Development and application of a patient-derived xenograft platform to test anti-cancer agents

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Gothenburg, Sweden, 2017
Cover illustration: Live imaging of a NOG mouse transplanted with a patient derived melanoma brain metastasis transduced with a luciferase expressing lentivirus.
Graphical work done by Siggeir F. Brynjólfsson

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http://hdl.handle.net/2077/51878

Printed in Gothenburg, Sweden 2017
By Ineko AB
This thesis is dedicated to
Krista and Styrmir

“Science seeks the truth, it does not discriminate. For better or worse it finds things out. Science is humble. It knows what it knows and it knows what it doesn’t know. It bases its conclusions on hard evidence — evidence that is constantly updated and upgraded. It doesn’t get offended when new facts come along. It embraces the body of knowledge. It doesn’t hold on to medieval practices because they are tradition.”
— Ricky Gervais
Abstract

Malignant melanoma is the most aggressive form of skin cancer and incidence rates are on the rise. Despite recent improvements in treatment options, the disease still remains lethal. Which calls for expedited solutions. In this thesis I will discuss three studies, which have not only contributed new knowledge to the research community but also led to development of novel tools used in cancer research.

In the first paper we developed a platform of patient-derived xenografts (PDXes) from metastatic melanoma patients. We show that PDXes can accurately predict clinical treatment responses and that the xenografts can be established in time to benefit the patients. Thus, the platform can be used for multiple pre-clinical and clinical purposes.

In the second paper we compared the transcriptome of cell line-derived xenografts (CDXes) and PDXes. The initial aim was to investigate if CDXes would be transcriptionally similar to PDXes and could therefore be used as in vitro surrogates for the PDXes. Instead, we identified a significant transcriptional difference between CDXes and PDXes, mainly explained by the pseudo hypoxia experienced by the cell lines once they are transplanted to the physiological environment.

In the third paper, we ran a pre-clinical trial in malignant melanoma PDX mouse models with the aim of identifying a predictive biomarker of the MTH1 inhibitor, Karonudib. By comparing the genomic and transcriptomic profiles of the responding and non-responding PDXes we identified that Karonudib has cytotoxic effect independent of those profiles. Also, we discovered that Karonudib causes cytotoxic effect beyond MTH1 inhibition.

Taken together, our data shows that PDX models predict clinical responses and can be used to test drugs pre-clinically, and argues that pre-clinical testing in PDX models is superior to cell line based drug testing.

Keywords
Malignant melanoma, patient-derived xenografts, cell line-derived xenografts, MTH1, Karonudib, pre-clinical research, clinical research, anti-cancer agents
Sammanfattning på svenska

Här brukar man skriva en populärvetenskaplig sammanfattning av avhandlingen; dess bakgrund, metoder och resultat. Helst inte mer än en sida.

This thesis is based on the following studies, referred to in the text by their Roman numerals.


Melanoma patient-derived xenografts accurately model the disease and develop fast enough to guide treatment decisions


II. Joydeep Bhadury, Berglind O. Einarsdottir, Agnieszka Podraza, Roger Olofsson Bagge, Ulrika Stierner, Lars Ny, Marcela Dávila López and Jonas A. Nilsson

Hypoxia-regulated gene expression explains differences between melanoma cell line-derived xenografts and patient-derived xenografts

*Oncotarget.* 2016 Apr 26;7(17):23801-11


The clinical MTH1 inhibitor TH1579 (Karonudib) has broad anti-melanoma effects in patient-derived xenografts

*Manuscript*
List of papers not included in the thesis


MTH1 inhibition eradicates cancer by preventing sanitation of the dNTP pool.


Validation and development of MTH1 inhibitors for treatment of cancer

*Annals of Oncology, 2016 27(12):2275-2283*
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Paper I

Histology, mutation status and expression profile is preserved when patient-derived tumor cells are grown as xenografts
PDXes can be used to predict treatment response
PDXes develop fast enough to guide treatment response

Paper II

Different transcriptome profile between CDXes and PDXes regardless of mutation status
Hypoxia-induced gene signatures characterize CDXes
Hypoxia response in cells cultured in 5% O₂
Reversing hypoxia-induced response using a miRNA decoy
miR210 inactivation reduces sensitivity to MEK inhibition in vivo

Paper III

Karonudib binds MTH1 in malignant melanoma cells
Karonudib has cytotoxic effect on melanoma cells in vitro
66% of PDX models respond to TH1579
TH1579 does not infer with T-cell mediated immunity
Cytotoxic effect of TH1579 is independent of driver mutations— but a potential inherent resistance could be identified
Synergistic effect of pgp-efflux pump inhibition and TH1579
TH1579 affects tubulin polymerization

Discussion

Paper I

Establishing PDXes and a bio bank for future usage
Low genomic, transcriptomic and histological drift in PDXes
PDXes predict clinical responses
PDXes can be established fast enough for clinical use

Paper II

Cell lines experience pseudo-hypoxia when grown in vivo
Genes involved in the hypoxia response
Inactivation of miR210 as a possible resistance mechanism

Paper III

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<tbody>
<tr>
<td>α-MSH</td>
<td>alpha-melanocyte stimulating hormone</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>BAD</td>
<td>BCL2 associated agonist of cell death</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<tr>
<td>CDX</td>
<td>cell line-derived xenograft</td>
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<td>CETSA</td>
<td>cellular thermal shift assay</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>FDA</td>
<td>The Food and Drug administration, USA</td>
</tr>
<tr>
<td>GEMM</td>
<td>genetically engineered mouse models</td>
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<td>GSEA</td>
<td>gene set enrichment analysis</td>
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<tr>
<td>HIF1</td>
<td>hypoxia inducible factor 1</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MCR1</td>
<td>melanocortin 1 receptor</td>
</tr>
<tr>
<td>MEK</td>
<td>mitogen-activated protein kinase kinase</td>
</tr>
<tr>
<td>MITF</td>
<td>microphthalmia-associated transcr. factor</td>
</tr>
<tr>
<td>MTH1</td>
<td>MuT homolog 1</td>
</tr>
<tr>
<td>mTOR</td>
<td>mechanistic target of rapamycin</td>
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<tr>
<td>MUTYH</td>
<td>mutY DNA glycosylase</td>
</tr>
<tr>
<td>NRF2</td>
<td>NF-E2 related factor 2</td>
</tr>
<tr>
<td>NUDT1</td>
<td>nudix hydrolase 1</td>
</tr>
<tr>
<td>OGG1</td>
<td>oxyguanine glycosylase</td>
</tr>
<tr>
<td>PDX</td>
<td>patient-derived xenograft</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
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<tr>
<td>UV</td>
<td>ultra violet radiation</td>
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Introduction

The cancer battle

Scientists have conducted cancer research for decades with the aim to cure patients of this complex yet fascinating disease. Our understanding of the multifaceted biology of the disease has escalated due to the hard work of dedicated researchers empowered by advances in biomedical technology. Due to the increased understanding, we now have multiple detection and treatment options extending the lives of patients and in some cases making cancer curable instead of deadly. But, despite recent breakthroughs, cancer is still one of the leading causes of mortality worldwide. Therefore, it is highly important to keep striving and work together to develop new tools and therapies to make cancer curable.

Skin cancer

Skin cancer, including both malignant melanoma (MM) and non-melanoma skin cancer (NMSC), is the most common malignancy in Caucasians. The most frequent form of NMSC is basal cell carcinoma (BCC), which develops from basal cells in

![Figure 1 Worldwide incidence rate of cutaneous malignant melanoma.](image)

World age-standardized rate (ASR) of melanoma of the skin, projected to 2012 and shown as per 100,000 habitants (1).
the epidermis and most often grows locally. Squamous cell skin cancer (SCC) is faster growing than BCC and originates from the keratinocytes in the epidermis. Other less common types of NMSC are Merkel cell carcinoma, Kaposi’s sarcoma and Bowen’s disease. Most cases of NMSC are easily treated and have good prognosis. On the other hand, malignant melanoma (MM) is the most aggressive and deadly form of skin cancer. MM originates from melanocytes and can thus form in any tissue containing melanocytes. The most common type is cutaneous malignant melanoma whereas other forms are uveal melanoma and mucosal melanoma (as reviewed in (2)).

Cutaneous malignant melanoma

Epidemiology

The incidence rate of cutaneous malignant melanoma has been rising for several decades, mainly in Caucasian populations, with the highest incidence rates in Australia, New Zealand, USA (Caucasians) and Northern Europe. The increase has been suggested to be due to ageing populations and better detection methods along with changes in sunbathing and tanning behaviour. Fortunately, there are signs of rates levelling off globally. (Figure 1) (1, 3).

In Sweden the incidence rates have been increasing for the last decades with no sign of levelling off. Between 1970 and 2014, the incidence rate increased from 6 to 40.33 per 100000 men and 7.85 to 38.60 per 100000 women. Mortality rate has not risen at the same pace but a slight increase has been registered (Figure 2) (4).

![Figure 2. Incidence and mortality rate of cutaneous malignant melanoma in Sweden.](image)

Age-standardized rate of incidence and mortality rate of melanoma of the skin in Sweden, shown as per 100000 habitants (4).
Etiology

Cutaneous malignant melanoma etiology is multifaceted and involves genetic, phenotypic, and environmental risk factors. Approximately 5-10% of malignant melanoma cases arise due to hereditary predisposition. Those subjects are usually diagnosed at a younger age but do not have a significantly different histology or survival (5). Amongst the most high risk hereditary melanoma genes are the cyclin-dependent kinase inhibitor 2A (CDKN2A), Telomerase reverse transcriptase (TERT) and Poly(ADP-Ribose) Polymerase 1 (PARP1) (6, 7).

Phenotypic risk factors include number of common and atypical naevi (8) and pigmentation traits like red or blond hair, blue or green eyes, and fair skin with low tanning ability (9). Pigmentation traits are determined by multiple genetic variants, for example MC1R and tyrosine (TYR). The most prominent and best studied environmental risk factor for malignant melanoma is sun exposure, where intermittent exposure poses particularly high risk (10). Fortunately, it is also well studied that the use of sunscreen can protect the skin from the damaging ultra violet radiation (11-13).

Subtypes and clinical classification

Cutaneous malignant melanoma can be divided in four basic categories. Superficial spreading melanoma (SSM) which is the most common type, lentigo maligna which is often found on chronically sun exposed areas of the body, and acral lentiginous melanoma which is usually found under nails, on palms or soles of the feet. These three subtypes can grow dermally for a long time before penetrating the deeper layers of the skin, but acral lentiginous melanoma can advance more quickly. The fourth is nodular melanoma (NM) which is the most aggressive subtype and often found on the trunk, legs, or arms (14).

A universal staging system is important to provide the most accurate diagnosis, which can be used to predict prognosis and treatment strategy. The American Joint Commission on Cancer (AJCC) published a staging system where the thickness of the tumor (Breslow’s depth), appearance of ulceration (not intact epidermis on top of tumor), and mitotic rate are posed as the most important factors when assessing the early stages of the disease (15). Melanomas are also divided in stages depending on how far the tumor cells have spread. Stage 0 tumors are non-invasive and superficial, stage I tumors have invaded the skin but to a low degree and are slow growing, stage II tumors are localized, larger (generally > 1mm thick) and may be ulcerated with high mitotic rate. Stage III tumors have spread to the lymph nodes and stage IV to distant organs (15).
The skin

Development of the skin

The skin is the largest human organ and serves as a barrier between vital organs and harmful factors from the environment. Important functions of the skin are thermo-regulation, restricting water loss, initiate an immune response, production of vitamin D and protection against ultraviolet (UV) radiation. The skin can be divided into three major layers, which are derived from different germ layers, the dermis, epidermis, and basement membrane. The dermis, which is derived from the mesoderm is the deepest layer of the skin and consists of epithelial tissue containing for example hair follicles, sweat glands, lymphatic tissue and blood vessels. The epidermis, which is derived from the ectoderm is the most exterior layer and is mostly composed of keratinocytes. Keratinocytes grow out from the dermal-epidermal junction as basal keratinocytes, as more basal keratinocytes are produced they are pushed towards the skin surface where they become terminally differentiated keratinocytes called corneocytes (16). At the junction of the epidermis and the dermis lies the basement membrane, a thin fibrous tissue anchoring down the epidermis to the loose epithelial tissue of the dermis. In the basement membrane, the pigment producing melanocytes are found, which are derived from the neural crest (17).

Melanocytes

Melanocytes are responsible for the production of melanin, the pigment that protects the body against UV radiation, and is one of the factors determining the color of the skin. During embryonic development, the melanocyte precursors, called melanoblasts, migrate in a tightly regulated manner from the neural crest to the skin, hair follicles, iris of the eye and to the inner ear of the human body (18). Melanocytes of the skin are found in the basement membrane where they are surrounded by keratinocytes. These two cell types, derived from different germ layers, have evolved a well orchestrated process to produce melanin, called melanogenesis.
Melanogenesis

Melanogenesis occurs when the skin is exposed to UV radiation, DNA damage occurs in the keratinocytes causing them to produce pro-opiomelanocortin (POMC). POMC is cleaved to produce hormones that are released into the blood stream having analgesic properties along with immune modulating effects, for example β-endorphin and α-melanocyte stimulating hormone (α-MSH). α-MSH is secreted by the keratinocytes stimulating the MC1 receptor (MC1R) on the neighboring melanocytes causing increased synthesis of cyclic adenosine monophosphate (cAMP). The cAMP binding protein (CREB) mediates up regulation of the microphthalmia transcription factor (MITF) leading to pigment production in melanosomes (20, 21). UV radiation also affects melanocytes directly to produce melanin but in a cAMP independent way (22). Melanosomes containing melanin are subsequently transferred to nearby keratinocytes protecting them from further DNA damage caused by the UV radiation (Figure 3).

Two types of melanin can be found in the human skin and hair follicle, eumelanin and pheomelanin. Eumelanin has pigments ranging from black to brown and pheomelanin has pigments ranging from yellow to reddish-brown. Both are derived from a tyrosinase-dependent pathway, giving rise to dopaquinone. From that step the eumelanin and pheomelanin productions diverge. Pheomelanin is derived from the conjugation of thiol-containing cysteine or glutathione and therefore...
more photolabile. Hence, when exposed to sunlight, pheomelanin produces hydrogen peroxide and superoxide, triggering oxidative stress and increased DNA damage (as reviewed in (23)). Eumelanin is produced when dopaquinone is converted to dopachrome, a precursor of 5,6-dihydroxyindole (DHI). Polymerization of DHI subsequently forms eumelanin (as reviewed in (24)).

**Ultraviolet radiation induced DNA damage**

Despite the UV protection provided by melanin, cells can still be damaged by too much exposure. UV radiation can be divided into three classes: UVA, UVB, and UVC. UVA has the longest wavelength (320-400 nm) and penetrates through the epidermis and down to the dermis. UVB has shorter wavelength (280-320 nm) and is absorbed in the epidermis. UVC has the shortest wavelength (100-280 nm) which is not sufficient to penetrate the atmospheric ozone layer. UVA and UVB have different effects on the human skin. UVB can cause direct DNA damage through, for example, formation of cyclobutane pyrimidine dimers (CPD) and pyrimidine-(6-4)pyrimidone photoproducts (64PPs) (25). Whereas, UVA can cause both direct and indirect DNA damage by increasing the level of reactive oxygen species (ROS). ROS can damage DNA directly by producing oxidative bases for example 8-hydroxyguanine (8OH-G). ROS can also damage the nucleotide pool, producing oxidized nucleotides 8-hydroxy-deoxyguanosine-triphosphate (8OH-dGTP) which can be incorporated into the DNA of a proliferating cell. Melanocytes have developed repair mechanisms responding to those mutations were the DNA excision repair pathways play an important role. But, when those pathways fail the cells can turn malignant resulting in the formation of melanoma.

**Biology of melanoma**

**The MAPK pathway**

The mitogen activated protein kinase (MAPK) pathway transfers signals from the cell surface to the nucleus via receptor tyrosine kinases (RTKs). The RTKs include the epidermal growth factor receptor (EGFR), KIT proto-oncogene receptor tyrosine kinase (c-KIT), platelet-derived growth factor receptor (PDGF-R), vascular endothelial growth factor receptor (VEGF-R), fibroblast growth factor receptor (FGFR) and fms-related tyrosine kinase-3 (FLT-3).
Once RTKs are activated upon ligand binding, they activate RAS family members. The RAS family members are comprised of NRAS, KRAS and HRAS. Normally, RAS switches between its active GTP-bound state and inactive GDP-bound state, which is controlled by GTPase activating proteins (GAPs) and nucleotide exchange factors (GEFs). GAPs (e.g. neurofibromin 1 (NF1)) stimulate the intrinsic GTPase of RAS keeping it inactive and in the cytosol while GEFs stimulate the exchange of GDP for GTP in RAS so it becomes active and can stimulate its downstream target RAF. NRAS was the first oncogene to be identified in melanoma and out of the three is the most commonly mutated (15-30%) (26, 27). The most common mutation found in NRAS is the activating Q61R substitution (27).

The human RAF protein family is comprised of BRAF, CRAF and ARAF. BRAF is the most frequently mutated gene in melanoma and is found in around 50%-70% of all cases (27). The most common BRAF mutation is the V600E activating mutation, having 10-fold higher kinase activity than the wild type (28). Activated RAF in turn phosphorylates and activates MEK1/2, which stimulates ERK1/2 to translocate to the nucleus leading to expression of genes involved in proliferation and differentiation (as reviewed in (29, 30)) (Figure 4).

The phosphoinositide 3-kinase pathway simulates cell survival, cell motility and growth and has been shown to be active in melanoma. Phosphoinositide 3-kinase (PI3K) is a lipid kinase, which becomes activated through RTKs or NRAS. PI3K phosphorylates the phosphatidylinositols in the plasma membrane leading to re-

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**Figure 4. MAPK pathway and PI3K pathway**

**PI3K pathway**

The phosphoinositide 3-kinase pathway simulates cell survival, cell motility and growth and has been shown to be active in melanoma. Phosphoinositide 3-kinase (PI3K) is a lipid kinase, which becomes activated through RTKs or NRAS. PI3K phosphorylates the phosphatidylinositols in the plasma membrane leading to re-
recruitment and activation of the protein Ser/Thr-kinase, AKT. The tumor suppressor PTEN is a phosphatase that does the opposite reaction. Downstream targets of AKT are amongst others, inhibition of GSK3β leading to stabilization of MYC, cyclin D phosphorylation leading to cell cycle entry, BAD phosphorylation leading to activation of Bcl-2 and cell survival, mTOR activation leading to translation and growth (31). Alterations of the members of the PIK3 pathway have been found in malignant melanoma cases. AKT has been found overexpressed in up to 40% of cases and deletion of PTEN has been found in ~20% (as reviewed in (32)).

Cell cycle regulation

Cell cycle regulation is tightly regulated in melanocytes, restricting their proliferation potential. Therefore, does dysregulation of the cell cycle promote melanomagenesis and is considered one of the hallmarks of cancer (33). The main players are the cyclins and their associate cyclin-dependent kinases (CDKs) that regulate transitions through the cell cycle. When CDKs bind cyclins they become an active complex and can promote cell cycle progression. CDK4/6 are central regulators of G1 to S transition and are activated by binding to cyclin D, which allows them to phosphorylate the tumor suppressor retinoblastoma (RB). Phosphorylated RB dissociates from the transcription factor E2F, allowing transcription of genes involved in G1 to S transition. CDKN2A encodes for p16INK4A, a tumor suppressor that binds CDK4/6 and prevents its interaction with cyclin D. Loss of function of CDKN2A due to deletion, mutation or methylation is observed in up to 60-70% of melanomas and has been found mutated in up to 25% of melanoma prone families (27, 34). In late G1 phase, after the restriction point (R), CDK2 binds to cyclin E enabling the cell to enter S phase where the DNA is replicated.

Figure 5. Pairing of cyclins with cyclin-dependent kinases
Once cells are in S phase, CDK2 switches cyclins and binds cyclin A allowing progression through the phase. Late in S phase, cyclin A switches CDKs and binds CDK1 and enters the G2 phase where the cell prepares to divide by synthesizing necessary proteins and by growing. Later in the G2 phase, CDK1 switches cyclins and binds cyclin B allowing the cell to go through (as reviewed in (35))(Figure 5).

**Hypoxia**

Hypoxia is often found within tumors and is linked to poor clinical outcome, therapy resistance and metastatic disease. The rapid expansion of tumor cells relies on constant supply of nutrients and oxygen to be delivered to them by the vascular system. In some cases the neovascularization cannot keep up with the rapid tumor growth leading to limited oxygen supply and thus hypoxia. In hypoxic conditions, the transcription factor, hypoxia inducible factor 1 (HIF1) is highly active, transcribing genes enabling the cell to survive in the new environment. HIF1 is a heterodimer composed of an alpha and a beta subunit (HIF-1α and HIF-1β) and the level of available HIF-1α controls its activation. In normoxia, HIF-1α is oxidized by the proline hydroxylase (PHD) allowing the tumor suppressor von Hippel-Lindau (pVHL) to bind to it, leading to poly-ubiquitylation and degradation in the proteasome, thereby disabling HIF1. PHD activity is dependent on available oxygen. Thus, in hypoxia, PHD does not oxidize HIF-1α and thereby causes restriction of the binding potential of pVHL to HIF-1α. Hence leaving HIF-1α available to form heterodimer with HIF-1β and active HIF1. HIF1 transcribes genes involved in increasing neovascularization (vascular endothelial growth factor (VEGF)), increasing erythropoiesis platelet-derived growth factor (PDGF), glycolysis, and glucose transport (as reviewed in (36)). One of the targets of HIF1 is the miR-210 host gene (mir-210HG). miR-210HG, often called the “hypoxia master regulator”, is a non-protein coding transcript expressing the mature miR-210 intronically when grown under hypoxia (37-39). miR-210 regulates multiple target genes involved in both hypoxia and normoxia and its expression has been linked to metastasis of both breast and melanoma tumors (40).
Oxidative stress

Melanocytes transform into melanoma cells through abnormal metabolic and signalling pathways causing imbalance in the redox homeostasis, leading to accumulation of oxidative stress. It becomes fundamental for them to regulate the redox homeostasis in order to survive. The cellular redox homeostasis is regulated by the constant balancing of reactive oxygen species (ROS) inducers and scavengers (Figure 6). The main reactive oxygen species are; superoxide anions (O$_2^-$), hydroxyl radicals (OH$^-$) and hydrogen peroxide (H$_2$O$_2$).

Intracellular ROS can be increased due to the high metabolic activity and uncontrolled proliferation of tumor cells, which require high amounts of ATP. ATP synthesis takes place in the respiratory chain on the mitochondria membrane and its by-products are superoxide anions (O$_2^-$) and hydroxyl radicals (OH$^-$). Hypoxia has
also been shown to increase the level of ROS by stimulating the respiratory chain of the mitochondria.

Tumor cells activate numerous pathways to maintain the redox homeostasis. One of the most central antioxidant regulator is the transcription factor nuclear factor erythroid 2-related factor 2 (NRF2). Under normal redox conditions, NRF2 is constantly degraded by Kelch-like ECH-associated protein 1 (KEAP1). But, once oxidative stress accumulates within the cells, KEAP1 is oxidized affecting its ability to bind NRF2. Unbound NRF2 is stabilized and translocates to the nucleus where it transcribes genes involved in producing glutathione (GSH). Even though it is commonly stated that ROS can be increased under oncogenic stress, there are studies showing that oncogenic KRAS and MYC can stabilize NRF2 (42). GSH is the most essential non-enzymatic antioxidant and exists both in reduced (GSH) and oxidized state (GSSH). Hence, alterations in the GSH synthesis are frequent in tumor cells. Tumor suppressors such as, forkhead box O (FOXO), retinoblastoma (RB), breast cancer susceptibility 1 (BRCA1) and TP53 have also been associated with antioxidant effects. Finally, dietary intake can affect the redox homeostasis, where selenium, beta-carotene, vitamin C and E are antioxidants that have been shown to affect the cellular redox system.

Antioxidants are often promoted as healthy dietary supplements and used by some cancer patient as an attempt to control the disease. But clinical trials testing the preventive effect of antioxidants have shown conflicting results, where some trials have shown increased cancer risk with antioxidant consumption (43, 44). Experimental studies using mouse models have also shown conflicting results, where antioxidants have been shown to either, reduce tumor initiation/burden or have tumor progressive effects (45-49). This inconsistency reveals the complexity of studying the redox homeostasis in cancer. Furthermore, it points towards difference in health benefit of antioxidants in tumor free individuals versus those that have already developed tumors.

DNA damage caused by ROS

If the redox system fails, oxidative damage can occur causing detrimental damage to lipids, proteins, and DNA. Oxidative damage to DNA can occur directly or indirectly. Direct DNA damage occurs when oxygen group is added on guanine, forming 8-oxoguanine (8-oxoG)(50). If the damage goes undetected, the 8-oxoG can accumulate in the genome causing mutations or single strand break (SSB). Indirect DNA damage occurs when free nucleotides (dNTPs) in the nucleotide pool are oxidized. Both dATP and dGTP are commonly found oxidized, forming 2-hydroxy-dATP (2-OH-dATP) and 8-hydroxy-dGTP (8-oxo-dGTP). When this
damage goes undetected, the oxidized nucleotides can be incorporated into the replicating DNA. Normally, cytosine (C) is incorporated against guanine (G) when the DNA is replicated. But, when guanine is oxidized, adenine (A) is incorporated forming a Hoogsteen–type base pair (8-oxoG/A) causing G>T transition (51, 52).

Cells have evolved a repair mechanism to counteract the DNA damage and thus protect the genetic material. The human DNA glycosylase OGG1 detects 8-oxoG and starts a highly evolutionary conserved DNA damage response called, base excision repair (BER) (53). Human OGG1 is found in two different isoforms, OGG1-1a containing a nuclear localization signal (NLS) and thus found in the nucleus, and OGG1-2a found in the inner membrane of the mitochondria (54). Once OGG1 recognizes the 8-oxoG, it hydrolyzes the N-glycosidic bond between the sugar moiety and the oxidized base to remove it, leaving abasic site called an apurinic/apyrimidinic (AP) site. Next, AP-endonuclease (APE1), DNA polymerase and DNA ligase process the AP site to insert guanine (G) and repair the DNA (as reviewed in (55)). If the 8-oxoG goes undetected and the DNA is replicated, adenine (A) will be inserted against the 8-oxoG. A human DNA glycosylase encoded by MUTYH, recognises the wrongly inserted A and excises it to replace it with cytosine (C) (56). To prevent the incorporation of 8-oxo-dGTP and 2-OH-dATP into the replicating DNA, the 7,8 dihydro-8-oxoguanine triphosphatase MTH1 hydrolyses the oxidized nucleotides (oxo-dNTPs) to their monophosphate form (oxo-dNMPs) (57-59) (Figure 7).

![Figure 7. Mutagenesis caused by 8-oxoguanine and defence system.](image)

Figure adapted and modified from Nakabeppu et al. (60).
MTH1

The human MutT homolog 1 (MTH1) is encoded by the nudix hydrolase family member, NUDT1 and was first identified and given its name by a Japanese research group in the year 1994 (57). The nudix hydrolase family is comprised of a variety of genes known to encode for proteins hydrolyzing a wide range of pyrophosphates and are found in all classes of organisms (61). MTH1 is dispensable during mouse development and Nudt1−/− mice appear physically normal and live for as long as the wild type mice. However, at the age of 18 months there is significantly more spontaneous tumor development in the Nudt1 knockouts compared to the wild type with a 2 fold higher mutation rate in the knockouts (62). Furthermore, MTH1 is overexpressed in many tumor types and suggested as a marker of oxidative stress (63-65). Therefore, it has been suggested that once cells become malignant they experience higher oxidative stress and therefore become dependent on MTH1 to prevent DNA damage caused by the oxidized nucleotides (66, 67).

MTH1 as a therapeutic target

Due to these observations, MTH1 has become an interesting anti-cancer target and in the year 2014 two papers (Gad et al. and Huber et al.) were published in Nature describing the first-in-class MTH1 inhibitors, TH588 and (S)-crizotinib, respectively (66, 68). Briefly, Gad et al, describe how MTH1 is required for cancer cell survival by depleting it using short interfering RNAs (siRNAs) causing DNA damage and reduced survival in U2OS cells. These effects could be rescued by expression of an RNAi resistant wild type MTH1, but not with a catalytically dead MTH1 protein. Furthermore, it was shown that MTH1 depletion using siRNA caused double stranded break (DSB), activating RAD51, phosphorylating DNA-PKcs and increased cleaved caspase 3 along with ATM dependent phosphorylation of p53 (S15) and up-regulation of p21. Thus, the MTH1 inhibitor TH588 was developed and shown to have cytotoxic effect on cancer cell lines while less toxic to primary/immortalized cells. The compound was also shown by us to have cytotoxic effect on a patient-derived xenograft from a multi resistant malignant melanoma patient (66).

Since TH588 was disclosed, others have published studies testing the inhibitor. In these studies, MTH1 as a target is questioned and an off-target suggested contributing to the cytotoxicity. Kettle et al. show how MTH1 knock down or inhibition using their MTH1 inhibitor does not have cytotoxic effect on U2OS cells. Furthermore they show how TH588, in consistence with Gad et al., activates DNA damage response but their MTH1 inhibitor does not. They also show how TH588 has cytotoxic effect on cells in which MTH1 had been knocked down (69). In an-
other study, published by Kawamura et al., a proteomic profiling on the cytotoxic effect of several different MTH1 inhibitors was performed. They observed that TH287 (chemically similar to TH588 (66)) clustered with known microtubule/tubulin targeting agents. Also, they show how TH287 and TH588 in concentrations over 30 µM inhibit the polymerization of microtubules in an in vitro assay (70). Furthermore, Wang et al. published a study where they found TH588 to be cytotoxic in melanoma, but did not find the sensitivity of the cells to be associated with MTH1 expression. Rather they observed the response to be dictated by the level of ROS. Also, they did not find that knock down of MTH1 affected the survival of melanoma cells (71). Recently, a lead-optimized form of TH588 was disclosed by Warpman-Berglund et al., called TH1579. In the study, multiple MTH1 inhibitors were tested (including the ones from Kettle et al.) but only TH588 and TH1579 were shown to increase the level of incorporated 8-oxoG into the DNA of the treated cells. Therefore it is argued that other MTH1 inhibitors do not have cytotoxic effect due to their failure to inhibit the hydrolysing potential of MTH1 (72).

The reason for this inconsistency remains to be explained, but it should be kept in mind that the experimental conditions in those studies were not the same. Nonetheless, it is clear that further research is needed. Both, it needs to be investigated whether MTH1 is a sufficient target to induce cancer cell death and if any possible off-targets of TH588/TH1579 are found. However, what cannot be argued is the high cytotoxic potential of TH1579 on various cancer cells and potent effect on patient-derived xenografts with no obvious side effects on the mice. This will be discussed further in this thesis.

**Malignant melanoma treatment**

**Current treatment options**

The first recorded successful operation performed on a melanoma patient was in the 1780s. Since then and with constant improvements, operation has been one of the main treatment options for malignant melanoma patients (73). The first FDA approved chemotherapy for malignant melanoma, Dacarbazine, was approved in the year 1975 (74). Today it is the only (along with its analog temozolamide) approved chemotherapy since others have failed to show additional clinical benefit. It works by adding a methylation group on guanine causing the DNA to stick together, making DNA replication impossible (74, 75). In the year 1998 the FDA approved a new treatment for malignant melanoma. After extensive research on IL-2
and its induced T-cell mediated tumor cytotoxicity, high-dose IL-2 was approved for clinical use showing good and sometimes durable responses (76).

After the completion of the Human Genome Project several significant melanoma mutations, such as BRAF and NRAS, were identified by targeted re-sequencing, enabling the development of targeted therapy. The first targeted therapy for malignant melanoma patients was the BRAF inhibitor vemurafenib, which was approved by the FDA in the year 2011 (77). Few years later, a new BRAF inhibitor, dabrafenib, and the MEK inhibitor trametinib were also approved as single and combination treatment (78-80). Malignant melanoma has long been considered a disease that could benefit from immunotherapy and today there are several approved immunotherapies available. In the year 2011, the checkpoint inhibitor ipilimumab, which targets the immune response inhibitor CTLA-4 and thereby activates the cytotoxic effect of T cells, gained FDA approval (81-83). Two more checkpoint inhibitors have received FDA approval, pembrolizumab and nivolumab, which are both PD-1 inhibitors used as single or combination treatment (84-87).

**Resistance**

Most malignant melanoma patients respond to the treatment they are assigned to, at least initially. Often, the tumors adjust to the new environment created by the treatment and develop resistance, either intrinsic or acquired. Resistance to chemotherapy has been reported in melanoma. The main resistance mechanisms being, increased expression of glutathione-S-transferase (GSTs), increased expression of the DNA repair enzyme O6-alkylguanin DNA alkyltransferase (AGT), activating RAS mutation, dysregulation of apoptosis activation and expression of drug efflux pumps (multi drug resistance proteins (MDRs). The main MDRs being the p-gp pump encoded by the \( \text{ABCB1} \) gene and the breast cancer resistance protein BCRP encoded by the \( \text{ABCG2} \) gene (as reviewed in (88)).

Resistance to BRAF mutated targeted therapy has also been a recurrent clinical problem. Several different resistance mechanisms have been identified, the most frequent being reactivation of the MAPK pathway. Increased expression of growth factors and RTKs can lead to pathway reactivation causing resistance (89-92). Amplification of mutant BRAF or mutations in NRAS or MEK can also cause resistance (89, 93), along with activation of other RAF kinases (94), and up regulation of COT (95). To try to overcome the reactivation of the MAPK pathway when treating with a BRAF inhibitor, double treatment with BRAF (dabrafenib) and MEK (trametinib) inhibitors has been approved by the FDA (96). The double treatment was shown to increase progression-free survival, showing better responses and less toxicity than when treated with the BRAF inhibitor dabrafenib alone.
Resistance to immunotherapy has also been observed. Resistance to checkpoint inhibitors and adaptive T cell transfer (ACT) can be due to lack of recognition by T cells due to absence of tumor antigens (98). Also, cancer cells can develop mechanisms to avoid antigen presentation on the surface by not expressing the MHC complex (99). Multiple mechanisms can drive resistance to immunotherapy, both tumor-cell-intrinsic and tumor-cell-extrinsic factors (as reviewed in (100)).

The cycle of cancer research – from bench to bedside – and back again

Cancer research is a multidisciplinary field spanning from basic research to pre-clinical research to clinical research. What defines these different terms is decided by aim of the research. Basic research often aims at increasing the general knowledge of a certain subject and understanding its nature, without the obligation of applying it to practical ends. Pre-clinical research often has the aim of evaluating potential therapeutic compounds in vitro or in vivo where effectiveness of a compound can be tested in a cohort of patient samples without tracking results back to each individual patient. Clinical research is more of a patient-oriented research were the research is conducted with a human sample and is traceable back to the same individual, for example studying the mechanism of a disease and clinical trials. Those fields are in most cases integrated and knowledge transferred multidirectionally, or translated from the bench to bedside and back in a process called translational research. Translational research covers all of the above terms and requires interaction of several disciplines to translate knowledge from one field to another, with the long-term aim of developing novel concepts and approaches to address important health issues (as reviewed in (101)).

Cancer research tools

Cell lines

Commercially available cell lines have historically played an essential role in the advancement of cancer research and drug discovery (as reviewed in (102)). They can be established from biopsies of animals or patients and continuously cultured in controlled environment making in vitro testing and screening fast and efficient. Furthermore, they can be distributed between researchers to standardize certain procedures and give investigators a chance to compare findings. Thus, they have been in
many cases the source of important research findings, which have furthered biological knowledge and clinical improvements (103, 104). Moreover, their value in functional studies is undisputable. However, they have their limitations as most other research models. The fact that they are continuously cultured in artificial environment has selective pressure on them, encouraging genomic, transcriptomic and metabolomics adaptation. In general, cells are cultured in 5% CO₂, 37 °C and ambient oxygen level (21%). The cells are most often cultured on plastic plates or flasks which they often become attached to which encourages a positive selection of attached cells while other can be washed away. Also, they need to be cultured in cell culture medium, which usually contains high-level glucose, growth factors and nutrients necessary for the cells to survive. Due to those factors, the gene ontology of cultured cells has been shown to diverse from their original tumor biopsy (105-107). Therefore, to circumvent the artificial culture environment, cell lines have been used to establish cell line derived xenografts (CDXes) with the aim of mimicking tumor growth in patients in a pre-clinical setting, with both convincing (108) and unconvincing results (109).

**Genetically engineered mouse models**

Multiple mouse models exist as tools in malignant melanoma research. Genetically engineered models (GEMMs) are widely used in both basic and pre-clinical research with good success (as reviewed in (110)). GEMMs can resemble tumorigenesis in many aspects since the tumors form spontaneously in immune-proficient microenvironment allowing the tumor cells to progress and metastasise. On the other hand, they often do not mimic the inter-patient heterogeneity. Therefore, they are likely better suited as a tool to investigate cancer formation and in proof-of-concept studies on the mechanism of action of anti-cancer agents.

**Patient-derived xenograft mouse models**

In pre-clinical and clinical research, where the aim is to predict a patient’s response to treatment or to take into consideration the inter-patient heterogeneity, tumor samples from patient can be tested as patient-derived xenografts (PDXes). Transplantation of human tumor tissue in host animals has been performed for decades. In the early days, before the availability of immunocompromised mice, the tissue was transplanted in immunologically privileged sites, with less chance of rejection. Also, transplantations were performed on animals that had undergone thymectomy or radiation, impairing the immune system. However, those methods were considered unpractical due to the fact that the animals were often fragile and
Immunosuppression often did not last more the 5-6 weeks. The biggest advance in developing patient-derived xenografts came with the use of athymic mice, which are inbred hairless mice exhibiting thymic aplasia, causing defects in T cell formation. The impaired immune system fails to recognize the human tissue as foreign and thus does not reject it (111, 112).

Today, there are several different immunocompromised mouse strains available. The Nude mice which have no functioning T cells due to the Foxn1null genotype, but have functioning B and NK cells. The non-obese diabetic/severe combined immunodeficiency (NOD-SCID) mice have a homozygous mutation in the Prkdcscid mutation causing non-functioning T and B cells, the mice also have impaired NK cells. The NOG/NSG (NOD/SCID/IL2Rγ-null) mice have in addition to the Prkdcscid mutation a knockout of the IL-2 γ chain receptor causing non-functional T and B cells, also they have non-functional NK cells.

For PDXes to be successfully used, it is important that the xenografts do not diverge from the patient biopsy. The high similarity of the PDXes to the original patient tumor has been verified for many cancer types (113-116). PDXes from malignant melanoma patients have been established successfully, and shown to maintain their histology and genomic profile (9, 117). Studies have shown a correlation between clinical responses and the response of the matched PDX to the same treatment (118, 119). Also, successful xenograft growth has been associated with worse clinical outcome in patients (120-122). Furthermore, it has been shown that the rate of spontaneously forming metastasis in subcutaneously transplanted NSG mice correlate with clinical outcome in the patient (120). Because of those factors, PDXes have been used successfully in a pre-clinical trial to screen for a predictive biomarker to Cetuximab in a cohort of colorectal PDXes (123). The pre-clinical trial was then developed even further to a one mouse per patient per treatment (1+1+1) setting to enable screening through multiple patient samples and treatments (124).

Despite the advances in cancer research due to the accuracy of PDX mouse models, they have their limitations. The biggest limitation of those models is the lack of a fully functional immune system in the recipient mice. Thus, hampering the use of them in testing anti-cancer immune therapy and vaccines. Additionally, the effect of anti-cancer agents on the immune system cannot be observed. Furthermore, there is difference in the microenvironment since the tumor cells come into contact with mouse stromal cells as PDXes instead of the autologous stroma they grew in originally.
Clinical trials

Clinical trials are the last step to bring new treatments to the clinic where it can serve the end users, the patients. The drawback is that most drugs fail in early phases of clinical trials, leading to only around 10-15% clinical approval success rate for cancer agents (125, 126). The main reasons for failure is insufficient efficacy and safety (126). This suggests that there is an insufficient predictive value of the pre-clinical models used today, and their failure to mimic the heterogeneity of cancer. Therefore, to increase the chance of a cancer drug to reach the clinic, it is important to recruit the right patient group for the early stages of testing. Not all drugs, entering clinical trials, have a known predictive biomarker. For these compounds, a pre-clinical step can be included where a predictive biomarker is screened for and used as inclusion criteria. That way, biomarker negative patients can be spared the enrolment and more biomarker positive patients given a chance to benefit from the treatment. This extra step comes at the cost of longer pre-clinical testing period and adds to the complexity of patient recruitment.
Aims

The aims of the papers included in this thesis are:

• Paper I: To develop a platform where tumor samples derived from malignant melanoma patients can be used to test anti-cancer agents

• Paper II: To compare the transcriptome and the applicability of xenografts derived from commercial cell lines or from patients

• Paper III: To pre-clinically test the anti-cancer drug Karonudib in a large cohort of patient-derived xenografts to investigate inter-patient responses and screen for a predictive biomarker
Methodology

Ethical permissions

All human tumor samples were handled and collected from consenting patients according to ethical approval (Regional Human Ethics Board of Västra Götaland, Sweden, #288-12). All animal studies were performed according to E.U. directive 2010/63 (regional animal ethics committee of Gothenburg approval #287/289-12 and #36-2014).

Establishing patient-derived xenografts
-from single cell suspension

Once a tumor material has been excised from the patient, it is best to keep it in a sterile tube on wet ice until it is processed. The tumor material should be processed in a clean environment using sterile tools. Start by cutting away all non-tumor tissue (necrotic, fat, etc.). If material for later analysis is needed, cut away pieces and snap freeze and/or formalin fix. Chop up the remaining tumor piece using tissue chopper or scalpel, suspend the tumor cells in culture medium and filter through a 70 uM cell strainer (use a plunger from a syringe to press the minced tissue through the strainer). Spin down the cells and aspirate the culture medium. If live cells are needed for later use, take part of the cells and freeze down for cryo-preservation in 50% FBS, 40% RPMI-1640 and 10% DMSO. Next, cells can be counted if certain cell number is preferred for injection. Mix the tumor cells intended for the transplantation in equal ratio of RPMI-1640 and Matrigel and transplant 100 µL subcutaneously into the flank of the mouse. Mix an extra volume of 100-200 µl, due to dead volume of syringe and volume lost in tube. This is particularly important when multiple mice are being transplanted, to make sure there is enough volume to equally transplant all the mice. It is good to shave the fur at the transplantation location before transplanting, as it makes tumor growth observation easier. Once tumors are visible/palpable, their growth can be monitored using a caliper or in vivo imaging if the cells have been transduced with fluorescent or luminescent markers. It is advisable to observe growth of the tumors for at least two consecutive measurements before starting treating the mice with compounds, to make sure the tumors are in an active growth phase.
RNA extraction

Tumor pieces were snap frozen once excised and kept in -80°C until analysed. The frozen pieces were homogenized in lysis buffer using a Bullet Blender® Homogenizer and RNA extracted using the Nucleospin® RNA extraction kit (Macherey-Nagel) according to manufacturer’s protocol.

RNA sequencing

Quality control was performed using Agilent 2100 Bioanalyzer and samples with RNA integrity number (RIN) values ≥ 8 submitted for RNA sequencing. RNA sequencing for papers I and II was performed at BGI China and for paper III at NGI-SciLifeLab, Sweden. The library was prepared using poly-A-enrichment and sequenced using pair-end sequencing. Raw reads were aligned to the human assembly hg19. Bioinformatics analysis can be seen in materials and methods for each of the papers.

CETSA

Cellular thermal shift assay (CETSA) on intact cells was performed by treating cells with DMSO, 5 µM TH1579 or 5 µM TH1827 for two hours before harvesting them, washing and re-suspending in PBS. Cells were divided in PCR tubes (1x10^6 cells per tube) and heated at temperatures ranging from 37°C to 67°C for 3 minutes and thereafter snap frozen. Proteins will denature and aggregate under their intrinsic melting temperature causing a gradual disappearance of the soluble proteins with increasing temperatures. Insoluble proteins do not denature and will therefore not be part of the analysis. Samples were analysed by liquid chromatography-mass spectrometry (LC-MS/MS) or western blotting. For LC-MS/MS, samples were lysed by freeze-thaw cycles, labelled and analysed. A fit curve was made from the intensity of the measured peptides to calculate the melting temperature (Tm) for each protein to identify differences in the melt curves indicating ligand binding. LC-MS/MS measurement and data analysis was performed at the Proteomics core facility at the University of Gothenburg. For western blot analysis, samples were lysed by freeze thawing and prepared for gel electrophoresis to identify stability of proteins across the different temperatures. CETSA was also performed on cell lysate and analysed using western blotting. The cells were lysed before adding DMSO or drugs and samples prepared and analysed in the same way as the intact cells.
Cell culture

All cell lines were cultured in 37°C, 5% CO₂, and 5% or 20% oxygen. Cell culture medium was supplemented with 10% fetal bovine serum and antibiotics (Gentamycin).

Virus production

Lentiviruses were produced by transfecting HEK293T cells using calcium phosphate precipitation. Briefly, plasmids needed for the virus production were mixed in buffered water and 2.5M calcium chloride (CaCl₂) added to the mix. The plasmid-CaCl₂ mix was added in 2x Hepes buffered salin while vortexing and the mix kept at room temperature for 20 minutes while the precipitates form. Next, the mix as added dropwise on top of the HEK293T cells. After 15-20 minutes of incubation, plate was moved under the microscope to check for precipitates, which are a positive sign of successful transfection. Twenty-four hours after transfection, medium was changed to the same medium as used for the target cells. The day after, virus was harvested three times and kept on ice over night until the last virus was harvested. All harvested virus was pooled and filtered through 0.45 µm filter and frozen (-80°C) down in aliquots. Details about each plasmid used can be found in the materials and methods for the respective paper.

Airyscanning

Cells transduced with EB3-GFP were used to image the polymerization of microtubules by airyscanning. Airyscanning is based on confocal laser scanning microscopy with improved signal-to-noise ratio enabling high resolution imaging. Cells were 3D scanned in ten consecutive cycles. Each scan was compressed from 3D to 2D image making ten 2D images. Each 2D image was color coded (one color per image) and all ten images assembled using maximum intensity projection. Given rise to multi colored 2D images were the polymerization of the microtubules can be traced by different colors appearing in different cycles. White color represents a signal that was at the same place in each of the cycles. The airyscanner LSM 880 (ZEISS) was accessed at the Cellular imaging core facility at the University of Gothenburg.
**Immunohistochemistry**

Fresh tumor pieces were fixed in 4% formalin, dehydrated and embedded in paraffin. Next, they were sectioned in 5 µm slices, mounted on glass slides and dried over night at 37°C. Rehydration and antigen retrieval was performed by pressure-cooking in citrate buffer. Staining was performed with an auto-stainer (Auto-stainer Link 48, Dako). Primary antibody staining was done for 60 minutes at room temperature, secondary staining was performed for 20 minutes and horseradish peroxidase (HPR) staining was performed for 20 minutes. DAB staining was used to stain DNA and counterstaining was done using hematoxylin. Finally, the slides were dehydrated, mounted with Pertex and imaged. See list of antibodies in the respective papers.

**Tubulin polymerization assay**

*In vitro* tubulin polymerization dynamics was measured according to manufacturer's instructions using the Tubulin polymerization assay kit (Cat.#BK011P, Cytoskeleton). Briefly, lyophilized porcine α-tubulin and β-tubulin was solubilized in reaction buffer to a final concentration of 2 mM and kept at 4°C. 10x stock solution of the test compounds, dissolved in DMSO were pre-warmed in a 96 well plate. The tubulin mix was added to the wells and the plate immediately transferred to the plate reader. The contents of the plate were mixed and the fluorescent signal (Ex 355, Em 460) read with one minute intervals for 60 minutes.
Results

Paper I

In this paper we asked the question if patient-derived xenografts (PDXes) were feasible in pre-clinical and clinical research. To answer this, we developed a PDX platform using tumor samples from melanoma patients treated at the Department of Surgery, Sahlgrenska University Hospital. First, some practical issues were addressed such as, transplantation method and finding the right mouse strain. Once the platform had been streamlined (Figure 8a), we applied it to address the following questions:

1. Do PDXes maintain the histology, mutation status and expression profile of the original tumor samples?
2. Can clinical treatment responses be predicted by using the platform?
3. Can the PDXes be established fast enough to benefit the patients?

Histology, mutation status and expression profile is preserved when patient-derived tumor cells are grown as xenografts

To address the first question we compared the expression of melanoma specific markers along with H&E staining of patient biopsies to the matched PDXes. Immunohistochemical staining of the samples revealed the high cellular growth pattern of both samples along with similar expression of the melanoma specific markers, indicating conserved melanoma characteristics (Figure 8c). Also, we compared the mutation status and expression profile of the original tumor and serially transplanted PDXes using RNA sequencing. The melanoma driver mutations BRAF-V600 and NRAS-Q61 were found in 46% and 31% of the samples, respectively (Figure 8b). Other well known but less frequent melanoma alterations were also observed for example mutations in PPP6C, TP53 and MAP2K1 and low expression of the tumor suppressor CDKN2A. The wide variety of genetic alterations demonstrates that the platform can be used for testing new compounds against these mutations and the possibility of screening out predictive biomarkers for compounds lacking one. To analyze if serial transplantation in mice results in detectable transcriptomic drift, we performed an unsupervised hierarchical clustering of the samples. Six of the samples had matching samples from different passage, with...
M121218 having the whole range from the biopsy to passage 3 (P3). All six samples clustered with their matching samples indicating low transcriptomic drift (Figure 8d). Despite the fact that the original tumor pieces, to a variable degree, contain immune cells that are not present in the PDXes, they clustered with their matching samples instead of clustering together. This suggests a stronger resemblance between the expression profiles of samples originating from the same patient than between fresh melanoma samples from the patients.

**PDXes can be used to predict treatment response**

The second question was if we could predict treatment responses in patients using the platform, to test that we describe two cases.

In the first case, we received a tumor sample from a stage III melanoma patient harboring NRAS-Q61 mutation. We cultured the cells *in vitro* and screened with a drug library containing 319 different compounds. More than one of the MEK inhibitors in the library had very good cytotoxic effect and therefore we established xenografts to test if these tumor cells would be sensitive to the inhibitor *in vivo* as well. Good response was observed when the mice were treated with the MEK inhibitor Trametinib, both by decrease in the physical size of the tumor and decrease of the human melanoma specific marker S100B, as measured in the plasma of the mice. To validate the target, PDXes from the same patient were treated with another MEK inhibitor (TAK-733) with the same results. As the ethical permit states that tumors cannot be grown over 10 mm on the shorter side, mice in the vehicle group had to be sacrificed while none of the mice in the Trametinib treatment group reached that limit.

In the second case, we received a tumor sample from a stage IV melanoma patient harboring a BRAF-V600 mutation. This patient was included in a double-blind clinical trial testing the combination of BRAF inhibitor (dabrafenib) and MEK inhibitor (trametinib) (97). To test the predictability of the platform, we established xenografts from this patient's biopsy and treated the mice with three different treatment arms, BRAFi, MEKi and the combination. Good response was observed in the PDXes in all the treatment groups as the physical size of the tumor decreased and the melanoma specific marker S100B decreased as was measured in the plasma from the mice. Indeed, the patient responded to the treatment as was observed in a CT scan and a decrease in S100B levels.
Figure 8. Overview of figures from paper I. a) Schematic of the PDX platform. Tumor biopsy from a malignant melanoma patient is processed. Tumor pieces are frozen down or fixed in formalin for later analysis. Also part is chopped up to make single cell suspension for cryo-preservation, in vitro culture and/or transplanting the cells in mice. The cells are then serially transplanted in mice to make a treatment group. Mice are next treated with anti-cancer agents and treatment response followed with caliper measurements or human melanoma specific markers in the mouse plasma. b) Frequency of BRAF and NRAS mutations in the melanoma samples included in this study. c) Immunohistochemical staining revealing the histological similarities between the patient biopsy and the matching PDX. d) Unsupervised hierarchical clustering of the transcriptome of patient biopsies and matching PDXes showing samples originating from the same patient clustering together. e) Kaplan-Meier plot showing that patient survival is longer than the time it takes to establish a PDX in treatment phase.
PDXes develop fast enough to guide treatment response

The third question addressed, was to see if PDX models develop fast enough to guide clinical decision making. To that end, we retrospectively investigated the time it took to establish a PDX in passage 3. This time was compared to the survival of the patients from when the biopsy was taken. In a Kaplan-Maier curve, the ratio of the established PDXes is compared to the ratio of patients still surviving at a given time point. This analysis revealed a statistically significant longer total survival of patients then the total time it took to establish the xenograft models (Figure 8e).
RESULTS

Paper II

To investigate if commercial cell lines could be used along with patient-derived xenografts in pre-clinical research, we compared the transcriptome of cell line derived xenografts (CDXes) and patient-derived xenografts (PDXes). We hypothesised that if unsupervised hierarchical clustering would group the samples according to their transcriptome profiles regardless of if it originated from a PDX or a CDX, we would be able to use CDXes to guide PDX treatment strategy. That way the easily cultured commercial cell lines would serve as surrogates for PDXes showing similar gene expression profile, making PDX treatment strategy faster. As it turned out, the samples did not inter-mix but instead the PDXes mainly cluster with the original biopsy and separately from the CDXes.

Different transcriptome profile between CDXes and PDXes regardless of mutation status

Ten widely used commercial metastatic melanoma cell lines and 23 patient-derived tumor samples originating from fifteen melanoma patients where transplanted in NOG mice to generate xenografts. The PDXes were both primary tumor samples (PR) and serially transplanted PDXes (P1-P3). Once tumors were harvested they were snap-frozen, the RNA extracted and sequenced. Both the CDXes and PDXes were found to harbour many of the well-known melanoma mutations (127). Eight of the CDXes harboured a BRAF-V600 mutation, a NRAS-Q61 mutation and a NF1 inactivating mutation were found in one CDX each. Eight (53%) of the fifteen patient samples harbour a mutation in BRAF-V600 and three (20%) samples harboured a mutation in NRAS-Q61. To investigate the similarities between the samples based on their transcriptome, both principal component analysis (PCA) and samples distance matrix analysis were performed. Both analyses revealed that CDXes and PDXes have different overall gene expression profiles and do not inter-mix. Comparing the samples based on the top 8000 expressed genes using an unsupervised hierarchical clustering revealed again the difference between PDXes and CDXes.
Hypoxia-induced gene signatures characterize CDXes

Next we investigated which genes were responsible for the difference in expression between PDXes and CDXes, and found that miRNA host genes differed significantly. A PCA plot based on the expression of the miRNA host genes revealed difference between PDXes and CDXes (Figure 9a) but notably not as evident as in the PCA based on the whole transcriptome. The most significant difference was the high expression of the hypoxia-regulated miR210HG in CDXes (FDR = 3.33E-06) (Figure 9b). To identify genes whose expression correlate with expression of miR210HG, Persons correlation was performed. Of the 40 genes with the highest correlation were many known to be either targets of HIF1 and/or involved in gly-
colysis, as predicted by Gene set enrichment analysis (GSEA) (Figure 9c). Taken together, the results indicate that the main difference between the transcriptome of PDExes and CDXes is due to the adaptation of cell lines to the physiological environment once they are transplanted in mice, where they experience pseudo-hypoxia compared to the artificial cell culture environment they had adapted to.

**Hypoxia response in cells cultured in 5% O₂**

To further investigate the role of miR210HG in the hypoxia-response, three melanoma cell lines were chosen based on their high, medium and low expression of miR210HG, according to the RNA sequencing data (Figure 9d). The cell lines were cultured in 5% or 20% oxygen and the expression of miR210HG analysed by qRT-PCR, showing increased expression in all three cell lines (Figure 10a). Furthermore, we detected increased expression of the genes correlating with expression of miR210HG when cells were cultured in 5% vs. 20% oxygen. Next, we examined hypoxia-response on protein level by analysing expression of Phosphorylated RB (pRB), geminin and CA9 as markers of G1 phase progression, S-G2 phase and target of hypoxia, respectively. Detection of all three markers was lower in two (SK-MEL-2 and MML-1) of the three cell lines when cultured in 5% vs. 20% O₂. Expression of total RB was also lower which could be explained by lower cap-dependent protein translation due to hypoxia-induced growth arrest. Indeed, the expression of the cap-dependent translation marker p4EBP1 was also low. One of the cell lines (A375) showed no difference in expression of any of these markers.

**Reversing hypoxia-induced response using a miRNA decoy**

To assess the role of miR210 in the hypoxia response we genetically engineered all the three cell lines to express a miR210 decoy which binds and inactivates miR210 (128). Expression of the miR210 host gene (miR-210HG) was not hindered by expression of the decoy in either 5% or 20% O₂ (Figure 10b). To assess the downstream effect of inactivating miR210 using the decoy we compared again the protein expression of pRB, geminin and CA9. Contrary to what we observed in the parental cells, all three markers were expressed in both 5% and 20% O₂ (Figure 10c), indicating that hypoxia response was hindered by the decoy. Expression of CA9 was induced in all three cell lines both in 5% and 20% O₂. Collectively, the data suggests a different hypoxia response between cell lines dependent on basal miR210HG.
Figure 10. Abrogation of miR-210 function reverses the hypoxia-response and makes melanoma CDXes less sensitive to MEK inhibition. a) qRT-PCR analysis showing induction of miR-210HG expression in three melanoma cell lines in response to hypoxia. b) qRT-PCR analysis showing induction of miR-210HG in three melanoma cells expressing the miR-210 decoy, in response to hypoxia. c) Western blot analysis showing difference in protein expression between parental and decoy expressing cell lines cultured for 24 hours in 20% or 5% oxygen. d) Growth of CDXes established from cell lines expressing the decoy, compared to the parental cells. e) Kaplan-Meier plot showing survival of mice carrying parental or decoy expressing cells treated with trametinib or vehicle. f) Western blot analysis showing protein expression in CDXes engineered with the decoy, compared to the parental cells.
miR210 inactivation reduces sensitivity to MEK inhibition in vivo

To test if inactivation of miR210 had any effect on growth rate we transplanted MML-1 cells engineered with the miR210 decoy or the parental MML-1 cells in the flank of immune compromised NOG mice and measured tumor growth by physically measuring the size using a caliper. MML-1 cells expressing the decoy grew significantly faster, giving rise to larger xenografts 24 days after the cells were transplanted compared to the parental MML-1 cells (Figure 10d). We also investigated if inactivation of miR210 would interfere with targeted therapy. By treating mice carrying xenografts originating from MML-1 cells either expressing the decoy or not, with Trametinib we observed reduced sensitivity to Trametinib in decoy-expressing xenografts (Figure 10e). Interestingly, suggested targets of miR210 are both ERK and AKT which both are downstream of MEK and would therefore not be affected by the MEK inhibition. Western blot analysis revealed increased expression of pAKT in 3/5 decoy-expressing xenografts and no marked difference in pERK1/2 intensity (Figure 10f).
Paper III

Karonudib is an inhibitor of MTH1 which we have been a part of validating pre-clinically using our PDX platform (66, 72). The compound has been accepted to start phase I clinical trial at Karolinska University Hospital. Since Karonudib targets a non-mutated form of MTH1, patient stratification for the trial could be challenging. We therefore, ran a pre-clinical PDX trial where we tested Karonudib in multiple PDXes derived from malignant melanoma patients. The aim was to investigate the inter-patient heterogeneity in treatment responses and to screen for a predictive biomarker. During our research we observed that Karonudib has a second target beside MTH1, contributing to the cytotoxicity.

Karonudib binds MTH1 in malignant melanoma cells

To test if the newly developed MTH1 inhibitors TH1579 (Karonudib) and TH1827 are selective to MTH1 in melanoma we performed a cellular thermal shift assay (CETSA). The malignant melanoma cell line SK-MEL-2 was treated with DMSO or 5 µM of TH1579 or TH1827 for 2 hours. The harvested cells were subjected to thermal proteome profiling by heating intact cells at different temperatures to induce protein denaturation. Soluble proteins where next extracted and quantified using mass-spectrometry. Protein quantity at each temperature was plotted to visualize differences in denaturation between the samples caused by ligand binding. Stability of MTH1 increased 10.4°C and 15°C when cells were treated with TH1579 and TH1827 compared to DMSO, respectively. The increased stability was then verified with Western blot on both intact cells and cell lysate.

Karonudib has cytotoxic effect on melanoma cells in vitro

To investigate the long-term effect of TH1579 on melanoma cells, we treated four malignant melanoma cell lines (SK-MEL-2, MEL-CLS-2, SK-MEL-28 and MeWo) for 7-10 days with concentration ranging from 0.05 to 5 µM, and measured colony outgrowth. Crystal violet staining revealed a complete cytotoxic effect of cells treated with 0.5 µM and 5 µM in all cell lines. Flow cytometry analysis of the cell cycle profile of the same cell lines identified apoptosis, G2/M arrest and polyploidization after 48 hours of TH1579 treatment in a dose dependent manner.
66% of PDX models respond to TH1579

To assess the efficacy of TH1579 in a cohort of malignant melanoma patient samples we conducted a pre-clinical trial in PDX models from 33 patients. Tumor biopsies were serially transplanted as described previously (129). Mice were treated with either TH1579 (90 mg/kg) or vehicle and tumor growth followed using a caliper. Once the mice had been treated for 18 days, the tumors were harvested and snap frozen. RNA was extracted from the vehicle tumors and the RNA sequenced with the aim of identifying a predictive biomarker.

The patient samples represent many of the known driver mutations observed in melanoma (27). 58% (n=19) harbour BRAF-V600 mutation, 24% (n=8) harbour NRAS-Q61 mutation, 12% (n=4) harbour neither, and 6% (n=2) harbour both BRAF (V600 or S465) and NRAS-Q61 mutations. Interestingly, when looking more closely at those samples harbouring both mutations we observe that they are both derived from patients that had received treatment before the biopsy was taken. One of those samples is derived from a patient treated with a BRAF inhibitor and the other form a patient treated with Temodal. Looking more closely at the RNA sequencing data from those samples we observe a homozygous NRAS-Q61 mutation and a heterogeneous BRAF-V600 mutation (data not shown). The samples included in the pre-clinical trial originate from patients with stage III (24%) and stage IV (76%) disease.

Treatment response was estimated as growth of the treated xenograft divided by the growth of the vehicle xenograft (ΔT/ΔC). In that way the inherent growth capacity of the tumor cells is normalized to the response of the treated xenograft. The samples were divided in three groups based on their treatment response. Treated PDXes, which grew >50% of the matching vehicle treated PDX were assigned to the “progression” group (ΔT/ΔC> 50%), treated PDXes which grew between 0% and 50% of matching vehicle treated PDX were assigned to the “suppression” group (ΔT/ΔC= 0-50%), and samples that decreased in size from treatment start were assigned to the “regression” group (ΔT/ΔC< 0%). According to this criteria, 11 (33.3%), 13 (39.4%), and 9 (27.3%) samples were categorised in the Progression, Suppression, and Regression groups, respectively. Taken together, we observed that 66.7% of samples responded (regression and suppression groups) to the TH1579 treatment while 33.3% did not respond (progression group).

TH1579 does not infer with T-cell mediated immunity

To investigate if TH1579 would infer with T-cell mediated anti-tumor response we utilized a recently developed mouse model (Jespersen & Lindberg et al., in revision) where patient-derived tumor-infiltrating lymphocytes (TILs) are expanded ex-
vivo and injected in mice carrying autologous xenograft. For this experiment we used a brain metastasis from a malignant melanoma patient, which was included in the pre-clinical trial and responded to the TH1579 treatment by regressing 17% in size from treatment start. The tumor cells were transduced with a luciferase expressing virus before being serially transplanted as described previously (129) and TILs were expanded ex-vivo from the primary tumor biopsy.

First, we performed an *in vitro* granulation assay where the anti-tumor potential of the TILs were estimated in the presence of TH1579. TILs were cultured in the presence or absence of autologous tumor cells, with or without TH1579. TILs stained with the degranulation marker CD107 were then quantified using flow cytometry. No degranulation was observed when TILs were treated with 0.5 µM TH1579. On the other hand, when TILs were cultured in the presence of autologous tumors cells, increased degranulation was detected, which was not impaired by 0.5 µM of TH1579.

Next we transplanted the tumor cells in immunodeficient mice, once the tumors were palpable, TILs were injected in half of them. At the same time, half of the mice that were injected with TILs and half of the mice not injected with TILs started TH1579 treatment, making four treatment groups. Treatment response was observed by both measuring the physical size of the tumors and by *in vivo* imaging of the luciferase expressing cells. After 9 days of treatment no significant difference was observed between the three treatment groups (TH1579, TILs, TILs+TH1579) but the vehicle treated xenografts were significantly bigger. After TH1579 treatment was stopped, xenografts on mice receiving TH1579, not injected with TILs increased in size but did not catch up with the vehicle treated, not even 4 weeks later. PDXes on mice injected with TILs continued to decrease with no impairment from the TH1579 treatment.

**Cytotoxic effect of TH1579 is independent of driver mutations— but a potential inherent resistance could be identified**

With the aim of identifying a predictive biomarker for TH1579, we sequenced the RNA from all 33 vehicle PDXes included in the pre-clinical trial. No significant correlation was observed between driver mutations and any of the response groups. However, a slight trend was observed between *DDX3X* mutation and the regression group, although not significant (FDR= 0.09). Further more, expression profile of the samples failed to predict response to TH1579. On the other hand, a difference in growth speed was observed between the response groups. Samples assigned to the suppression group grew faster then samples in the other two groups. The repressed growth of the treated xenografts in the “suppression” group could also be
observed by immunohistochemistry with less Ki67 staining in the TH1579 treated samples compared to the vehicle treated. On the other hand, when comparing the differentially expressed genes in the different response groups using gene set enrichment analysis (GSEA) we identified the KEGG module “ABC transporters” as associated with the Progression group. High expression of the well-characterized multi-drug resistance gene \(ABCB1\), expressing the pgp-drug efflux pump, was observed in few samples categorized in the progression group.

**Synergistic effect of pgp-efflux pump inhibition and TH1579**

To further explore the connection between the pgp-drug efflux pumps and TH1579 we treated cells with the combination of elacridar (inhibitor of pgp-pumps) and TH1579. First, we examined the expression of \(ABCB1\) in ten commercially available cells lines. According to RNA sequencing previously done in the lab, SK-MEL-1 was the only cell line with high expression of the gene. Furthermore when adding Rhodamine-123 (p-gp substrate) on three of those cell lines, SK-MEL-1 was the only one able to pump it out, though not very efficiently. The rat glioma cell lines C6 was however able to efficiently pump out Rhodamine-123, which was possible to inhibit using elacridar. When treating those cells with the combination of elacridar and TH1579, synergistic cytotoxicity was observed.

To test the combination treatment in vivo, we established PDXes from patient sample M150330, which was in the progression group in the pre-clinical trial and expressed high levels of the drug efflux pumps MDR1 (\(ABCB1\)) and BCRP (\(ABCG2\)). The mice were divided into four treatment groups receiving, TH1579, elacridar, TH1579 and elacridar, or vehicle. However, the mice in the combination needed to be sacrificed prematurely, due to >10% of loss of body weight and decrease in blood counts. Analysis of the tumors, revealed synergistic effect then treating with elacridar and TH1579, observed with the high proportion of cells in sub-G1 phase measured with flow cytometry.

**TH1579 affects tubulin polymerization**

We also observed high expression of \(TUBB8P12\) in samples belonging to the progression group. \(TUBB8P12\) is a \(\beta\)-tubulin pseudogene with 90% sequence identity to \(\beta\)-tubulin. This observation along with the G2/M arrest, polyploidization and change in morphology of cells treated with TH1579 is an indication of TH1579 affecting polymerization of tubulin. To further explore that, we tested if polymerization of tubulin was affected in the presence of TH1579 in a test tube. We observed a dose dependent delay in the polymerization of tubulins with increased
concentration of TH1579. To visualize the effect of TH1579 on tubulin polymerization in live cells, we transduced SK-MEL-2 and MEL-202 cells with a lentivirus expressing EB3-GFP. EB3 is a microtubule end binding protein, which when labelled with GFP enables the tracking of microtubule growth by 3D live cell imaging. After 4 hours of treatment with TH1579, vinorelbine or paclitaxel, disruption of microtubule dynamics was observed. Furthermore, SK-MEL-1 cells were treated with TH1579, vinorelbine or paclitaxel for 4 hours and harvested. Cells were lysed and free tubulin (soluble fraction) or microtubule bound tubulin (insoluble fraction) was extracted. Next the tubulin was analysed using Western blot analysis, revealing increased tubulin staining in the soluble fraction and decreased tubulin staining in the insoluble fraction when the cells were treated with TH1579 or vinorelbine. Taken together, we show that TH1579 affects the microtubules dynamics both in a test tube and in live cells.
Discussion

Paper I

Establishing PDXes and a bio bank for future usage

When developing a PDX platform to be used in pre-clinical and clinical analysis it is important to standardize each step. Two different transplantation methods have been reported when transplanting human tumors subcutaneously in mice. The more common one is when a tumor piece is surgically implanted subcutaneously while the mouse is under anesthesia (123). Less frequently, the tumor piece is chopped making single cell suspension and the cells injected subcutaneously using a syringe. We tested both methods which yielded a very similar take rate. Hence we used the single cell suspension technique since it is superior practically. Others have also reported no difference in the take rate of those two techniques in Nude mice transplanted with endocrine tumor cells (130). Injecting single cells has the advantage that the tumor cells can be transplanted very quickly and without having the mouse under anesthesia. Furthermore, the cells can be cryopreserved for later usage. Thereby, we are able to cryopreserve cells from every passage. It is also important to choose the right mouse strain to get the best take rate. Other papers have reported the use of Balb-c, Nude and NOGs with the best take rate in NOGs (131), which is consistent with our tests. We observed the best take rate in NOGs and therefore used them in the development of the platform.

Low genomic, transcriptomic and histological drift in PDXes

Low genomic, transcriptomic and histological drift is an essential factor when this type of platform is used in pre-clinical and clinical analysis. It has been show that PDXes recapitulate the genomic and histological features of the patient samples for multiple tumor types (111, 114). In this paper, we show for the first time, using RNA sequencing the low transcriptomic drift between the malignant melanoma patient samples and their matched PDXes. Since this paper was published, others have shown the same in a larger scale study where PDXes were established from malignant melanoma along with multiple other tumor types, showing low
transcriptomic drift in all of them (124). The wide variety of genetic alterations as detected by RNA sequencing demonstrates the feasibility of the platform for testing compounds against those mutations and the possibility of screening out predictive biomarkers for compounds lacking one (123, 124).

**PDXes predict clinical responses**

Patient derived xenografts have been successfully used to predict clinical responses (121). Here, we describe a case where we screen for the appropriate treatment with *in vitro* drug screen. However, we also raise concern in that regard since not every patient sample can be grown in the high level of oxygen and on artificial material. Also, the artificial culture environment can stimulate different gene expression in the cells and thereby give misleading information about the sensitivity of the tumor cells when grown in their normal environment. Therefore, we recommend keeping patient sample for as short time as possible ex vivo and only propagate the samples *in vivo* when the samples are intended for translational purpose. We also predicted a clinical response in a patient, who was enrolled in a double bind clinical trial by treating the matching PDXes simultaneously. The three-arm trial tested the MEK inhibitor trametinib, the BRAF inhibitor dabrafenib and the combination of the two. Since the trial was double blinded, we divided the PDXes in all possible treatment groups and observed response in all groups. Later the treatment groups were disclosed revealing that the patient had received the combination treatment.

Due to the predictability of the platform, we suggest it can be used in several purposes. It can be used as inclusion criteria for clinical trials testing compounds lacking a predictive biomarker. Where a positive response of a PDX would serve as a marker for the matched patient to be included in the trial. That would mean an extra step in the clinical trial procedure, but might benefit more patients. Also, this platform can be used to screen out a predictive biomarker to use as inclusion criteria to clinical trials. In that case, multiple patient samples would be established as PDXes and treated with the compound. Afterwards, biological difference between the response groups can be screened out. The benefit of that process is that drugs that could fail in clinical trials due to incorrect patient strategy have higher chance of passing, that way benefitting the subgroup of patients showing that predictive biomarker.
PDXes can be established fast enough for clinical use

One burning question regarding the use of PDXes is if they can be established fast enough to guide cancer treatment or to identify patients suitable to be included in clinical trials for drugs lacking a predictive biomarker. We showed in this paper, for the first time, that the patient survived longer than it took to establish the models. Thus, showing that the models are a feasible option. It needs to be taken into account that this data was produced by retrospectively looking at the time it took to establish the models. Thus, the models were not established in the fastest way possible since this was done simultaneously as the platform was being developed and streamlined. Today, we have improved the injection method along with harvesting the tumors at a smaller size to serially transplant, which speeds up the process. Moreover, patients now can benefit from durable or partial responses to anti-PD1 immune therapy, which was not approved when we conducted this study. Therefore, if this comparison would be repeated with current technical improvements, the difference would be even greater. High engraftment potential of a tumor has been shown to predict shorter survival of the patient, where patients whose tumors failed to engraft had 81% reduced risk of death (121). This is in accordance with our observation of the longer survival than engraftment time. We also analysed the inter-patient difference in treatment response by treating melanoma PDXes derived from eight different patients with the MEK inhibitor trametinib. This analysis revealed response rate from -6% to -98%. Since then, trametinib has been FDA approved and has shown good clinical responses (80). On the other hand, the activity of trametinib in NRAS mutant melanoma is not predictable (80), but could be screened for using this PDX platform.

Paper II

Cell lines experience pseudo-hypoxia when grown in vivo

In this study we observed difference in the transcriptome of xenografts originating from cell lines and from patient tumor samples. Previously, it has been shown that CDX of NSCLC diverge from both the cell lines (culture for 6 months) they originate from and from PDXes established directly from the matched original tumor, and that the rewiring of the cells once cultured in vitro is irreversible (107). The irreversible rewiring of cells once grown in vitro has also been observed in melanoma, where the transcriptome of CDXes correlates better to the cell lines they are derived from then the original tumor biopsy (132). However, the difference of CDXes and PDXes has not been investigated in malignant melanoma. In this study
we show that the main difference is due to the pseudo hypoxia experienced by the cell lines once transferred from cell culture environment to physiological environment. This was identified by high expression of hypoxia response genes in CDXes compared to PDXes, with miR-210HG as highly expressed, which is an indication of the pseudo-hypoxia experienced by the cells once transferred from cell culture to physiological environment.

**Genes involved in the hypoxia response**

Owing to the well-established role of miR210 in hypoxia, we looked at the genes correlating with its expression and identified several well-established hypoxia-induced genes, one of them being Carbonic Anhydrase 9 (CA9). CA9 is a membrane protein and a known HIF1 target, which has been shown to be increased on RNA and protein level with increased hypoxia (133).

Even though we observed higher expression of miR-210HG in the CDXes compared to the PDXes, we can’t say if the observed expression in CDXes is increased or decreased compared to when the cells were grown *in vitro*. To address this we compared the expression of miR-210HG in cell lines cultured in either 5% or 20% O₂ and measured the level of miR-210HG expression using qRT-PCR. The increased expression in 5% O₂ in all three cell lines strongly suggests that expression of hsa-miR-210HG is increased due to the pseudo-hypoxia response. To further investigate the hypoxia-response we compared protein expression of CA9 between cells cultured in 5% and 20% oxygen, and observed decreased protein expression in SK-MEL-2 and MML-1, but unchanged level of CA9 in A375. It has been shown that A375 does not respond to hypoxia by increasing HIF1A and CA9 expression (134). Interestingly, we observed inconsistency in mRNA and protein expression of CA9 when comparing culture in 5% and 20% O₂. mRNA expression increased but protein expression decreased when the cells were grown in 5% compared to 20% O₂ which remains to be explained (compare Figure 3c and S2). The low detection of geminin and pRB was an indication of low transcription activity of the cells, interestingly we observed that the total RB expression was also low.

**Inactivation of miR210 as a possible resistance mechanism**

Expression of miR-210HG has been shown to be associated with aggressive tumor growth and poor prognosis across malignancies making it or downstream targets interesting candidates therapeutically (135-137). Contradicting those previous observations, we find that miR-210 functions more like a tumor suppressor in melanoma cells, keeping in mind the over-all low cap-dependent transcription when
mir-210HG is highly expressed, and the fact that xenografts expressing the decoy grow at a faster rate compared to the parental cells. Noman et al. (137) showed how miR-210 is elevated in myeloid derived suppressor cells (MDSC) located in the tumors of mice compared to the MDSC located in the spleen of the mice. Furthermore, they show how the miR-210 high expressing MDSC inhibits the cytotoxic effect of T cells and how targeting the hypoxia-induced miR-210 was sufficient to decrease MDSC function against T cells. Taking together, this finding and our finding demonstrate how targeting miR-210 has different effect depending on if it is targeted in tumor suppressing or tumor promoting cells.

Trametinib is a FDA approved MEK inhibitor which has been shown to be efficient against malignant melanoma (129), but like with many targeted treatments the tumor cells become insensitive to the treatment by finding a way to survive in the presence of the compound. Here we show that impairment of miR-210 makes malignant melanoma CDXes become less sensitive to Trametinib. Out of numerous miR-210 targets we chose to validate ERK and AKT, as both are downstream of MEK and could potentially, be responsible for the decreased sensitivity. Indeed, we observed increased AKT phosphorylation in CDXes engineered with the miR-210 decoy, compared to little difference in the levels of phosphorylated and total ERK. The PI3K/AKT pathway is known to contribute to malignant progression (138). Also, it has been shown that CAP-dependent translation is affected by stress, such as hypoxia (139, 140). Therefore, we hypothesize that by impairing miR-210, AKT inhibition is relieved causing activation of cap-dependent translation and cell survival.

Paper III

TH1579 targets MTH1 and microtubule dynamics

TH1827 and TH1579 (Karonudib) are inhibitors designed to inhibit the 8-oxo-dGTPase MTH1. The selectivity of TH1579 to MTH1 has been previously shown in the osteosarcoma cell line U2OS (72), and here we observe the same for both inhibitors in the malignant melanoma cell line SK-MEL-2 using CETSA. It is important to keep in mind that only soluble proteins are extracted from the samples and identified by the Mass Spectrometer therefore this method cannot detect insoluble proteins for example protein complexes and cytoskeletal proteins (141).

In recent studies, no cytotoxic effect was observed using siRNA or CRISPR targeting MTH1 (69, 71). Furthermore, potent and selective MTH1 inhibitors devel-
oped by others failed to demonstrate the same phenotype as reported for TH588 (69).

Kawamura et al. proposed that the cytotoxic effect of TH588 (a different version of TH1579) was due to its affinity to tubulin and thereby disrupting polymerization of microtubules (70). In that study they performed a tubulin polymerization assay where they showed that the polymerization of α and β tubulin subunits was interrupted in the presence of 30 µM or more of TH588 in a test tube. We have performed the same assay using TH1579, and did not observe as profound effect as Kawamura et al. On the other hand, we do observe a G2/M arrest measured by flow cytometry, disturbance in microtubule dynamics by live cell imaging and increased free tubulin when cells are treated in vitro with TH1579, which suggests microtubule targeting activity.

**Anti-tumor effect of TILs not hampered by TH1579**

It is well established that increased amounts of tumor infiltrating lymphocytes (TILs) is a positive prognostic marker (142). Therefore, it is important to investigate if treatment with TH1579 could impair the TIL anti-tumor effect. Especially since it has been proposed that activated T cells reprogram their metabolic pathways to fulfill the bioenergetics and biosynthetic demand of proliferation, much like tumor cells (143). Neither the *in vitro* nor the *in vivo* experiments indicated that TH1579 would interfere with the anti-tumor effect of the TILs.

**Pre-clinical trial to estimate responses to TH1579**

Given how many compounds fail at clinical trials due to insufficient efficacy, we investigated the inter-patient response to TH1579 in malignant melanoma patient-derived xenografts. Since TH1579 is an inhibitor designed to the un-mutated form of MTH1, a clear predictive biomarker is not apparent. Therefore, we did what we proposed in paper I, namely ran a pre-clinical trial in PDXes to estimate inter-patient response and screen for a predictive biomarker.

Malignant melanoma samples from 33 patients were included in the trial. This is the sample size needed, according to power calculations, to gain robust information with probability of type one error (α) = 0.05 and power of 0.8 (144, 145).

How samples are categorized in response groups, is dependent on which response criteria are used. Treatment response in patients is often evaluated using the Response Evaluation Criteria in Solid Tumors (RECIST), were patients are divided in response groups based on change in size of the tumor/s compared to treatment
start (146). In the clinic there is of course no way to control for how much a tumor would have grown would it not have been treated. When testing drugs in PDXes, that can however be controlled for. Therefore, can we take the growth speed of the untreated tumor into account when estimating response to a treatment. We evaluate response as the ratio (%) of growth of the treated PDX divided by the growth of the matching vehicle treated PDX, that way the inherent growth speed of the tumor is normalized.

Interestingly, we observed that vehicle treated samples in the suppression group grow at a faster rate than the samples in the regression and progression groups. Using immunohistochemistry, we observed less expression of the proliferation marker Ki-67 and more dead and dying cells in the treated PDX compared to the vehicle treated PDX in the suppression group. Hence, the difference in size between the TH1579 treated and vehicle treated xenografts is greatest in the suppression group. If response would have been measured by the difference in growth between the TH1579 treated and vehicle treated tumors, the suppression group would have shown the best response. But with that type of calculations, tumors actually decreasing from treatment start would not be categorised as responding simply because of how slowly the vehicle treated xenograft grew. Therefore, like stated before, it is obvious that response calling method is very important to think of when conducting PDX pre-clinical trials.

**TH1579 targets the phenotype – not the genotype**

We did not identify a clear mutation or expression profile predicting treatment response to TH1579, indicating that the compound targets the phenotype independent of genomic and transcriptomic profiles. Although a trend towards an association between a missense mutation in *DDX3X* and the regression group was observed, which could indicate an importance for *DDX3X* in the redox balance or in the damage response due to inactive MTH1.

We did observe good response in the samples categorised in the suppression group, even though they did not regress. But when comparing the treated PDXes to the matched vehicle a very profound size difference is evident. Those samples are also the fastest growing samples and therefore it is possible that TH1579 will affect faster growing tumors more than the slow growing. Those samples also, have the highest expression of MTH1 both in RNA and protein level. Wang et al. (71) published a study where they test the MTH1 inhibitor TH588 *in vitro* on malignant melanoma cell lines and patient derived cells. They conclude that the cytotoxic effect of TH588 on malignant melanoma cells is not due to MTH1 inhibition since overexpression of MTH1 did not rescue the cells. Furthermore they show that sen-
sitivity of cells to TH588 is correlated to the endogenous level of ROS where treating cells with the ROS enhancer elesclomol increased the cytotoxic effect of TH588. This points to a dual function of TH588/TH1579, one of which is involved the ROS-MTH1 axis and the other involves disrupting the microtubule dynamics.

TH1579 as a substrate of drug efflux pumps

Expression of the drug efflux pumps p-gp (ABCB1) and BCRP (ABCG2) enables the cells to pump out compounds and thereby reducing the intracellular accumulation of the compounds rendering the cells resistant. Here we show that melanoma cells expressing high level of drug efflux pumps are able to pump out p-gp substrates and that inhibiting the pumps along with treating with TH1579 has cytotoxic synergistic effect both in vitro and in vivo. But, further investigation is advised should this combination be used clinically. Due to the fact that drug efflux pumps are expressed widely in the body and that TH1579 binds the wild type form of MTH1 along with affecting microtubule dynamics.

Further investigation on combination treatment with TH1579 is on-going in the lab. We have identified synergistic cytotoxicity when treating melanoma cell with TH1579 and several compounds in vitro. These combinations will be tested in vivo to investigate if they are beneficial.
Conclusion and future directions

From this work I conclude that PDXes should be used more widely in order to benefit cancer patients, either to guide treatment strategies or to match the right patients to the right clinical trials. It occurs too often that patients have to go through several rounds of treatments before finding the right one, often with difficult side effects. But screening for the right treatment demands a wide variety of approved drugs to screen through. With the current status, many drugs fail early stage clinical trials, limiting the amount of approved drugs to choose from. Therefore, using the PDXes to pre-screen response to the drugs could lead to more variety in approved drugs. Furthermore, PDXes should be preferred over CDXes in that type of pre-clinical testing due to the difference in their transcriptome. Furthermore, I conclude from this work that Karonudib is an effective anti-malignant melanoma drug. Like most other cancer agents available, it is likely that the cells will find a way to survive in the presence of the drug. If so, further research is needed on how to manage that, for example by combination treatment.

The future direction of the project presented in paper III is to better establish how Karonudib impacts the microtubule dynamics, also to further investigate the redox homeostasis and the extent of reactive oxygen species, and thus the importance of MTH1 in late stage malignant melanoma.
Acknowledgement
References


REFERENCES


Appendix

Paper I