579. Ko so bile kontrolne celice približno 50% konfluentne, smo tudi njim dodali inhibitor. Rezultati so pokazali, da konfluenca močno vpliva na občutljivost celic na inhibicijo MTH1. Starševske celice, ki so bile 50% konfluentne, so namreč rasle tudi z dodanim inhibitorjem čeprav pri manjši konfluenci niso. Do neke mere lahko ta efekt pojasnimo z dejstvom, da program za analizo upošteva površino prekrito s celicami, tako da lahko tudi mrtve celice in ostanke odmrlih celic šteje kot rast. Vendar pa po analizi slik še vedno opazimo rast pri višji konfluenci. To lahko kaže na signaliziranje med celicami, ki na nek način ščitijo druga drugo pred inhibicijo, na primer z izločanjem parakrinih dejavnikov ali medceličnimi stiki.

Da bi preverili kako stabilna je odpornost, smo celice gojili en teden brez inhibitorja in nato testirali njihovo rast z in brez inhibitorja. Ugotovili smo, da je odpornost stabilna, saj so odporne celice ostale odporne tudi po tednu dni brez inhibitorja. Ti rezultati

kažejo, da gre za stabilno spremembo, ki je verjetno na genetskem ali epigenetskem nivoju. Ker smo opazili razlike v morfologiji med različnimi linijami odpornih celic ter odpornih celic in starševskih celic smo preverili, ali celice res izhajajo iz enake linije. Ena od možnih razlag za opažene razlike bi namreč lahko bila kontaminacija z drugo celično linijo. Da bi preverili ali gre res za celice HCT116 smo izolirali DNA in poslali vzorce na analizo kratkih tandemskih ponovitev (ang. *short tandem repeats*, STR). Opazili smo precej odstopanj med linijami, vendar gre pri večini odstopanj za slabšo kvaliteto analize, kar pomeni da so rezultati bolj nezanesljivi. Tako sicer ne moremo z gotovostjo trditi, da gre za isto celično linijo, vendar je zelo malo verjetno da bi šlo za okužbo, saj so razlike minimalne. Različno morfologijo pa bi lahko pojasnilo tudi dejstvo, da je lahko populacija celic heterogena že pred začetkom pregleda in tako z inhibitorjem le izberemo celice, ki so že vnaprej odporne. Tako bi bilo potrebno preučiti tudi, ali je odpornost že prirojena zaradi heterogenosti ali pa jo celice pridobijo zaradi nestabilnosti genoma.

Da bi ugotovili vzrok odpornosti, smo postavili in testirali nekaj različnih hipotez, ki bi vse lahko pojasnile odpornost. Prva hipoteza je bila, da bi prekomerno izražanje MTH1 pomenilo, da njegova inhibicija ne bi bila več učinkovita in celice bi tako postale odporne. Druga hipoteza je bila, da bi prekomerno izražanje nekaterih ATP-odvisnih črpalk, ki črpajo zdravila iz celic povzročilo odpornost, saj bi se celice na ta način znebile inhibitorja preden bi ta uspel delovati. Tretja hipoteza je bila, da celice spremenijo nivo ROS in se zato manj zanašajo na MTH1.

Spremembe v izražanju MTH1 in treh najbolj pogostih ATP-odvisnih črpalk (MDR1, MRP1 in BCRP) smo preverili s prenosom po Westernu. Izolirali smo proteine iz odpornih in starševskih celic, jih kvantificirali s poskusom z bikinojsko kislino in normalizirali. Proteine smo nato ločili z elektroforezo SDS-PAGE in prestavili na membrano. To smo inkubirali s primarnim protitelesom, sprali in inkubirali s sekundarnim protitelesom. Vizualizacijo smo opravili preko kemiluminiscence. Pri poskusu s protitelesom proti MTH1 smo opazili veliko nespecifičnega ozadja, kar otežuje interpretacijo rezultatov. Kljub temu lahko glede na velikost proteina sklepamo, kje gre za specifično liso in tako sklepamo, da ni opaznih razlik med odpornimi celicami in starševskimi celicami. Nespecifično ozadje predstavlja problem tudi pri poskusu s protitelesom proti MDR1, kjer je izražanje na splošno nizko, vendar prav tako ne opazimo razlik. MRP1 je prav tako izražen v manjših količinah in se ne razlikuje med celičnimi linijami, specifičnost pa smo potrdili z uporabo kontrole. Tudi v tem primeru nismo opazili razlik v izražanju. Proteina BCRP nismo zaznali, ker verjetno ni izražen v celični liniji, ki smo jo uporabili. Za kontrolo nanosa smo uporabili hišni protein HSP90. Ti rezultati kažejo, da prekomerno izražanje MTH1 ali ATP-odvisnih črpalk ne pojasni opažene odpornosti.

Z naslednjim poskusom smo preverili nivoje ROS v odpornih celicah in starševskih celicah. Nivoje ROS smo izmerili s specifičnim barvilom CellROX GReen, ki postane fluorescentno po reakciji z ROS in ga lahko izmerimo s pretočno citometrijo. Za pozitivno kontrolo smo uporabili tert-butil-hidroperoksid (TBHP), ki sproži nastanek ROS, za negativno pa smo celice najprej inkubirali z N-acetil-cisteinom, ki je

antioksidant in varuje pred ROS ter nato s TBHP. Celice smo en teden gojili brez inhibitorja ali z inhibitorjem ter nato izmerili nivo ROS. Naši rezultati so pokazali, da ni bilo razlik med odpornimi celicam in starševskimi celicami ali med celicami gojenimi z ali brez inhibitorja. Kljub temu pa je težko z gotovostjo trditi kako specifično in natančno je uporabljeno barvilo. Nivoji ROS se lahko morda spreminjajo zelo hitro in le majhne spremembe že pomenijo vliv na celičnem nivoju. Alternativen pristop bi zato vključeval merjenje ROS z drugo metodo, na primer preko merjenja karboniliranih proteinov.
Kljub temu, da nismo potrdili nobene od hipotez, bi lahko nadaljnji poskusi preverili še nivo izražanja proteinov, ki delujejo kot antioksidanti, na primer superoksid dismutaza. Povečano izražanje le-teh bi namreč lahko znižalo nivo ROS in predstavljalo mehanizem odpornosti na inhibicijo MTH1.

Poleg raziskovanja mehanizmov spontane odpornosti pa smo ugotavljali še vpliv inhibicije MTH1 na normalne celice. Uporabili smo normalne humane urotelijske celice (ang. *normal human urothelial cells*, NHUCs), ki so bile spremenjene s telomerazo hTERT in s tem pripravljene za gojenje v laboratoriju. Primerjali smo jih z isto celično linijo, ki pa je bila dodatno spremenjena z SV40 LT antigenom – NHUC-LT. Glede na dosedanje študije, ki so bile izvedene na liniji fibroblastov BJ smo pričakovali, da bodo celice NHUC manj občutljive na inhibicijo MTH1 kot NHUC-LT. Rezultati prejšnjih raziskav so namreč pokazali, da se v tem koraku občutljivost spremeni, saj tako normalne celice postanejo rakave. V nasprotju s pričakovanji smo opazili, da so bile celice NHUC enako ali še bolj občutljive na vse tri inhibitorje, kot NHUC-LT. Ti rezultati torej kažejo, da normalne celice niso manj občutljive na inhibicijo MTH1, kar bi lahko predstavljalo velik problem pri načrtovanju terapije. Prav tako je potrebno poudariti da so celice, na katerih so bili ti poskusi izvedeni v preteklosti fibroblasti, ki morda niso zadovoljiv predstavnik normalnih celic. Po drugi strani so NHUC epitelijske celice, ki bolje predstavljajo normalne celice in rakave, ko jih spremenimo z hTERT in LT. Zato so potrebne nadaljnje raziskave, da se popolnoma razjasni vpliv inhibicije MTH1 na normalne celice, na primer s poskusi na 3D modelih in živalih.

Poleg tega bi za pojasnitev opažene odpornosti lahko preverili morebiten porast v izražanju proteinov, ki sodelujejo pri odgovoru na poškodbe DNA, saj bi bolj učinkovito popravljanje poškodb zaradi oksidiranih nukleotidov lahko predstavljalo mehanizem odpornosti. Nadaljne iskanje genov udeleženih pri odpornosti, bi lahko olajšali tudi s funkcionalnim genetskim pregledom, ki uporablja tehnologijo CRISPRa. Gre za tako imenovani aktivacijski CRISPR, ki uporablja katalidično mrtev protein Cas9, ki nima več endonukleazne funkcije. Nanj je lahko vezanih več transkripcijsko aktivacijskih domen, ki vpokličejo transkripcijski kompleks in sprožijo izražanje tarčnega gena, ne glede na njegovo stopnjo izražanja prej. Tak pregled bi omogočil identifikacijo genov, katerih čezmerno izražanje bi zagotovilo odpornost. Da bi ugotovili, če obstajajo geni, ki so sintetično letalni z MTH1, kar bi bilo uporabno za načrtovanje kombiniranega zdravljenja bi lahko izvedli tudi pregled osipa s sistemom CRISPR/Cas9 ali majhnimi lasničnimi RNA (ang. *short hairpin* RNA, shRNA). V tem primeru bi ugotovili kateri gen v kombinaciji z izgubo MTH1 povzroči celično smrt.

Identifikacija in razumevanje mehanizmov odpornosti na inhibitorje encima MTH1 bi nam lahko omogočila razvoj biooznačevalcev, ki bi predvideli potek zdravljenja pri bolnikih. Prav tako bi lahko pomagala pri razvoju kombiniranega zdravljenja, ki bi preprečilo razvoj odpornosti. Zaskrbljujoče je, da rakave celice lahko postanejo odporne na inhibicijo MTH1. Kljub temu da opažena odpornost ni močna in zato verjetno manj relevantna za klinične študije, je pomembno da odkrijemo mehanizme, ki stojijo za odpornostjo. Potrebne bodo nadaljnje študije, predvsem na živalskih modelih, ki bodo pokazale, ali se lahko odpornost pojavi tudi *in vivo* ter ali se mehanizmi razlikujejo. Morda pa je zaskrbljujoče dejstvo, da so tudi normalne celice lahko občutljive na inhibicijo MTH1, kar zahteva posebno pozornost preden zdravilo začne s kliničnimi poskusi.

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SUPPLEMENTARY

Suplementary figure 1: Sequences used for gRNA validation
Priloga 1: Sekvence gRNA uporabljene za validacijo

hsa-mir-21	HGLibA_58207	CTTGTGGAAAGGACGAAACACCGCTCATGGCAACACCAGTCGAgtttaagagctagaaatagcaag	66	CTCATGGCAACACCAGTCGA
hsa-mir-21	HGLibA_58208	CTTGTGGAAAGGACGAAACACCGGTCTGATAAGCTACCCGACAgtttaagagctagaaatagcaag	66	GTCTGATAAGCTACCCGACA
hsa-mir-21	HGLibA_58209	CTTGTGGAAAGGACGAAACACCGATGTCAGACAGCCCATCGACgtttaagagctagaaatagcaag	66	ATGTCAGACAGCCCATCGAC
hsa-mir-21	HGLibA_58210	CTTGTGGAAAGGACGAAACACCGATGTTGACTGTTGAATCTCAgtttaagagctagaaatagcaag	66	ATGTTGACTGTTGAATCTCA
PPP1CC	HGLibA_38010	CTTGTGGAAAGGACGAAACACCGTTTACCGATAGCAGCCATCGgtttaagagctagaaatagcaag	66	TTTACCGATAGCAGCCATCG
PPP1CC	HGLibA_38011	CTTGTGGAAAGGACGAAACACCGTCTTATATGTAGAGCCCATCgtttaagagctagaaatagcaag	66	TCTTATATGTAGAGCCCATC
PPP1CC	HGLibA_38012	CTTGTGGAAAGGACGAAACACCGGACCCCGATAAAGATGTCTTgtttaagagctagaaatagcaag	66	GACCCCGATAAAGATGTCTT
CXorf65	HGLibA_11958	CTTGTGGAAAGGACGAAACACCGTTCCACCTTACAAACGTAGTgtttaagagctagaaatagcaag	66	TTCCACCTTACAAACGTAGT
CXorf65	HGLibA_11959	CTTGTGGAAAGGACGAAACACCGCGTTTGTTCACACAAATCGAgtttaagagctagaaatagcaag	66	CGTTTGTTCACACAAATCGA
CXorf65	HGLibA_11960	CTTGTGGAAAGGACGAAACACCGCAACACGACGACAGCACAGTgtttaagagctagaaatagcaag	66	CAACACGACGACAGCACAGT
CLASP2	HGLibA_09795	CTTGTGGAAAGGACGAAACACCGAAGACAGTCGACGCGCTCACgtttaagagctagaaatagcaag	66	AAGACAGTCGACGCGCTCAC
CLASP2	HGLibA_09796	CTTGTGGAAAGGACGAAACACCGGGCGCCGAGTTTGACTAGTTgtttaagagctagaaatagcaag	66	GGCGCCGAGTTTGACTAGTT
CLASP2	HGLibA_09797	CTTGTGGAAAGGACGAAACACCGTTCATCTCGAACCTTGTCTTgtttaagagctagaaatagcaag	66	TTCATCTCGAACCTTGTCTT
PITPNA	HGLibA_36740	CTTGTGGAAAGGACGAAACACCGACCCACGTTTGTTCGAATGCgtttaagagctagaaatagcaag	66	ACCCACGTTTGTTCGAATGC
PITPNA	HGLibA_36741	CTTGTGGAAAGGACGAAACACCGAGGCTTTCTCGTGTATATTCgtttaagagctagaaatagcaag	66	AGGCTTTCTCGTGTATATTC
PITPNA	HGLibA_36742	CTTGTGGAAAGGACGAAACACCGTTTCTCACCGTCCTTCTCGTgtttaagagctagaaatagcaag	66	TTTCTCACCGTCCTTCTCGT
JUND	HGLibA_24008	CTTGTGGAAAGGACGAAACACCGCGCGGCGCGGACTCAGTACGCgtttaagagctagaaatagcaag	66	CGCGCGCGGACTCAGTACGC
JUND	HGLibA_24009	CTTGTGGAAAGGACGAAACACCGTTCGCGTAGACAGGCGCTTCgtttaagagctagaaatagcaag	66	TTCGCGTAGACAGGCGCTTC
JUND	HGLibA_24010	CTTGTGGAAAGGACGAAACACCGCCTACCCCCCCTGCGCGCCGAgtttaagagctagaaatagcaag	66	CCTACCCCCCCTGCGCGCCGA
P2RX5	HGLibA_34857	CTTGTGGAAAGGACGAAACACCGTCCTTGGAGCACGCGCCATCgtttaagagctagaaatagcaag	66	TCCTTGGAGCACGCGCCATC
P2RX5	HGLibA_34858	CTTGTGGAAAGGACGAAACACCGAAGGACAGCGACTGCCACGCgtttaagagctagaaatagcaag	66	AAGGACAGCGACTGCCACGC
P2RX5	HGLibA_34859	CTTGTGGAAAGGACGAAACACCGTGAAGTCTTCGGCCTCCTTCgtttaagagctagaaatagcaag	66	TGAAGTCTTCGGCCTCCTTC
KRTAP3-3	HGLibA_25745	CTTGTGGAAAGGACGAAACACCGGCCATCCATTAGCAGCCTCTgtttaagagctagaaatagcaag	66	GCCATCCATTAGCAGCCTCT
KRTAP3-3	HGLibA_25746	CTTGTGGAAAGGACGAAACACCGTCTGACAAATCCTGCCGCTGgtttaagagctagaaatagcaag	66	TCTGACAAATCCTGCCGCTG
KRTAP3-3	HGLibA_25747	CTTGTGGAAAGGACGAAACACCGTGGGGGGACAGTTGTCACAGCgtttaagagctagaaatagcaag	66	TGGGGGACAGTTGTCACAGC
PPP1CC	HGLibB_37961	CTTGTGGAAAGGACGAAACACCGGCTTCAAGTTCTAGTAGGATgtttaagagctagaaatagcaag	66	GCTTCAAGTTCTAGTAGGAT
PPP1CC	HGLibB_37962	CTTGTGGAAAGGACGAAACACCGTCCATAAATTCTGTTGATGCgtttaagagctagaaatagcaag	66	TCCATAAATTCTGTTGATGC
PPP1CC	HGLibB_37963	CTTGTGGAAAGGACGAAACACCGGAATATCTTCTCATCCACGAgtttaagagctagaaatagcaag	66	GAATATCTTCTCATCCACGA
CXorf65	HGLibB_11946	CTTGTGGAAAGGACGAAACACCGCTCAGATAATCAGCAGTTTCgtttaagagctagaaatagcaag	66	CTCAGATAATCAGCAGTTTC
CXorf65	HGLibB_11947	CTTGTGGAAAGGACGAAACACCGTGTGCCTCTCCTCAAGAATCgtttaagagctagaaatagcaag	66	TGTGCCTCTCCTCAAGAATC
CXorf65	HGLibB_11948	CTTGTGGAAAGGACGAAACACCGTCTGTAAGCATTCTCCAGCCgtttaagagctagaaatagcaag	66	TCTGTAAGCATTCTCCAGCC
CLASP2	HGLibB_09786	CTTGTGGAAAGGACGAAACACCGTAACTTACCCATTGCTACATgtttaagagctagaaatagcaag	66	TAACTTACCCATTGCTACAT
CLASP2	HGLibB_09787	CTTGTGGAAAGGACGAAACACCGCTTTAAATCCTATGTAGCAAgtttaagagctagaaatagcaag	66	CTTTAAATCCTATGTAGCAA
CLASP2	HGLibB_09788	CTTGTGGAAAGGACGAAACACCGTGTAGCTTTAATAGACAGAAgtttaagagctagaaatagcaag	66	TGTAGCTTTAATAGACAGAA
PITPNA	HGLibB_36693	CTTGTGGAAAGGACGAAACACCGAATGAGCCCTACGAGAAGGAgtttaagagctagaaatagcaag	66	AATGAGCCCTACGAGAAGGA
PITPNA	HGLibB_36694	CTTGTGGAAAGGACGAAACACCGAAGCGACATGGTGCTGCTCAgtttaagagctagaaatagcaag	66	AAGCGACATGGTGCTGCTCA
PITPNA	HGLibB_36695	CTTGTGGAAAGGACGAAACACCGGCACTCACCGGTTCTGCAGTgtttaagagctagaaatagcaag	66	GCACTCACCGGTTCTGCAGT
JUND	HGLibB_23974	CTTGTGGAAAGGACGAAACACCGGGCGGGCTCTCGCCGAAGCTgtttaagagctagaaatagcaag	66	GGCGGGCTCTCGCCGAAGCT
JUND	HGLibB_23975	CTTGTGGAAAGGACGAAACACCGCGCGAACCTGAGCAGCTACGgtttaagagctagaaatagcaag	66	CGCGAACCTGAGCAGCTACG
JUND	HGLibB_23976	CTTGTGGAAAGGACGAAACACCGCTCCTGCGTGTCCATGTCGAgtttaagagctagaaatagcaag	66	CTCCTGCGTGTCCATGTCGA
P2RX5	HGLibB_34811	CTTGTGGAAAGGACGAAACACCGGCACCAGGCAAAGATCTCACgtttaagagctagaaatagcaag	66	GCACCAGGCAAAGATCTCAC
P2RX5	HGLibB_34812	CTTGTGGAAAGGACGAAACACCGACCCCTGCAGGAGTGAAGACgtttaagagctagaaatagcaag	66	ACCCCTGCAGGAGTGAAGAC
P2RX5	HGLibB_34813	CTTGTGGAAAGGACGAAACACCGTTTCAGGGAGCCATTCCTGAgtttaagagctagaaatagcaag	66	TTTCAGGGAGCCATTCCTGA
KRTAP3-3	HGLibB_25709	CTTGTGGAAAGGACGAAACACCGGGGGACACTGCAGCCTCGAGgtttaagagctagaaatagcaag	66	GGGGACACTGCAGCCTCGAG
KRTAP3-3	HGLibB_25710	CTTGTGGAAAGGACGAAACACCGGGATTTGTCAGAGGAGCAGAgtttaagagctagaaatagcaag	66	GGATTTGTCAGAGGAGCAGA
KRTAP3-3	HGLibB_25711	CTTGTGGAAAGGACGAAACACCGATGGATTGCTGTGCCTCTCGgtttaagagctagaaatagcaag	66	ATGGATTGCTGTGCCTCTCG

Supplementary Table 1: Primers used for MTH1 PCR
Priloga 3: Oligonukleotidni začetniki uporabljeni za MTH1 PCR

Exon	Left primer	Right primer	Product size
1	CAAACCCATTTACCCTGTCTG	GGACCAGTGATCCTTCTTGG	857
2	CCCACTGCCTCCCAGAG	CCTGCTCCAGGTCACTTAGC	375
3	CCCTGGGCTGTGTGTAGATG	CCCTGGCACTCAGAGATGG	345
4	GTCAGTGTACGTTTGGGCTG	TGTGCTGGTGTCTGGAGAAG	1197